Repairing injured articular cartilage: Investigation of potential mechanisms using mesenchymal stem cells

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Abstract

Traumatic cartilage injuries are a known risk factor for development of osteoarthritis (OA). Classically cartilage is seen as poorly repairing, however, cartilage injuries in very young animals have been observed to heal although the mechanism of this repair is unknown. The aim of this project was to investigate if mesenchymal stem cells (MSCs) in the joint tissue can respond to cartilage injury and are involved in the potential cartilage repair mechanisms.

Fluorescently-labelled synovial fluid-derived MSCs (SF MSCs) specifically adhered along the edges of the injured cartilage. SF MSCs and bone marrow-derived MSCs (BM MSCs) migrated in significantly greater numbers to injured cartilage compared to non-injured cartilage. Incubation of freshly chopped articular cartilage with serum –free media gave an injury conditioned medium (IJCM) which significantly stimulated cell migration of both SF and BM MSCs in classical 'Scratch' assays. Migration of MSCs in response to IJCM was significantly reduced by pre-treating the IJCM with immobilised heparin or using neutralising antibodies to TGFβ3, FGF2 and CXCL12, and the CXCR4 receptor inhibitor AMD3100. No SF MSC migration was observed with IJCM in cell exclusion zone migration assays (no 'wound' is created in the cell layer in this assay protocol). However, migration in response to IJCM was observed in this system when a cell injury was created (by scraping off a thin line of cells) close to the cell exclusion zone. Creating the 'scratch' wound caused release of μM amounts of ATP from the injured cells as determined by an ATP-bioluminescence assay. Supplementation of IJCM with ATP or UTP or the stable purine analogues ATP-γS or UTP- γS stimulated MSC migration in the cell exclusion zone assays, suggesting a role for a putative P2 receptor in priming of SF MSCs for migration. Furthermore, IJCM-stimulated SF MSC migration could be completely inhibited in scratch assays using the P2 purine receptor antagonist suramin.

The results suggested that articular cartilage released ATP, CXCL12, TGFβ3 and FGF2 following injury and these factors together stimulated MSC migration to injured cartilage. Extracellular nucleotides such as ATP and UTP are required to prime the SF MSCs before CXCL12 and TGFβ3 stimulates cell migration to the injury site. In contrast, BM MSCs do not require any priming signal before their migration along the chemotactic gradient of CXCL12, FGF2 and TGFβ3.

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Abbreviations

3D	Three dimensional
ACI	Autologous chondrocyte implantation
ATP	Adenosine triphosphates
ATPγS	Adenosine-5'-(γ-thio)-triphosphate tetralithium salt
FGF2	Basic fibroblast growth factor 2
BM	Bone marrow
BM MSC	Bone marrow mesenchymal stem cells
BSA	Bovine serum albumin
CIJM	Cell injury conditioned media
DMEM	Dulbecco's modification of Eagle's medium
ECM	Extracellular matrix
FCS	Foetal Calf Serum
GAG	Glycosaminoglycan
HSC	Haematopoietic stem cells
IJCM	Injury conditioned media
IMS	Industrial methylated spirit
MACI	Matrix assisted chondrocyte implantation
MCP	Bovine metacarpophalangeal joint
MSC	Mesenchymal stem cells
OA	Osteoarthritis
PBS	Phosphate buffered saline
SF	Synovial fluid

SF MSC	Synovial fluid mesenchymal stem cells
TBS	Tris-buffered saline
TGFβ3	Transforming growth factor β3
UTP	Uridine triphosphates
UTPγS	Uridine-5'-(γ-thio)-triphosphate tetralithium salt

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Dedication

To my superhero, my fairy godmother, my sister: Sadia Afreen Sarkar Sarah.

Chapter 1

Introduction

1.1 Rationale for the study

Osteoarthritis (OA) is the most common form of arthritis [1] (approximately 1 in 5 people over the age of 50 have symptomatic OA in one or both knees [2]) and poses a major problem for the ageing populations worldwide. Due to increasing life expectancy, OA is predicted to be the 4th leading cause of functional disability in the ageing global population by 2020 [3]. OA, also known as degenerative joint disease is characterised by gradual loss of cartilage structure and function accompanied by alterations in the subchondral bone and limited intra-articular inflammation and synovitis [4]. OA also involves deformity, limited joint movement and pain which in turn lead to restricted motion and chronic disability. Due to this, OA has a sizeable economic impact on the healthcare system of 1-2% of GDP in industrialised countries [5, 6].

Current surgical interventions in place that attempt to stimulate regeneration of injured cartilage include joint washout, tissue debridement and microfracture technique [7] to release the mesenchymal stem cells (MSCs) from the underlying bone marrow. These procedures provide some symptomatic relieve from pain but the repair tissue is fibrocartilaginous in nature which is unable to withstand compressive load over time. Therefore, these procedures do not arrest joint degeneration and loss of joint function over time. Ultimately, patients may need to resort to joint replacement surgery. Joint replacement has a high success rate in hip and knee OA [8]. Over 79,000 primary knee replacements and more than 71,000 primary hip replacements were performed in the England and Wales during 2011, at an estimated cost of £1.65 billion [8]. However, joint prostheses have a limited lifespan (20-25 years) and around 5% of the patients suffer prosthesis loosening which requires further invasive revision surgery [8, 9]. Loosening is particularly problematic in younger patients under the age of 60 who tend to place higher demands on their prostheses due to their active lifestyles. Since, a limited number of revision surgeries are recommended and population longevity is increasing, there is both a clinical and societal need to prolong the life of the natural joint. Newer regenerative medicine technologies involving autologous chondrocyte or MSC implantation have been developed over the past decade to regenerate cartilage and improve the functional life of damaged joints [10, 11]. These treatments have largely been directed at patients less than 50 years of age with focal cartilage defects rather than OA. Several studies have reported safe and effective clinical outcome, although none have included a placebo control [12-14]. Autogenous re-implantation is expensive, requires multiple surgical procedures, and is associated with donor site morbidity. Tissue outcome is variable with one study showing 78% of patients had fibrocartilaginous repair [15].

The question of whether cartilage is capable of repairing itself, so called “intrinsic” repair, is an important one. Although, once believed to be a tissue that had little reparative

activity, a number of recent studies in human and animals show that repair does occur. Young patients with isolated partial thickness injuries or full- thickness injuries in an otherwise healthy joint regained full knee function without requiring any aggressive treatment approach [16, 17] suggesting that these focal lesions repair spontaneously. A 14-year clinical and radiographical follow-up of 28 young athletes with isolated partial thickness injuries showed that 22 of these patients were able to return to their pre-injury level sport activities [16]. Spontaneous healing of partial thickness injuries has been seen on arthroscopy [18]. Furthermore, regrowth of cartilage is seen in advanced OA patients when the joint is off-loaded by "joint distraction" (6 week treatment with an external metal fixator) [19, 20] or by realignment osteotomy [21, 22]. Intrinsic repair and integration with the surrounding cartilage has also been consistently observed in the cartilage donor site in patients who are undergoing ACI treatment for lesions elsewhere in the joint [23]. Repair of experimentally-induced partial-thickness injuries have also been observed in animal models such as rabbits [24], foetal lambs [25] and pigs [26].

Increasing evidence suggests the importance of mesenchymal stem cells (MSCs) as a source of repair cells. Indeed, in microfracture the repair is largely mediated by the proliferation and differentiation of bone marrow derived MSCs [27]. MSCs have been identified in synovial fluid (SF) [28-30] which are increased 7-fold in arthritic patients compared to normal controls suggesting that synovial fluid MSCs may play a role in attempted homeostatic repair. Furthermore, a recent study by McGonagle *et al* suggested that knee joint distraction promoted intrinsic repair of cartilage by favouring adhesion of synovial fluid derived MSCs to the cartilage surface [31]. There is also evidence of intrinsic repair in young animals mediated by MSCs. Using a rabbit model of partial thickness injury, Hunziker *et at* found that topical application of growth factors such as FGF2 or TGFβ1 stimulated MSC migration from the synovial membrane and subsynovial space into the defect void [32]. Similarly, it was suggested that spontaneous healing of full-thickness injuries seen in DBA/1 mice strain was promoted by mesenchymal progenitor cells populating the wound [33]. This paper was particularly interesting because it shows that repair is not only strain dependent (genetic influence) but also age dependent (older mice failed to repair).

Therefore, the purpose of this study was to investigate if MSCs in the joint tissues respond to articular cartilage injury and are involved in potential repair mechanisms. If successful, this knowledge can be used to harness the power of the intrinsic repair mechanism used by the cartilage to develop therapeutic strategies for promoting cartilage regeneration.

Chapter 2

Literature review

2.1 Synovial joints

Synovial joints are complex biomechanical structures which makes pain-free movement possible. They are formed by multiple tissues including joint capsule, ligaments, menisci, synovium, hyaline articular cartilage and the subchondral bone (Figure 2.1). The tissue that confers the most to the exceptional functional capacities of the synovial joints is the articular cartilage covering the surfaces of the articulating bones. Articular cartilage serves two main functions; it act as a 'biological shock absorber' so to limit the stress applied to the bone extremities and provide a smooth low friction surface which is necessary for efficient joint function and pain-free locomotion [34]. Even though the cartilage consists of the same components in all synovial joints and mediates the same function, it varies in thickness, mechanical properties, cell density and matrix composition within the same joint, among different joints in the body and among the same joints in different species [35]. Although, its thickness is at most only a few millimetres, it has extraordinary stiffness to resilience and compression and extraordinary ability for load distribution which reduces stress on the underlying subchondral bone. Furthermore, under normal physiological conditions it has exceptional durability and can mediate normal joint functions for over 80 years or longer.

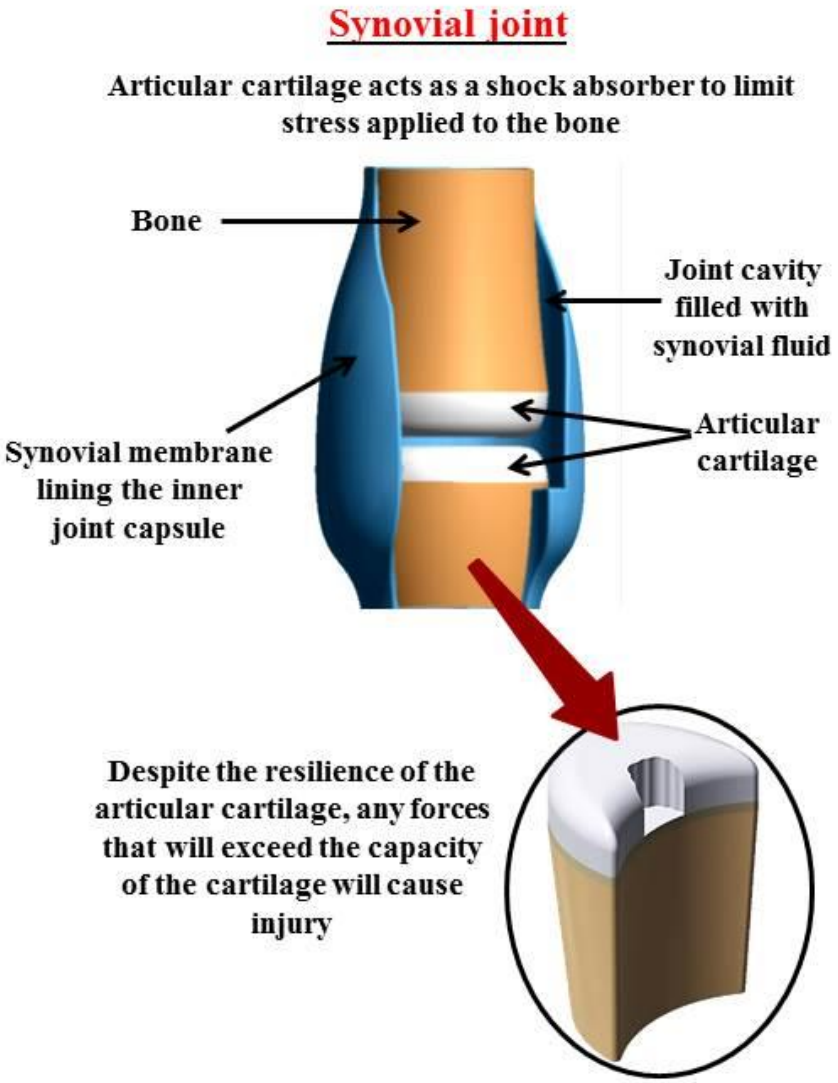


Figure 2.1: Schematic of a synovial joint. Articular cartilage is found in between two articulating bones where it acts as a shock absorber, allowing bones to move on top of each other with low frictional resistance and thereby enables a smooth, painless joint movement.

2.2 Articular cartilage composition

Articular cartilage appears to be a simple inert tissue upon gross examination. Light microscopic examination shows that the cartilage primarily consist of extracellular matrix (ECM) with sparsely distributed cells and devoid of blood vessels, lymphatics and nerves [36].

2.2.1 Extracellular matrix

The principle components of the cartilage ECM are tissue fluid and the structural macromolecules which provide the tissue with its characteristic form and stability. Furthermore, it is the interaction between the tissue fluid and the macromolecules that gives the cartilage its mechanical stiffness and resilience. 65-80% of cartilage is composed of water which is important for the load-dependent cartilage deformation. Other than water, tissue fluid also contains small proteins, gases, metabolites and a high concentration of positively charged ions such as sodium (Na^+; 250-350 mM), potassium, (K^+; 8-10 mM) and calcium (Ca^{2+}; up to 20 mM) to balance the negatively charged proteoglycans [37]. The behaviour and volume of water within the cartilage is mainly dependent on its interaction with the local proteoglycans which also helps to maintain the osmolarity of the cartilage [35, 37].

The structural macromolecules of the cartilage are proteoglycans, collagens, non-collagenous proteins and glycoproteins [35, 37]. Proteoglycans consist of a protein core with covalently attached chains of glycosaminoglycans (GAGs). GAGs are long chain heteropolysaccharides formed from repeating disaccharide units. The disaccharide unit is formed by glycosidic bond between a hexosamine and a non-nitrogenous sugar molecule. Different types of GAG found in the cartilage include chondroitin sulphate, hyaluronic acid, keratan sulphate and dermatan sulphate. The concentration of GAG molecules varies between different locations within the cartilage and can also be altered with age, injury and in disease states.

Cartilage proteoglycans are further divided into two major classes: large aggregating molecules called aggrecans and smaller proteoglycans including fibromodulin, biglycan and decorin. Furthermore, type IX collagen is also

sometimes considered a proteoglycan due to its GAG component [35]. Aggrecans consist of high numbers of keratan sulphate and chondroitin sulphate chains covalently linked to the protein core. 90% of the total proteoglycan mass is formed by aggrecans which fill the interfibrillar space within the cartilage matrix. The remaining 10% is formed by the large non-aggregating proteoglycans.

The aggregan molecules non-covalently interact with hyaluronic acid (hyaluronan) to form proteoglycan aggregates. These interactions are critical for stabilising proteoglycans within the ECM and to prevent the proteoglycans from being displaced by cartilage deformation during movement. Furthermore, these interactions are also important for allowing interaction between the collagen network and the proteoglycans. The smaller proteoglycans do not directly contribute to the mechanical properties of the cartilage. Instead, they influence the functions of chondrocytes and bind to macromolecules within the cartilage. For example, fibromodulin and decorin binds with type II collagen and this interaction may play a role in stabilisation of the collagen meshwork. Similarly, biglycan which is highly concentrated within the pericellular matrix also binds to type VI collagen. Furthermore, these small proteoglycans may also bind to growth factors and cytokines and allow these factors to sequester in the matrix.

Cartilage matrix also contains multiple types of genetically distinct collagen. These include type II, VI, IX, X and XI. Type II is the major collagen type in the cartilage. It is responsible for the tensile strength of the tissue and confines the degree of swelling of aggrecans to allow the tissue to develop swelling pressure. This swelling pressure is essential to enable the cartilage to withstand compressive loading [37, 38]. Type VI collagen is found in the pericellular matrix, surrounding the chondrocytes where they help chondrocytes to attach to the matrix. Type X collagen is found in the hypertrophic zone of the growth plate and close to the cells that forms the calcified zone. This suggests that type X collagen might have a role in the cartilage mineralisation.

Matrix component of the articular cartilage varies based on the topography of the tissue. For example, the superficial layer of the cartilage contains the highest amount of collagen which gradually decreases with the cartilage depth. In contrast, keratan and chondroitin sulphate are highest in the deeper layers of the cartilage and are lowest towards the outermost layers [38].

2.2.2 Zones

The smallest unit of the articular cartilage is called a chondron. It consists of a singular chondrocyte linked to the pericellular glycocalyx. This pericellular matrix is in turn surrounded by a fibrillary pericellular capsule [39]. The pericellular capsule is surrounded by the territorial matrix which is in turn surrounded by the interterritorial compartments going from near to the chondrocytes to further removed from the chondrocytes. Articular cartilage contains three unmineralised zones, although a clear boundary among the different zones cannot always be precisely defined; nevertheless, the organisational differences among the zones are important for the cartilage function. These zones are however, sharply delineated against the fourth zone called the calcified zone, by the tidemark. The three unmineralised zone are: superficial, middle, and deep [35, 36, 39]. Calcified zone is separated from the bone along the osteochondral junction by an interface that has staining properties similar to cement lines in bones [38].

The superficial zone is the thinnest and the outermost layer of the articular cartilage and is composed of two layers. The outer layer is acellular and formed by thin fibrils with little polysaccharides. The second layer contains flattened dics-shaped chondrocytes that are arranged with the long axis parallel to the articular surface [35]. The matrix of the superficial zone contains a high concentration of collagen, fibronectin and water and low concentration of proteoglycans compared to the other zones. The dense meshwork formed by the high collagen concentration at the superficial zone is important for determining the mechanical properties of the cartilage and exchange of molecules between the cartilage and the surrounding fluid. For example, one of the first changes observed following experimental induction of OA is the disruption and remodelling of the collagen network in the superficial zone.

This suggests that the changes in the biocmechanical properties and permeability of the tissue arising from the changes in the superficial zone might play an important role in the development of OA. Moreover, the tangential orientation also allows this zone to act as a filter for cell nutrients from the synovial fluid and as a barrier to tissue damage from the larger molecules of the immune system. In the middle zone, chondrocytes show rounded or spherical cellular profiles and are randomly distributed in a matrix with high proteoglycan content. The collagen

fibres decussate in oblique directions to provide a transitional network which is an intermediate between the superficial zone and the radial deep zone. Within the deeper zone, the cells increase further in size, where the rounded chondrocytes are arranged in columns surrounded by matrix which has highest proteoglycan content, lowest water content and largest diameter collagen fibres relative to the other zones [38, 39].

2.2.3 Chondrocytes

Cartilage contains only one type of cell called the chondrocyte which occupy 1-10% of the matrix volume and are responsible for the production, organisation and maintenance of the ECM [37, 40]. The chondrocytes are remarkable cells; they can maintain the ECM and produce unique ECM components far away from the nutritional sources or any clear control mechanisms. All chondrocytes have a deeply crenated contour which increases the surface area : volume ratio but have variations in distribution, size and shape depending on the area of the tissue. Chondrocyte of the adult cartilage have a low respiratory activity and show both regional and local diversity in rates of synthesis and types of secretory products they produce. Chondrocytes are sparsely spread within the matrix and the cells in their lacunae are separated from one another by variable amount of matrix, lying either singly or in small clusters [37].

Due to the lack of precise boundaries between each zone, it is difficult to define cell densities at each zone. However, cell density decreases going from the outermost layer to the deeper layers of the cartilage [41]. Histological assessment suggests that the highest cell density is found in the superficial zone where these cells might act as a metabolic centre to regulate the environment of the entire tissue. Ultrastructural and autoradiographic analysis suggests that even though all chondrocytes are involved in production of the ECM molecules, some cells containing higher endoplasmic reticulum (ER) primarily synthesise collagen whereas glycogen-rich chondrocytes mainly synthesise proteoglycans [42].

2.2.4 Extracellular matrix receptors

Interaction between chondrocytes and the ECM is important for chondrocyte function and metabolism and these interactions are partly mediated by a family of matrix receptors called the integrins [43]. Integrins are transmembrane receptors made of an α subunit and a β subunit. To date, 15α and 8β subunit have been reported whose variations in pairing creates a multitude of integrin receptors. Whereas, some integrin receptors specifically bind to some matrix molecules, other integrins are promiscuous in their matrix protein interactions. Binding of the ligands to the integrin molecules either increases the affinity of the receptor for other ligands or stimulates chondrocyte activation.

Chondrocytes express a number of integrin receptors which binds to collagen type II and VI (α1β1, α2β1, α11β1), fibronectin (α5β1) osteopontin and vitronectin (αVβ1) and laminin (α6β1). Interaction of the chondrocytes with the collagen via the integrin receptors have been shown to be critical for chondrocyte survival. Integrins are also found in the matrix itself where they help to organise the matrix and take part in the matrix development. Other important matrix receptors include the hyaluronan receptor CD44 which also affects chondrocyte functions and cartilage tissue homeostasis.

2.2.5 Cartilage matrix and soluble mediators

Cartilage binds, store and releases soluble mediators such as growth factors and cytokines which are important for both regulating cellular behaviour and maintaining tissue homeostasis [43]. One soluble mediator found abundantly in the cartilage matrix is TGFβ which is produced in a latent form by the chondrocytes and stored in the matrix in this form. Small proteoglycans such as decorin, fibromodulin and biglycan regulates the activity of TGFβ by sequestering the growth factor within the matrix [44]. Another important growth factor is FGF2 which binds tightly to heparin forming a heparin-growth factor complex [45]. Formation of this complex is important for the activity of the growth factor as this complex protects FGF2 from being degraded. Furthermore, binding to heparin might also increase the activity of the growth factor by preventing its sequestration in the matrix [45]. The physiological significance of binding soluble mediators by the matrix appears to be that it establishes a reservoir of molecules

in the matrix to be either used by the chondrocytes or released following mechanical injury as a signal to indicate that damage has occurred [46]. Furthermore, it can also partially explain the differentiation-inducing and growth-promoting effects of the matrix.

2.3 Osteoarthritis

Osteoarthritis (OA) also known as degenerative joint disease is characterised by gradual loss of cartilage structure and function accompanied by alterations in the subchondral bone and limited intra-articular inflammation and synovitis [4]. Along with cartilage degeneration and structural alterations of the synovial joint, OA also causes joint deformity, limited joint movement and pain which in turn lead to restricted motion and chronic disability. OA can affect any synovial joint in the body including knee, hip, spine, foot, hand and the temporomandibular joint. Traumatic joint injuries, aging, genetic defects and obesity are all risk factors for development of OA [4].

2.3.1 Effect of traumatic cartilage injuries on development of OA

Despite the resilience of the articular cartilage, any forces that will exceed the capacity of the cartilage to maintain tissue homeostasis and structural integrity will cause injury to cartilage resulting in lesions in the cartilage structure [36]. Such cartilage lesions can be caused as a result of direct or indirect trauma (e.g. intraarticular fracture and high-intensity impact) or mechanical injuries during surgical management of the tissue [47]. Cartilage lesions/defects are classified according to the depth of the defect, as either osteochondral (involving the cartilage and underlying bone) or chondral in which only the cartilage is affected [40]. Articular cartilage has poor intrinsic tissue repair capacity. The degree of intrinsic repair depends on whether the lesion is confined within the cartilage layer (partial thickness defects) or extends through the cartilage and through the subchondral bone into vascular BM (osteochondral and full-thickness chondral defects).

Partial thickness defects have a very poor healing capacity with little endogenous cartilage tissue formation. Furthermore, using a goat defect model it was

demonstrated that larger osteochondral injuries extending to greater than 6mm in diameter also rarely heals and leads to progressive cartilage degeneration around the injury [48]. Full thickness defects which reach the underlying subchondral bone can undergo a reparative process in which the defect becomes filled by a fibrocartilage and not hyaline cartilage [48-50]. Using a rabbit model of full-thickness injury it was demonstrated that following creation of injury, the newly synthesised repair tissue was fibrocartilaginous. Moreover, even though at 12 weeks, under light microscopy, the repaired tissue looked stably integrated with the existing tissue, by 48 weeks the connection had ruptured leading to cartilage degeneration in almost every test animal [27]. Hence, the new tissue cannot withstand load-bearing functions. Under such conditions there is an increase in load transfer and changes in the pattern of load distribution which can lead to development of OA.

The cellular response to biomechanical alterations includes upregulation of the metabolic activity or increase in the production of inflammatory cytokines. Studies investigating the effect of injurious mechanical loading reported that inappropriate static compression induces proteoglycan depletion, collagen meshwork damage, reduces the production of cartilage matrix proteins and increases the synthesis of proteinases that degrade the cartilage [51]. Furthermore, following matrix degradation by proteinases, resulting products positively feedback to upregulate the cellular events causing further cartilage damage.

2.4 Clinical procedures for cartilage repair

In the UK, over 10,000 cartilage injuries occur annually which warrant repair, of which around 40% are predicted to lead onto the development of OA [2]. Moreover, even though the primary clinical symptom of OA is pain, the structural degeneration of the synovial joint which characterises the disease is the major contributor to the impaired quality of life and disability of the patient. Therefore, joint repair and restoration is important to improve patients' quality of life both before and after the development of OA. Major challenges involved in repair of cartilage include restoration of the 3D ECM structure and integration of the repaired tissue with the original tissue [4].

Current clinical interventions in place for the repair of the injured cartilage include joint washout, tissue debridement and microfracture technique to release the mesenchymal stem cells (MSCs) from the underlying BM. These procedures provide symptomatic relieve from pain but the repair tissue is fibrocartilaginous in nature which is unable to withstand compressive loading over time and therefore will degenerate. Ultimately, patients may need to replace the arthritic joint with a prosthetic joint to restore joint mobility. Joint replacement surgeries have a high success rate with over 150,000 primary knee and hip replacements carried out per year in England and Wales [2]. However, joint prostheses have a limited lifespan (20-25 years) and around 5% of the patients suffer prosthesis loosening which requires further revision surgery. Loosening is particularly problematic in younger patients under the age of 60. Since, a limited number of revision surgeries are possible per patients, those patients who can no longer undergo revision surgeries will be wheel chair bound. In the UK, over 92000 knee replacement surgeries (on average £10000/surgery) and 15000 revision surgeries are done per year [2]. Therefore, there is both a clinical and societal need to prolong the life of the natural joint.

New techniques have been developed over the past decade to improve the integrity of the repair tissue. These include cell grafting using either autogenous or allogenous chondrocytes (autologous chondrocyte implantation; ACI) or MSCs at the focal injuries to enhance repair by providing added number of cells [50, 52-54] (Figure 2.2). Whereas allografts have the risk of transmission of infectious diseases and host immune reaction, autogenous re-implantation have disadvantages relating to cost, multiple surgical procedures, donor site morbidity and chondrocyte de-differentiation during *in vitro* culture. Further, studies assessing the success of ACI treatments have reported repair tissue of varying characteristics; one study reported 78% patients showed fibrocartilaginous repair [15]. Similarly, using an equine femoropatellar joint model it was demonstrated that MACI (matrix assisted chondrocyte implantation) only improved the early healing response and not the long-term repair [55]. Nevertheless, these cell-based therapies do show that regeneration of hyaline cartilage is possible.

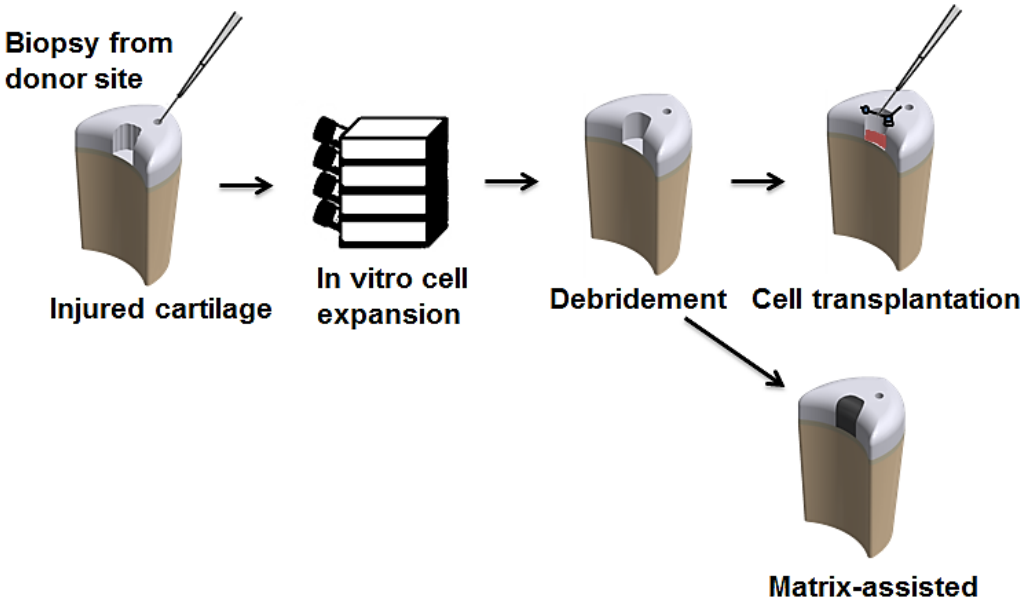


Figure 2.2: Principles of autologous chondrocyte transplantation (ACI) and matrix assisted chondrocyte implantation (MACI). This novel therapeutic approach involves taking a biopsy from a donor site, growing the chondrocytes *in vitro* and transplanting them back into the injured region either directly or through assistance of a matrix (MACI).

Increasing evidence suggests the importance of MSCs as a source of repair cells in wound healing and inflammatory diseases. MSCs are pluripotent precursor cells of the connective tissues such as bone, muscle, cartilage, tendon and fat. Microfracture technique where the repair is mediated completely by the proliferation and differentiation of BM MSCs [27] demonstrates the importance of BM MSCs as a source of repair cells for cartilage injury. More recently, MSCs have been identified in synovial fluid (SF) [28-30] which increased 7-fold in arthritic patients compared to normal patients. This suggests that SF MSCs may also play a role in homeostatic repair during cartilage diseases.

Even though classically, cartilage is seen as a poorly repairing tissue, more recent evidence suggested that cartilage injuries in very young animals can repair. For example, application of FGF2 promoted cartilage repair in partial-thickness injuries in immature rabbits [24] and spontaneous repair of superficial cartilage injuries were also observed in foetal lambs [25]. It is possible that these repairs

were mediated by SF and BM MSCs. The exact mechanism of *in situ* tissue regeneration by endogenous MSC migration and recruitment is still unknown.

2.5 Mesenchymal stem cells (MSCs)

In 1970 it was demonstrated that BM contains a rare population of plastic-adherent cells along with non-adherent hematopoietic cells. The plastic-adherent cells formed colonies derived from single cells and proliferated into fibroblastoid cells when cultured in media supplemented with 10% foetal calf serum (FCS). This observation let to the coining of the term Colony Forming Unit- fibroblasts (CFU-F). These fibroblast forming colonies differed little in morphology depending on the tissue of origin but differed in their properties. When the BM derived adherent cells were transplanted back *in vivo*, they formed bone tissue whereas under the same conditions adherent-cells from spleen cultures formed reticular tissues. These experiments led to the suggestion that fibroblasts from the cultures created a specific microenvironment which is characteristic of the corresponding hematopoietic organ. They were also pioneers in demonstrating that the heterogeneous adherent-cells could proliferate and differentiate into mature cells of mesenchymal lineages such as chondrocytes or osteoblasts [56-58].

Further phenotypic characterisation and proliferation studies established that culture expanded CFU-F retain their full developmental potency to differentiate into chondrocytes, adipocytes, osteoblasts and even myoblasts. Frequency of CFU-F varied between BM donors and was age dependent along with how healthy the donors were. Caplan referred to these cells as MSCs because they have the capacity to differentiate into variety of non-hematopoietic cells of the mesenchymal lineages [59]. He further defined the fundamental aspects of multilineage pathways in the mesengenic process. Since, MSCs originate from the BM supporting structures and may be used as a feeder layer for haematopoietic stem cells (HSC) cultures; MSCs have also been referred as marrow stromal cells.

The precise definition of MSCs, whether to consider them as progenitor cells with multipotentiality or true stem cells is still under debate. Currently, they are defined as ‘plastic-adherent cell population that can be directed to differentiate *in*

vitro into cells of osteogenic, chondrogenic, adipogenic, myogenic and other lineages'[60]. Since, MSCs can divide to form new cells with the same phenotype and gene expression pattern, thereby maintaining their stemness, the ability to self-renew and differentiate are the two characteristics that defined MSCs as stem cells. This definition of MSCs, however, is only based on the *in vitro* analysis of culture expanded MSCs. And no clear descriptions have been reported to date of the characteristics shown by non-cultured MSCs. The role and location of MSCs in their native tissues have also not been identified. This is because unlike HSCs that can be isolated based on their surface marker (CD34), MSCs lack a specific surface marker for unambiguous identification [61].

Human BM MSCs have been isolated using the CD105 surface marker (endoglin). Such approach allowed characterisation of freshly isolated BM MSCs. However, few studies have suggested that phenotypic alterations during *in vitro* culture where MSCs do not express some surface markers and express new ones [62]. For example, it was reported that freshly isolated BM MSCs expressed CD31 and CD45 whose expression reduced following culture expansion, suggesting differences in abilities and phenotypes between *in vivo* and *in vitro* MSCs. Alteration of their natural microenvironment and exposure to chemical and physical growth conditions may play a role in the differences observed. Growing interest in MSC-based cell therapies demand identification of MSCs in a definitive way for clinical and regulatory requirements. In general,, cultured MSCs are characterised either based on their cell surface marker or validated based on their multipotency. In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society of Cellular Therapy proposed that the minimal criteria for MSC identification are: 1. Plastic-adherence under standard culture conditions and formation of CFU-U; 2. Expression of CD105, CD73 and CD90 surface markers and absence of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules as assessed by FACS analysis; 3. Multipotency [63].

MSCs have been isolated from several species which includes cows, mice and humans and several adult tissues [30]. These tissues include the BM, synovial membrane [64], SF [29], articular cartilage, adipose tissue [65] and peripheral blood. These cells show marked differences in their biological properties such as

phenotype, proliferative capacity and differentiation potential along with different growth abilities and expression profile of common surface markers based on their tissue of origin.

MSCs need to be isolated from tissues and their multipotency validated before their use in experimental work. Adherent cells of hematopoietic origin present during the first few days of *in vitro* culture poses a challenge in isolating MSCs based on their plastic adherence. One way to bypass this problem is to isolate MSCs based on their cell surface markers (immunoisolation). This could be either positive selection where MSCs are selected [62] or negative selection [66] where other cells types such as HSCs are removed. However, this is also difficult due to lack of specific MSC markers and the *in vitro* phenotypic plasticity demonstrated by MSCs. CD271 (LNGFR) commonly expressed by BM MSCs and CD49a along with others reported by International Society for Cell Therapy are currently used to define an MSC [67]. Studies are ongoing to identify marker sets for both positive and negative selection which would allow MSC enrichment and ideally purification from native tissues for consistent and clinically required potency (a pre-requirement for developing standardised, GMP-compliant clinically applicable cell-therapy).

Stem cells are characterised by their multipotency. Previous studies reported that MSC can differentiate into osteoblasts and chondrocytes depending on the surrounding environment. For example, differentiation into osteoblasts required close proximity to vasculature whereas chondrocytes did not have this requirement [57, 59]. Chondrogenic differentiation was dependent on the coordination between two fundamental parameters: growth factors and cell density. Therefore, chondrogenic differentiation can only be performed in three-dimensional (3D) culture systems such as pellet cultures or micromasses. During differentiation, MSCs secreted ECM component of the hyaline cartilage including proteoglycans, aggrecans and type II collagen. Glucocorticoids such as dexamethasone played an important role in this by stimulating collagen II upregulation and inducing the matrix components dermatopontin, collagen XI and aggrecan. Induction was detected by identifying cells which expressed cartilage-specific proteins such as GAGs using alcian blue staining or using antibody for matrix markers aggrecan (ACAN) and collagen 2A1 (COL2A1) [68, 69].

Similar to chondrogenesis, *in vitro* differentiation of MSCs down an osteogenic lineage requires multiple soluble inducers. Dexamethasone is an essential component and its continual supplementation is required, but the mechanism by which it promoted osteogenesis is poorly understood. Ascorbic acid enhances collagen production and β-glycerolphosphate provides favourable conditions for culture mineralisation. This mineralisation can be detected at the cell membrane as calcium deposition. The enzyme alkaline phosphatase is essential for ECM mineralisation and is a marker of an osteoblastic phenotype and therefore detection of alkaline phosphatase can also be used for identifying osteogenesis [69, 70]. In adipogenesis, differentiated cells accumulate lipid-rich vacuoles and express markers which are consistent with adipocytes. These markers include perioxisome proliferation-activated receptor-γ2 (PPARγ2), fatty acid binding protein ap2 and lipoprotein lipase. Therefore, adipogenic differentiation of MSCs can be identified by detecting the appearance of lipid vacuoles inside the cells using Oil red O staining [69, 71].

MSC differentiation is mainly dependent on two molecular pathways: first intracellular pathway is that utilised by the TGFβ family and the second pathway is the canonical Wnt I pathway. Wnt I are soluble glycoproteins that bind to receptor complexes composed of Lrp5/6 proteins and stimulate intracellular cascades which mediates cell proliferation and differentiation. The canonical Wnt signalling promotes osteoblast proliferation and supresses chondrocyte formation via β-catenin [72]. The TGFβ pathway on the other hand regulates MSC differentiation into chondrocytes by upregulating chondrocyte specific genes including ERK1/2 (extracellular-signal regulated kinase), SMAD proteins, MAPK (mitogen activated protein kinase), p38 and JNK. Several other regulatory molecules including EGF (epidermal growth factor), PDGF (platelet derived growth factor) and FGF (fibroblast growth factor) have been reported to regulate MSC differentiation, probably through their interaction with the Wnt and TGFβ signalling pathways [73].

2.6 Factors regulating MSC migration

Previous study has shown that MSCs express a number of chemokine receptors namely CXCR1, CXCR2, CXCR4, CXCR5, CXCR6, CCR1, CCR2, CCR7 and CCR9 [74] which are a class of receptors shown to be involved in homeostatic

trafficking of leukocytes. The same study also showed that chemokines corresponding to these respective surface receptors could induce migration of BM MSC in transwell assays. Further, addition of CXCL12 in particular induced F-actin polymerisation into organised filamentous stress fibres associated with cell mobilisation [75]. These findings support the hypothesis that chemokines, particularly CXCL12 may play a role in BM MSC migration. Another study investigating the effect of cytokines on MSC migration to damaged tissues compared 26 growth factors and cytokines using human and rabbit MSCs in microchemotaxis chamber. They reported that platelet-derived growth factor (PDGF-BB and PDGF-AB), TGF-α, insulin growth factor (IGF-I), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF-2) in particular reproducibly induced MSC migration [76]. Further, since combination of these growth factors enhanced migration, it was suggested that maybe combination of growth factors is important to elicit optimum chemotactic migration. Other studies have reported metalloproteinases such as MMP-2 and MMP membrane type I (MT1-MMP) and tissue inhibitor of MMP-1 (TIMP-1) to be involved in MSC migration. Inflammatory cytokines including monocyte chemotactic protein 1 and 3 (MCP-1 and MCP-3) have also been suggested by the same study to affect MSC migration.

Furthermore, extracellular nucleotides such as adenosine triphosphates (ATP) and uridine triphosphates (UTP) have recently been characterised as important chemotactic factor for hematopoietic stem cell (HSCs) functions. For example, activation of purinoceptors by ATP and UTP stimulates HSC migration in synergy with other cytokines. Moreover, in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, preincubation with UTP expanded the number of marrow-repopulating HSCs [77].

Previous studies have shown that human BM derived MSCs express almost all P2R subtypes identified to date with $P2X_4R$ showing the highest expression. The role of this subtype outside the nervous system is unknown. In the nervous system it is thought to be one of the main mediators of neuropathic pain and is upregulated in the spinal cord following injury. Human BM MSCs also expressed $p2X_7R$ which acts as a cytotoxic receptor in different cell types. However, ATP did not have any cytotoxic effect on BM MSCs as seen from the lack of release of cell death markers and changes in mitochondrial network following incubation

with ATP. ATP-treated MSCs however, showed decreased expression of cell-cycle related genes and increased expression of growth arrest genes thereby having an inhibitory effect on the cell proliferation. Therefore, it is possible that extracellular nucleotides also play a role in the migration of MSCs [78].

2.7 Migration assays

The term migration is used to define any directed cell movement in the body, allowing cells to alter their position either between two tissues or within the same tissue [79, 80]. Only a small proportion of highly specialised cells in the body can actively and autonomously migrate. These include tumour cells, leukocytes, sperm cells, fibroblasts and stem cells. Only recently, studies have focused on migration of stem cells since it has been reported that stem cells not only reside in tissues such as the BM but also migrate to the other sites where they take part in functional *in situ* tissue regeneration. A range of assays have already been developed to study cell migration in details, each having its own advantages and disadvantages. Therefore, before choosing a certain type of migration assay, it is first important to consider the aims of the experiment.

Most migration assays are end-point experiments and does not allow investigation of the intermediary stages of the cell migration. These onsets to end-point assays are advantageous for screening studies and not suitable for studies requiring constant observation of cells during the time of the migration. Moreover, some of these assays are catered towards studying directed cell movement (chemotaxis) over undirected random migration (chemokinesis) [81]. In our study, we used three different types of cell migration assays to investigate MSC migration. These are: Transwell assay (to study chemotaxis), scratch assay (to study intermediate stages of migration at different time points) and cell exclusion zone assay (to study migration in the absence of cell injury).

2.7.1 Transwell assay

Transwell assay was first developed by Boyden to study leukocyte chemotactic responses. Briefly, there are two wells separated by a porous membrane through which cells can move. The size of the pore is dependent on the cells being studied

to avoid unspecific dropping of the cells. Membranes with different pore sizes ranging from 3 to 12 µm are commercially available. Cells are usually seeded in the top well in serum free medium and can migrate vertically through the pore towards an attractant present in the bottom well. Incubation time to allow cell migration needs to be determined as this can vary between different cell types [81].

Cell migration can be detected and quantified by two different methods. With the first method, cotton swab is used to remove the non-migrated cells from the top side of the membrane and the migrated cells are fixed on the bottom side of the membrane and stained with cytological dyes such as toluidine blue, haematoxylin or crystal violet. Stained cells are then counted to quantify the number of migrated cells. Alternatively, migrated cells can be fluorescently stained, removed from the membrane using trypsin and fluorescence can be quantified using a microplate reader. Light transmission from the non-migrated cells can also be blocked using dark porous membranes which negates the need to remove the migrated cells from the upper side of the membrane. Dark porous membranes (FluoroBlok) are commercially available from Becton Dickson. Given the range of commercially available culture inserts with various pore sizes, the short lasting chemotactic gradient between the two wells and the lack of need for any specialised equipment besides the culture inserts, transwell assays are powerful techniques to study cell migration.

2.7.2 Scratch assay

Scratch assay is based on the principle that upon creation of an artificial gap (wound/scratch) on a confluent cell monolayer, cells respond to the disruption of the cell to cell contact by migrating towards the gap to heal the wound [82]. Such migration not only reflects cell behaviour on individual levels but also the sheet of cells as a surrogate tissue. Wound is generally created using a pipette tip or syringe needle and the assay is performed either on a multi-well tissue culture plate or on a cover slip. The closing of the gap can be observed within 3 to 24 hrs, where cells at the edge of the wound polarise and protrude towards the gap and migrate to close the wound. Migration can be monitored either by imaging wells at certain time points or by time-lapse microscopy. As a result scratch assays can

be used to study various cell behaviours such as matrix remodelling, cell polarisation, cell migration, validation of molecules that affect migration, metastasis and angiogenesis. For example, the role of Rho family GTPases, CDc42, Rho and Rac in cell polarity and actin cytoskeletal regulation have been studied using scratch assays. Further, involvement of p53 in cell migration and orientation of the microtubule organisation centre (MTOC) and the Golgi apparatus have also been studied using scratch assays [81].

2.7.3 Cell exclusion zone assay

An alternative form of scratch assay is cell exclusion zone assay where the wound is created by seeding cells in two separate wells [81]. This produces sharp borders and avoids problems such as unevenly thick gap created by cell scraping and reattachment of the floating cells in the wound area. Further, stoppers increase assay reproducibility as the wound width remains the same with each repeat. This assay also allows study of migratory behaviour and interaction between two different cell types, seeded in separate wells of the stopper. Similar to scratch assays, migration can be observed at various time points using light or time-lapse microscopy. It also allows quantification of migration using microplate reader if the cells are fluorescently labelled prior to seeding. This is done by masking the region where the cells are seeded and detecting the cells once they leave the masked region and into the gap. Cells are detected as fluorescent signals which are then quantified using the microplate reader. Schematics of this technique can be found in the Materials and Methods sections on pages 68-69 (Figure 3.12 – 3.13).

Aim

The overall aims of this study were to:

1. Investigate the potential role of MSCs in the joint tissues in the healing of cartilage defects when articular cartilage is damaged by trauma.

2. Investigate the potential migration and repair mechanisms used by MSCs to regenerate hyaline cartilage lesions caused by trauma.

Objectives

1. To establish protocol for *in vitro* isolation of synovial fluid-derived (SF) and bone marrow-derived (BM) mesenchymal stem cells (MSCs).
2. To establish and optimise *in vitro* migration assays for MSCs.
3. To determine if SF and BM MSCs will migrate to articular cartilage *in vitro* in response to non-injured, experimentally –induced injured and dead cartilage.
4. Investigate whether *in vitro* injured articular cartilage releases agents (i.e. 'injury factors') which promote migration of SF and BM MSCs and characterise any 'injury factors' which are released by articular cartilage injured *in vitro.*
5. Investigate if SF and BM MSCs need to be primed before migration in response to the injury factors.

Chapter 3

Materials and Methods

Materials

Table 3.1: Joint dissection and tissue collection

Materials	Source	Information
Bovine metacarpophalangeal joints	Local abattoir	Healthy and skeletally matured animals aged less than 30 months. Collected within 4h of commercial slaughter
Dissection tray, scalpel, scalpel handle, tweezers and spatula	Scientific Laboratory Supplies, UK	No. 11 and 22 scalpel blades attached to no.3 and 4 scalpel handles respectively
Post mortem knife	Swann Morton, Sheffield, UK	No.44 blade
Aluminium foil	Sainsbury, UK	Standard kitchen foil
Trigene	Scientific Laboratory Supplies, Nottingham, UK	Disinfectant
70% Industrial methylated spirit (IMS)	Genta Medical, York, UK	Diluted 7 parts IMS with 3 parts distilled water
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich, Poole, UK	Without calcium chloride and magnesium chloride.
50 ml universal tubes	BD Biosciences, Oxford, UK	
Pasteur pipette	Fisher Scientific Loughborough, UK	
RJ162VK reciprocating saw	Ryobi, UK	
Petri dish	BD Biosciences, Oxford, UK	
Class 2 laminar flow cabinet	Walker Safety Cabinets Ltd, UK	
Sturat mini orbital shaker	Scientific Laboratory Supplies, UK	Used at 29 RPM
Cell strainer (sterile)	BD Biosciences, Oxford, UK	70 μm pore size, nylon mesh
Bacterial collagenase	Sigma-Aldrich, Poole, UK	From clostridium histolyticum type I; 2 mg/ml in complete media. Filter sterilised before use.
Hammer and Chisel	Homebase, UK	
Dremel	Homebase, UK	
Dremel cut-off wheel blades	Homebase, UK	
Biopsy punch	Selles Medical, UK	Disposable

Table 3.2: SF and BM MSC and chondrocyte expansion

Materials	Source	Information
Class 2 laminar flow cabinet	Walker Safety Cabinets Ltd, UK	
Galaxy R Plus CO_2 incubator	Eppendorf, UK	37°C, 5% CO_2, 95% humidity
Dulbecco's modification of eagle's medium	Sigma-Aldrich, Poole, UK	Low glucose (1000 mg/l) and high glucose (4500 mg/l). Both were sodium bicarbonate buffered without L-glutamine and sodium pyruvate
MSC specified foetal bovine serum	Life Technologies Ltd Paisley, UK	Used only for culturing MSCs. Batch no. 12662029
Foetal calf serum	Sigma-Aldrich, Poole, UK	Used for culturing chondrocytes. Batch no. F9665
L-alanyl-Lglutamine	Sigma-Aldrich, Poole, UK	200 mM stock solution. 2 mM used for preparation of cell culture media
Penicillin-Streptomycin	Sigma-Aldrich, Poole, UK	10 000 units/ml penicillin with 10 mg/ml streptomycin stock solution. 10 µl/ml used for preparation of cell culture media
HEPES buffer	Sigma-Aldrich, Poole, UK	100x stock. 10 µl/ml used for preparation of cell culture media
MEM non-essential amino acids	Sigma-Aldrich, Poole, UK	1 M stock. 10 µl/ml used for preparation of cell culture media
Human fibroblast growth factor 2 (FGF2)	PeproTech, London, UK	1 µl/ml stock. Final concentration used in the culture media: 5 µg/ml for MSC culture media and 10 µg/ml for chondrocyte culture media
Syringe filter	Nalgene, Hereford, UK	0.2 µm pore size, cellulose acetate membrane
20 ml Luer syringe	BD Biosciences, Oxford, UK	
T25 and T75 tissue culture flasks	Sarstedt	Yellow capped. Used only for MSC culture
T75 tissue culture flasks	Greiner Bio-One, Stonehouse, UK	Red capped. Used for chondrocyte culure

Table 3.2: SF and BM MSC and chondrocyte expansion

Materials	Source	Information
Trypsin/EDTA	Sigma-Aldrich, Poole, UK	
Centrifuge		
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich, Poole, UK	Without calcium chloride and magnesium chloride.

Table 3.3: Materials used for migration assay

Materials	Source	Information
Corning® Costar® Transwell® culture inserts	Sigma-Aldrich, Poole, UK	8 μm pore size, non-treated membrane, 12 inserts in a 24 well plate
24 well plates	Greiner Bio-One, Stonehouse, UK	
Ibidi culture inserts	Thistle Scientific, UK	Creates 500 μm cell free gap
Oris™ Cell migration assembly Kit- FLEX	Platypus technologies	96-well format
Fibronectin	Sigma-Aldrich, Poole, UK	6.5 μg/ml
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Poole, UK	Crystalline grade, from bovine serum
MSC specified foetal bovine serum	Life Technologies Ltd Paisley, UK	Used only for culturing MSCs. Batch no. 12662029
Formaldehyde	Sigma-Aldrich, Poole, UK	
Crystal Violet	Sigma-Aldrich, Poole , UK	0.1% Crystal violet in distilled water
Distilled water		
Glass slides	Thermo Scientific, USA	Superfrosted
Glass coverslips	VWR International, UK	
DPX slide mounting medium	Fisher Scientific, Loughborough, UK	
Scalpel, scalpel handle, tweezers and spatula	Scientific Laboratory Supplies, UK	No. 11 scalpel blades attached to no.3 scalpel handles

Table 3.4: Materials used for chondrogenic differentiation assay

Materials	Source	Final concentration
Dulbecco's modification of eagle's medium (DMEM)	Sigma-Aldrich, Poole, UK	High glucose (4500 mg/l) with sodium bicarbonate buffer without L-glutamine and sodium pyruvate
TGFβ1	PeproTech, London, UK	10 ng/ml
Dexamethasone	Sigma-Aldrich, Poole, UK	100 nM
Insulin transferrin selenium (ITS)	Sigma-Aldrich, Poole, UK	10 µg/ml insulin + 5.5 µg/ml transferrin + 0.5 µg/ml selenium + 4.7 µg/ml linoleic acid + 4.7 µg/ml oleic acids
Ascorbic acid	Sigma-Aldrich, Poole, UK	50 µg/ml
Alcian blue	Sigma-Aldrich, Poole, UK	1 g Alcian blue dissolved in 100 ml of 3% acetic acid (pH 2.5)
30% hydrogen peroxide	Sigma-Aldrich, Poole, UK	
Tris buffered saline (TBS)	Sigma-Aldrich, Poole, UK	6.1 g/l tris (hydroxymethyl aminomethane, plus 8.1 g/l NaCl and 0.86% (v/v) 5 M HCl in distilled water
TBS/Tween20®	Sigma-Aldrich, Poole, UK	TBS supplemented with 0.05% Tween 20®
Triton X100	Sigma-Aldrich, Poole, UK	
Methanol	Fisher Scientific, Loughborough, UK	
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich, Poole, UK	Without calcium chloride and magnesium chloride.
Hyaluronidase	Sigma-Aldrich, Poole, UK	From bovine testes, 618.4 units/mg activity
Pronase	Sigma-Aldrich, Poole, UK	From Streptomyces griseus, 5.55 units/mg activity
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Poole, UK	Crystalline grade, from bovine serum
BSA blocking solution	Sigma-Aldrich, Poole, UK	PBS +1% BSA+ 1% NHS + 0.05% Triton x100

Table 3.5: Materials used for chondrogenic differentiation assay

Materials	Source	Final concentration
Type II collagen primary antibody	Southern Biotech, UK	Anti-bovine collagen type II, produced in goat. 0.4 mg/ml diluted 1 in 100 parts in TBS/Tween20® buffer
Rabbit anti-goat IgG	Vector Laboratories, UK	
ABC reagents	Vector Laboratories, UK	Avidin biotinylated enzyme complex
DAB kit (diaminobenzidine tetrahydrochloride peroxide substrate)	Vector Laboratories, UK	

Table 3.6: Materials used for osteogenic and adipogenic differentiation assay

Materials	Source	Final concentration
Dulbecco's modification of eagle's medium	Sigma-Aldrich, Poole, UK	Low glucose (1000 mg/l) with sodium bicarbonate buffer without L-glutamine and sodium pyruvate
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Poole, UK	Crystalline grade, from bovine serum
Dexamethasone	Sigma-Aldrich, Poole, UK	10 nM for osteogenesis; 10 μM for adipogenesis
Ascorbic acid	Sigma-Aldrich, Poole, UK	50 μg/ml
B-glycerol phosphate	Sigma-Aldrich, Poole, UK	10 mM
MSC specified foetal bovine serum	Life Technologies Ltd Paisley, UK	Used only for culturing MSCs. Batch no. 12662029
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich, Poole, UK	Without calcium chloride and magnesium chloride.
Methanol	Fisher Scientific, Loughborough, UK	
SIGMA *FAST*™ BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium)	Sigma-Aldrich, Poole, UK	1 tablet in 10 ml distilled water
IBMX	Sigma-Aldrich, Poole, UK	100 μM indomethacin + 0.5 mM 3-isobutyl-1-methyl xanthine
2% Gluteraldehyde		
Oil Red O	Sigma-Aldrich, Poole, UK	0.5 g Oil Red O in 100 ml isopropanol. Sterilised

Table 3.7 Materials used for characterisation of injury conditioned media (IJCM)

	Materials	Source	Final concentration
Growth factors	TGFβ3	PeproTech, London, UK	10 ng/ml
	FGF2	PeproTech, London, UK	5 ng/ml
Cytokines	CCL2	Sigma-Aldrich, Poole, UK	30 ng/ml
	CCL3	Sigma-Aldrich, Poole, UK	50 ng/ml
	CCL5	Sigma-Aldrich, Poole, UK	50 ng/ml
	TNFα	Sigma-Aldrich, Poole, UK	50 ng/ml
	CXCL12	Sigma-Aldrich, Poole, UK	400 ng/ml
Antibodies against cytokines and growth factors	CX3CL1	R&D Systems, UK	1.5 μg/ml
	CXCL12	Sigma-Aldrich, Poole, UK	3.6 μg/ml
	TGFβ3	Sigma-Aldrich, Poole, UK	0.3 μg/ml
	FGF2	Sigma-Aldrich, Poole, UK	5 μg/ml
Receptor antagonists	CCL3	Sigma-Aldrich, Poole, UK	10 μg/ml
	CCL5	Sigma-Aldrich, Poole, UK	7 μg/ml
	AMD3100	Sigma-Aldrich, Poole, UK	6.29 μM
Extracellular nucleotides	ATP	Sigma-Aldrich, Poole, UK	250 μM
	UTP	Sigma-Aldrich, Poole, UK	250 μM
	ATPγS	Sigma-Aldrich, Poole, UK	250 μM
	UTPγS	Sigma-Aldrich, Poole, UK	250 μM
P2 Receptor blocker	Suramin	Tocris Biosciences	1 mM
Heparin-treatment of IJCM	Heparin	Sigma-Aldrich, Poole, UK	50 μg/ml
	Heparin-binding plates	Becton Dickson	96-well format. Wells plasma polymerised with allyl amine

Table 3.7: Materials used for characterisation of injury conditioned media (IJCM)

	Materials	**Source**	**Final concentration**
Protein inhibitor	Cycloheximide	Sigma-Aldrich, Poole, UK	10 μg/ml
Matrix metalloproteinase inhibitor	Marimastat	R&D Systems, UK	10 μg/ml

Table 3.8: Materials used for fluorescently labelling cells

Materials	**Source**	**Final concentration**
Dulbecco's modification of eagle's medium	Sigma-Aldrich, Poole, UK	High glucose (4500 mg/l) with sodium bicarbonate buffer without L-glutamine and sodium pyruvate
CMFDA cell tracker Green^{TM} (5'-chloromethylfluorescein diacetate)	Invitrogen Ltd, Paisley, UK	Dissolved in 20 μl dimethyl sulphoxide (DMSO). Final concentration:10 μM
PI (propidium iodide)	Invitrogen Ltd, Paisley, UK	Final concentration: 10 μM
Zeiss Axioskop LSM510 Confocal laser scanning microscopy	Carl Zeiss AG, Germany	
LSM image browser software	Carl Zeiss AG, Germany	
Formaldehyde	Sigma-Aldrich, Poole, UK	
PKH67 Green Fluorescent Cell Linker Kit	Sigma-Aldrich	

Table 3.9: Materials used for histological assessment of MSC migration

Materials	Source	Information
Mayer's Haematoxylin	Fisher Scientific, Loughborough, UK	
Eosin Y stain	Fisher Scientific, Loughborough, UK	
Automated H&E staining line		
Formaldehyde	Sigma-Aldrich, Poole, UK	
Glass slides	Thermo Scientific, USA	Superfrosted
Glass coverslips	VWR International, UK	
DPX slide mounting medium	Fisher Scientific, Loughborough, UK	
Leica CM3050S cryostat	Leica Biosystems, Milton Keynes, UK	
Optimal cutting temperature sample mounting media (OCT)	VMR International, UK	
20 mm cork	Fisher Scientific, Loughborough, UK	
Aluminium foil	Sainsbury, UK	
Liquid nitrogen		

Table 3.10: Materials used for ATP Bioluminescence assay

Materials	Source	Information
ATP Bioluminescence assay kit HS II	Sigma-Aldrich, Poole, UK	Reagents prepared according to manufacturer's instructions
Infinite® M2100 Microplate reader	Tecan, Switzerland	

Methods

3.1 Media composition for culturing different cell types

Five different cell types were used. Three of them were primary cell types which were isolated from the bovine metacarpophalangeal joints (MCPs). These were chondrocytes, BM and SF MSCs. The osteoblastic MG-63 cell line was purchased from Sigma-Aldrich and human BM MSCs were purchased from PromoCells. Different compositions of media were used for culturing the different cell types to provide optimal culture conditions. Both BM and SF MSCs were cultured in basic medium [Dulbecco's Modification of Eagle's Medium, DMEM (low glucose formulation containing 1000 mg glucose/litre; Sigma-Aldrich, UK) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml; Sigma-Aldrich, UK) and non-essential amino acids (NEAA, Sigma Aldrich, UK).

Unless stated otherwise, all DMEM with a formulation of 1000 mg glucose/litre were supplemented with the above mentioned concentration of penicillin, streptomycin and NEAA and will be known as low glucose DMEM. MSC qualified foetal calf serum (FCS) was batch tested for MSC viability and proliferation and will be known as MSC serum. Unless stated otherwise, all cell lines of MSCs from both bovine and human tissues were cultured in low glucose DMEM supplemented with 10% MSC serum and 5 ng/ml fibroblast growth factor 2 (FGF2) and will be known as MSC complete media.

Chondrocytes and cartilage explants were cultured in basic media [Dulbecco's Modification of Eagle's Medium, DMEM (high glucose formulation containing 4500 mg glucose/litre; Sigma-Aldrich, UK)] supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml; Sigma-Aldrich, UK), 10 mM HEPES buffer pH 7.4 (Sigma-Aldrich, UK) and non-essential amino acids (NEAA, Sigma-Aldrich, UK). Unless stated otherwise, all DMEM with a formulation of 4500 mg glucose/litre were supplemented with the above mentioned concentration of penicillin, streptomycin, HEPES and NEAA and will be known as high glucose DMEM. Foetal bovine serum (FBS) was batch tested for chondrocyte viability and proliferation and will be known as chondrocyte serum. Chondrocytes were cultured in high glucose DMEM supplemented with 10% chondrocyte serum and 10 ng/ml of FGF-2 and this media composition will be called chondrocyte

complete media. MG-63 cells were cultured in basic media in chondrocyte complete media.

3.2 Tissue culture flasks used for different cell lines

Due to differential adherence capacity of the cell lines used, cells were cultured in different tissue culture flasks. Corning® cell culture flasks with canted neck and red vented caps were used for chondrocytes and MG-63 cell lines. Sarstedt cell culture flasks with yellow vented caps were used for all types of MSCs.

3.3 Collection of bovine chondral explants

3.3.1 Source of chondral explants

Metacarpal phalangeal joints (MCP) from cows aged less than 30 months were obtained from a local abattoir within 4 hours of slaughter. Joints were sprayed with TriGene disinfectant, skinned using a P40 blade attached to a post-mortem knife and further disinfected using IMS (industrial methylated spirit). Joints were opened under aseptic condition inside a Class II sterile cabinet. A small incision was made into the joint cavity using a number 22 scalpel blade attached to a number 4 scalpel handle and the SF was aspirated. Joints were opened by reflecting the distal limbs to expose the proximal articular surface. Condylar cartilage was collected either as chondral flakes or as osteochondral explants.

3.3.2 Development of an in vitro cartilage injury model

To study the effect of cartilage injury it was important to first develop an *in vitro* chondral model. The four proximal sesamoid bones of bovine MCP joints were used to create osteochondral explants. In our preliminary experiments a hammer and a chisel were used (Figure 3.1) to harvest the osteochondral explants. This produced curved osteochondral explants with irregular edges which could not be studied due to its inconsistent shape and the issues with reproducibility. A dremel was then used to improve the reproducibility of the collection of osteochondral explants. To do this, at first, the four proximal sesamoid bones were separated from the joint using a Ryobi RJ162VK Reciprocating Saw. The separated bones were immediately wrapped in gauge soaked in phosphate buffered saline (PBS)

solution (PBS) to prevent the cartilage from drying out. Then the osteochondral explants were collected using 8 mm diameter cutting disc attached to a hand dremel.

The cartilage- drill edge was flushed with PBS at 1 min intervals to cool the cutting edge and four explants were collected per MCP joint. The explants were cut to approximately 5 mm in length and washed in DMEM. Each explant was then placed in individual wells in a 24 well plate containing complete chondrocyte media and kept overnight in culture media to verify sterility. To prevent the release of substances from the injured bone in to the media it was first required to excise the cartilage from the osteochondral explants. This method was also not reproducible, producing cartilage explants of various sizes and further injured the cartilage (Figure 3.1).

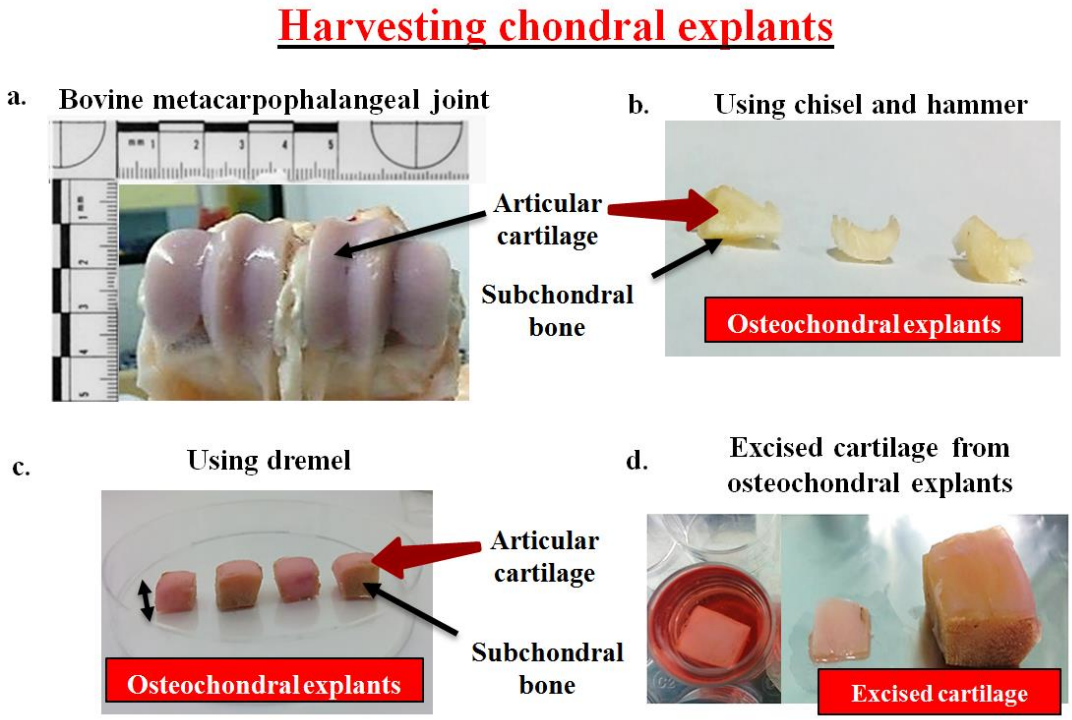


Figure 3.1: Development of a model to harvest chondral explants for studying MSC migration in response to injured cartilage. In our preliminary experiments various tools were tested for collection of chondral explants with consistent thickness and height. **(a)** Bovine metacarpophalangeal joints were opened under sterile conditions but following dissection explants were harvested under non-sterile conditions. **(b)** Chisel produced osteochondral flakes with inconsistent cartilage to bone ratio with curvature in the middle or at the edges. **(c)** Dremel reproducibly produced consistent square osteochondral explants. However, prolonged exposure of cartilage to air and increased temperature at the cutting edge of the saw led to cartilage drying and heat-induced injury respectively. **d)** It was possible to excise the cartilage from the osteochondral explants however; it was difficult to reproducibly excise cartilage with the same thickness and height.

Next the chondral flakes were harvested using no.11 scalpel blades attached to a no.4 scalpel handle. Chondral flakes with irregular edges were collected which were then trimmed to obtain regular, rectangular shaped chondral flakes. However, there was inconsistency in the thickness of the flakes collected and it was not possible to reproducibly collect flakes with the same thickness (Figure 3.2).

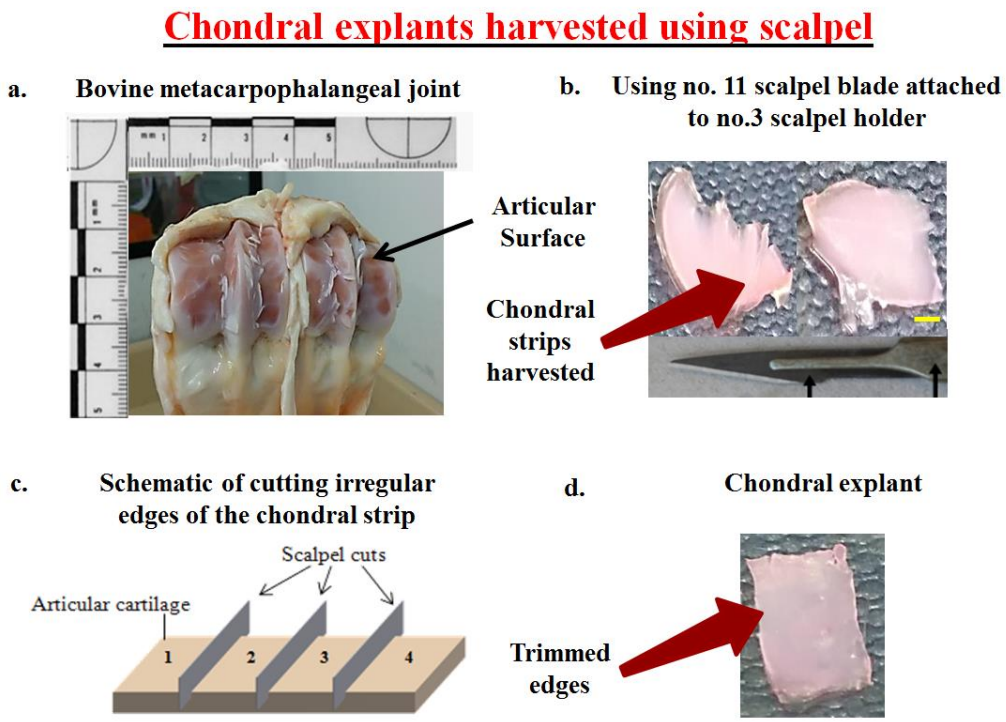


Figure 3.2: Use of scalpel to harvest chondral explants. (a) Bovine metacarpophalangeal joint was opened under sterile conditions. **(b)** Chondral flakes with irregular edges were collected using no. 11 scalpel blades attached to no.4 scalpel handle. Even though blades were frequently replaced to maintain blade sharpness, it was not possible to ensure that all chondral flakes had similar level of blunt and sharp injuries. **(c)** Schematic of cutting irregular edges of the chondral strips using a No.11 scalpel blade to obtain trimmed strips with four straight edges. Each flake was re-cut into four stripes and strip 1 and 4 were discarded. **(d)** Following trimming, chondral explants with four straigh edges were obtained.

A 6 mm biopsy punch was used to reproducibly collect 6 mm × 1.5 mm cartilage discs without attached subchondral bone. The cartilage discs were then 'rested' by incubating in media supplemented with 10^{-7} M dexamethasone for 72 h before being used in experiments (Figure 3.3).

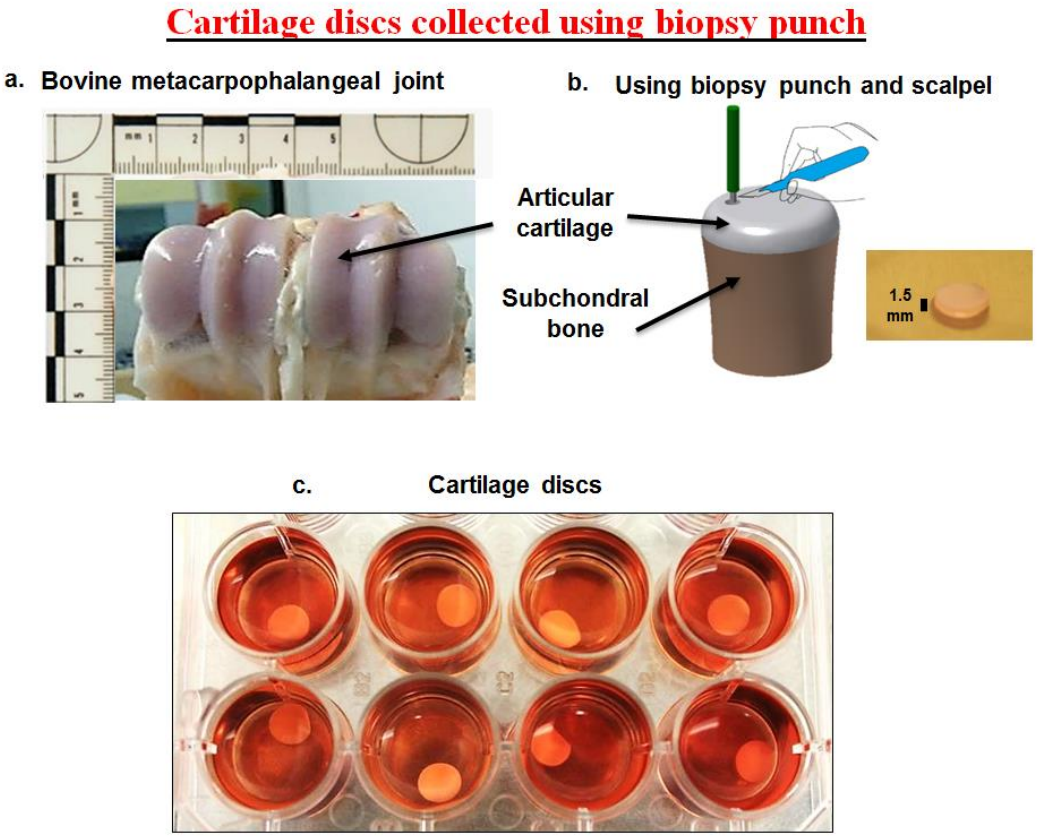


Figure 3.3: Collection of cartilage discs using 6mm biopsy punch. (a) Bovine metacarpophalangeal joints were opened and discs were collected under sterile conditions. **(b)** 6mm biopsy punch was used to punch the cartilage and discs were cut from the joint using no.11 scalpel blade. **(c)** Cartilage discs of 6 mm in diameter and 1.5 mm thickness were consistently obtained by this method.

3.4 Processing of bovine articular cartilage to obtain non-injured, injured and dead cartilage

3.4.1 Non injured cartilage

Full thickness hyaline cartilage with no attached subchondral bone was harvested from the bovine MCP using no. 11 scalpel blade. Initially the chondral strips were processed using the following method. The chondral strips were immediately placed in complete chondrocyte media (refer section 3.1). For experiments using 'non-injured cartilage' cartilage discs were initially maintained in an incubator at 37°C in a humidified 95% air 5% CO_2 for 72 h in complete chondrocyte culture medium to rest the tissue. For later experiments the method of resting the cartilage was changed to 72 h incubation in complete chondrocyte medium containing 10^{-6}m dexamethasone. The 72 h resting period was to allow any areas of the cartilage that were potentially injured during the harvesting procedure to recover to a basal non-injured state.

3.4.2 Injured cartilage

Rested, non-injured cartilage flakes were injured by cutting individual cartilage discs 5 times with no.11 scalpel blade.

3.4.3 Devitalised/dead cartilage

To obtain 'dead cartilage', cartilage discs were treated in initial experiments to 2 cycles of freezing the tissue at -20°C followed by thawing it to ambient temperature. The free-thawed tissue was then stored at -20°C.

In later experiments the devitalising procedure was optimised and freshly isolated cartilage discs were treated with microwave irradiation for 30 sec at power 30 (Figure 3.4). This killed the cartilage instantaneously by denaturing and precipitating out the cartilage proteins. Following irradiation, the cartilage flakes were frozen down at -20°C.

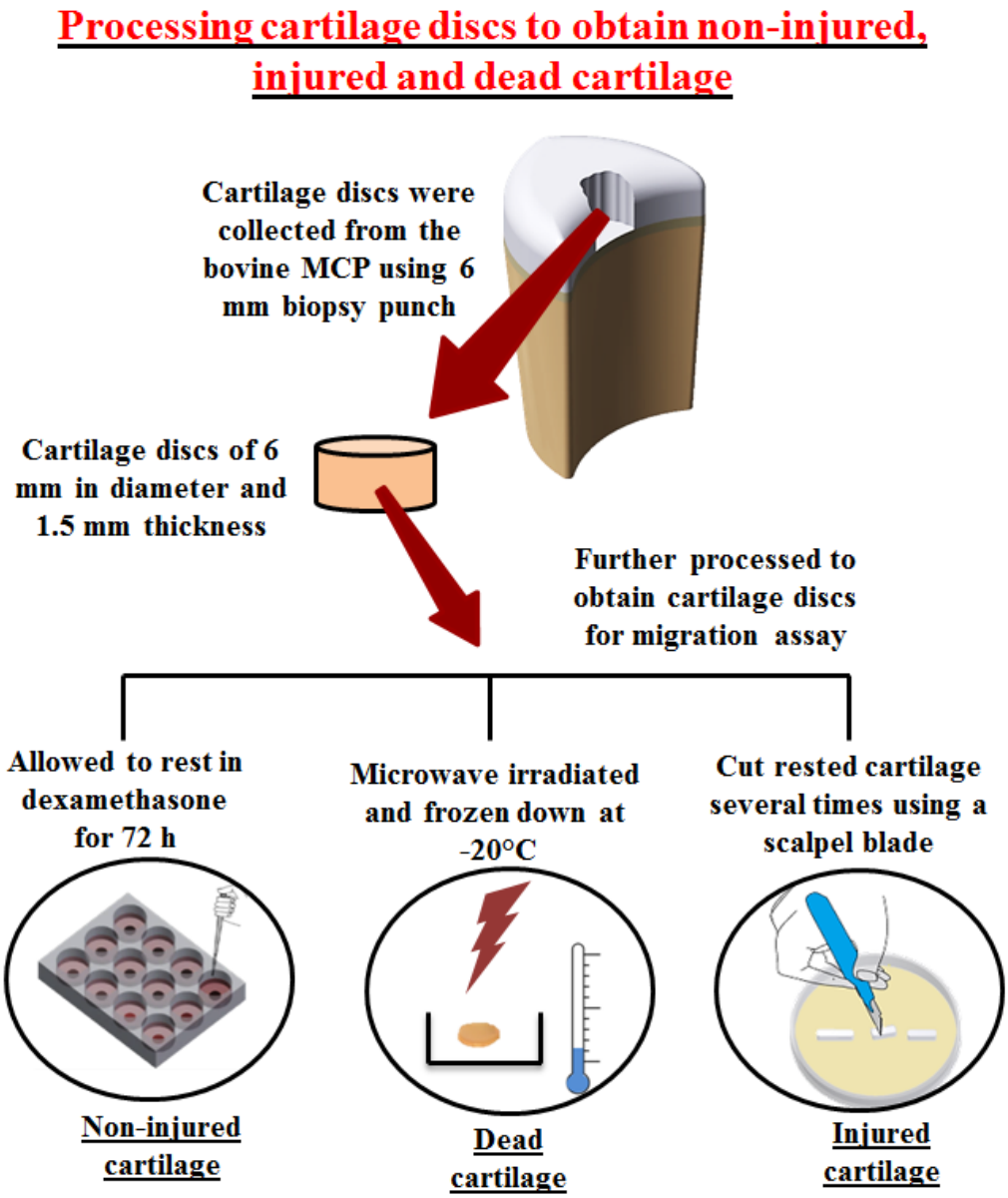


Figure 3.4: Processing cartilage discs to obtain non-injured, injured and dead cartilage. Following collection, cartilage discs were either allowed to rest in DMEM supplemented with chondrocyte serum and dexamethasone to obtain non-injured cartilage or irradiated with microwave radiation and subsequently frozen down at -20°C to obtain dead cartilage. Injured cartilage was obtained by re-cutting rested cartilage several times with scalpel blade.

3.5 Assessing cartilage viability using PrestoBlue®

Viability of 'non-injured', 'injured' and 'dead' cartilage was assessed using PrestoBlue® which is a commercially available preparation of the resazurin dye. Rezazurin enters the living cells and is reduced to resorufin in the mitochondria. In this form resorufin can freely diffuse out of the cells and can be flurometrically detected in the media. Following termination of transwell migration assay to investigate MSC migration to chondral pieces, cartilage samples were moved from the transwell plates to fresh 24 well plates. 900 µl of DMEM* supplemented with 0.1% BSA and 100 µl of PrestoBlue® was added to each cartilage sample. The samples were incubated in the PrestoBlue for an hour and following hour incubation 200 µl of the supernatant from each sample was transferred into a 96-well plate. Data was analysed as fluorescent intensity measured using the microplate reader at an excitation wavelength of 570 nm and emission wavelength of 600 nm.

3.6 Isolation and culture of bovine synovial fluid mesenchymal stem cells (bSF MSCs) and bone marrow mesenchymal stem cells (BM MSCs)

The method of Jones *et al* [29, 62] was used. All tissues were dissected under aseptic conditions. A small incision was made at the skin surrounding the proximal sesamoid bones using a no. 22 scalpel blade attached to a no. 4 scalpel handle. SF was aspirated through the incision using a sterile Pasteur pipette. Approximately 5-10 ml of SF was collected on average per MCP. The fluid was then diluted with PBS at a 1:3 ratio. Diluted fluid was filtered using a cell strainer to remove the synovial membrane debris. Filtered fluid was centrifuged for 10 mins at 300 *g* to isolate the SF MSCs. The supernatant fluid was discarded and the remaining cell pellet was re-suspended in PBS and centrifuged again for 10 mins at 192 *g*. The supernatant was discarded and the cell pellet was re-suspended in 1 ml of complete MSC media. The cell suspension was added into the cell culture flask and further 3 ml of complete media was added to the flask. For a single experiment SFs were collected from five different animals and cultured in separate flasks to reduce the risk of contamination. Cultures of SF-MSCs were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed by changing the media after 4 days. Following the initial 4 days, media was changed every 5-7 days and the cells passaged when 70%

confluent. Following the first expansion after isolation (passage 0), SF MSCs were pooled and cultured till passage 3 before using them for experiments. Therefore, a single experiment was performed using SF MSCs from five different animals.

The femoral cavities of the MCPs were exposed by drilling and the BM was drawn from the femoral marrow cavity using a sterile spatula. BMs were digested in collagenase for 4 h to increase the recovery of the MSCs. Digested BMs were centrifuged for 10 mins at 300 *g*. The supernatant fluid was discarded and the remaining cell pellet was re-suspended in PBS and centrifuged again for 10 mins at 192 *g*. The supernatant was discarded and the cell pellet was re-suspended in 1 ml of complete MSC media and cultured using the same protocol used for SF MSCs. For a single experiment BMs were collected from five different animals and cultured separately to avoid contamination. Following the first expansion after isolation (passage 0), BM MSCs were pooled and cultured till passage 3 before using them for experiments. Therefore, a single experiment was performed using BM MSCs from five different animals.

3.7 Differentiation assays

3.7.1 Chondrogenesis assays

Chondrogenesis was assessed using 3D micromass and pellet cultures.

3.7.1.1 Micromass cultures

To form micromass cultures, MSCs (5×10^5 cells/ 50 µl) were placed as a 50 µl droplet in the middle of the wells in 24 well culture plates and cells were allowed to attach over 24 h. Following incubation, the medium was carefully removed from each well and replaced with serum-free standard chondroinductive medium consisting of high glucose DMEM, 1 mg/ml BSA, 100 nM dexamethasone, 10 µg/ml insulin, 5.5 µg/ml transferrin, 0.5 µg/ml selenium and 4.7 µg/ml each of linoleic and oleic acids (ITS) 50 ug/ml ascorbic acid and 10 ng/ml transforming growth factor-1 (TGFβ-1) [83]. For control experiments, micromasses were incubated in complete MSC media. Micromasses were incubated in the specified medium and cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 21 days by replacing with fresh medium every 2 days. Following 21 days culture,

each micromass was washed with sterile PBS and fixed in methanol at -20°C for 30 min. Fixed micromasses were further washed twice with PBS and sulphated glycosaminoglycans (GAG) were visualised by staining with 0.5% alcian blue for 18 h. This consisted of incubating cells with 3% (v/v) acetic acid for 1 min followed by 1% (v/v) alcian blue (1 g alcian blue in 100 ml 3% v/v acetic acid, pH 2.5) for 18 h at ambient temperature, counterstained with 0.05% (w/v) neutral red (0.5 g neutral red in 100 ml distilled water). Stains were removed and cells washed five times with distilled water before imaging using light microscopy.

3.7.1.2 MSC pellet cultures

For preparation of MSC pellets, aliquots of 5×10^5 cells in 1 ml of low glucose DMEM supplemented with 10% FCS were spun down at 250 *g* for 5 mins in 20 ml universal tubes with conical bottoms. Tubes containing the cell pellets were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Following incubation, the medium was carefully removed from each tube and replaced with serum-free standard chondroinductive medium consisting of high glucose DMEM, 1 mg/ml BSA, 100 nM dexamethasone, 10 µg/ml insulin, 5.5 µg/ml transferrin, 0.5 µg/ml selenium and 4.7 µg/ml each of linoleic and oleic acids (ITS) 50 ug/ml ascorbic acid and 10 ng/ml transforming growth factor-1 (TGFβ-1). For control experiments, pellets were incubated in complete MSC medium. Pellets were incubated in the specified medium and cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 21 days by replacing with fresh medium every 2 days.

3.7.1.3 Measurement of proteoglycan content

Pellets were assessed for proteoglycan content by quantifying the level of sulphated glycosaminoglycans (GAGs) using 1,9-dimethylmethylene blue (DMB; Sigma-Aldrich, UK) assay [84]. DMB binds to glycosaminoglycans generating a metachromatic shift that peaks at A525-530 and can be quantified spectrophotometrically. Each pellet was digested with papain solution consisting of 200 mM phosphate buffer, 1 mM EDTA, 0.05% w/v % papain and 0.96 w/v% N-acetyl cysteine and incubated at 60°C for 4 h. Following the 4 h digestion, the supernatant was collected and stored at 4°C until GAG assay was carried out. 20 µl of chondroitin sulphate standard solutions prepared at concentrations from 0-50

µg/ml and 20 µl of each cell pellet sample were placed in a 96-well plate. 250 µl of DMB solution was added to each well and the optical density was quantified at 530 nm using a microplate reader.

3.7.1.4 Collagen Type II immunoassay for assessment of chondrogenesis

3D micromasses were fixed with formaldehyde. Following fixation, micromasses were treated with 10 mg/ml hyaluronidase in PBS for 30 mins followed by washing in PBS. The PBS was removed and 3mg/ml pronase in PBS added to the micromasses and incubated at 37°C for 30 mins. 3% v/v hydrogen peroxide in methanol and PBS was used to quench the enzymatic activity for 5 mins. To inhibit any non-specific hydrophobic binding interactions, 1% BSA in TBS/Tween20® buffer (blocking solution) was applied for 1h at ambient temperature. The blocking solution was then removed and primary collagen II antibody was added (0.4 mg/ml diluted 1 in 100 parts in TBS/Tween20® buffer) to each micromass and incubated at 4°C. Following an overnight incubation at 4°C, all sections were washed three times with TBS/0.05% Tween 20® with each wash lasting for 5 mins. The secondary antibody was added to all wells and incubated for 1h in ambient temperature. The ABC reagent was prepared according to the manufacturer's instructions and left to stand for 30 mins. Then micromasses were incubated in ambient temperature for 30 mins with the ABC reagent. All wells were washed three times with TBS/0.05% Tween 20® with each wash lasting for 5 mins. DAB reagent which was prepared according to the manufacturer's instructions was applied to each well and left for 10 mins. All wells were washed with distilled water and imaged using light microscopy.

3.7.3 Osteogenesis

MSCs were plated out at a density of 5×10^5 cells per well in a six-well culture plates in low glucose DMEM supplemented with MSC serum for 72 h to allow cell adhesion and formation of confluent monolayer. Osteogenic differentiation was induced by subjecting triplicate cultures to standard osteoinductive media consisting of low glucose DMEM, 2% FCS, 1mg/ml BSA, 10 nM dexamethasone, 50 ug/ml ascorbic acid and 10 mM β-glycerophosphate [70]. Control media contained low glucose DMEM supplemented with 10% MSC serum. The cultures

were maintained for 14 days with change of medium every 2 days. After 14 days cells were washed with sterile PBS and fixed in cold methanol at -20°C for 30 minutes. Fixed cells were washed twice with distilled water and SIGMA *FAST*™ BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) tablets were used as immunohistochemical stains for detection of alkaline phosphatase activity.

3.7.4 Adipogenesis

For adipogenesis, MSC were plated out at a density of 5×10^5 cells per well in a six well plate in low glucose DMEM supplemented with 10% MSC serum for 72 h to allow cell adhesion and formation of confluent monolayer. MSCs were cultured in triplicate in either standard adipo-inductive media or control media for 72 h. Adipo-inductive media contained low glucose DMEM, 2% FCS, 1 mg/ml BSA, 10 µM dexamethasone, 100 µM indomethacin and 0.5 mM 3-isobutyl-1-methyl xanthine (IBMX) [71]. Control media contained low glucose DMEM supplemented with 10% MSC serum. Following 72 h, adipo-inductive medium containing cultures were washed with PBS and replaced with maintenance medium consisting of low glucose DMEM, 1mg/ml BSA and 10 µg/ml of insulin while control cultures were maintained in control media for 5 days. Following the 5 days, cultures were washed with sterile PBS and fixed with 2% gluteraldehyde for 30 min at room temperature. Fixed cells were then washed twice with distilled water and subsequently stained with 0.5% oil red O (0.5 g Oil Red O in 100 ml Isopropanol) for 2 min to detect adipogenic differentiation. Stains were removed and cells were washed five times with distilled water before imaging.

3.8 Collection of bovine chondral explants

Chondral flakes were collected from the four proximal sesamoid bones of fresh MCP joints for isolation of chondrocytes and collection of injury conditioned media (IJCM). Full thickness hyaline cartilage with no attached subchondral bone was harvested using a no. 22 scalpel blade attached to a no.4 scalpel handle. Flakes were processed in two different ways depending on their use. For isolation of chondrocytes, flakes were immediately placed in complete chondrocyte media. For IJCM, flakes were placed in low glucose DMEM supplemented with 0.1%

bovine serum albumin (BSA). Table below summarises the different ways chondral flakes were processed following collection.

Table 3.40: Chondral flakes were collected for isolation of chondrocytes, collection of injury conditioned media and migration assays involving cartilage. Therefore, following collection they were placed in different media combinations. DMEM: Dulbeccos modified Eagles medium; FCS: Foetal calf serum; BSA: bovine serum albumin.

	Reason for collection of chondral flakes	Constituents of complete media
Chondral flakes	Isolation of chondrocytes	DMEM (high glucose formulation containing 4500 mg glucose/litre) supplemented with 10% FCS
	Collection of injury conditioned media (IJCM)	DMEM (low glucose formulation containing 1000 mg glucose/litre) supplemented with 0.1% BSA

3.9 SF MSC labelling with PKH67

SF MSCs were labelled with green fluorescent cell linker PKH67 using previously described procedure [85]. Briefly, PKH67 dye was prepared according to the instructors manual immediately prior to staining and added to 1 ml of re-suspended SF MSCs (0.4 µM PKH67/ 2×10^6 cells). Cells were incubated with the fluorescent dye for 25 mins at ambient temperature following which the staining reaction was terminated using serum free DMEM.

3.10 Use of fluorescent probes to assess chondrocyte viability following mechanical injury of cartilage

CMFDA cell tracker Green^{TM} (5'-chloromethylfluorescein diacetate) and PI (propidium iodide) was used to stain live and dead cells respectively. As a hydrophobic dye, CMFDA can freely pass through the cell membrane of an intact cell. Once the dye enters the cell, it is hydrolysed by intracellular esterases which break down the diacetate group to form CMF (chloromethyl-fluorescein). In this form, the dye is hydrophilic and therefore cannot leave the cells. CMF is a

fluorescent compound and fluoresce green, thereby enabling the visualisation of live cells. In contrast, PI can only enter the cell when the cell is no longer intact and the cell membrane is disintegrated. Inside the cell it binds to intra-nuclear DNA of dead cells and makes the nucleus fluoresce red.

CMFDA (Invitrogen Ltd, Paisley, UK) was dissolved in 20 µl of dimethyl sulphoxide (DMSO) and added to DMEM (high glucose formulation; 4500 mg glucose/litre) to give a final concentration of 10 µM. 10 µl of PI was added to the DMEM to give a final concentration of 10 µM. Osteochondral and chondral strips were incubated in the DMEM containing the CMFDA and PI for 1 h at room temperature. Following 1 h incubation, strips were fixed in formaldehyde solution (4% v/v; Fisher Scientific, UK) for 30 mins at room temperature and stored in PBS at 4^{o}C overnight before microscopy.

3.11 Confocal laser scanning microscopy (CLSM)

Fluorescently labelled and fixed cartilage explants were placed on a microscope slide with the articular surface uppermost. An upright Zeiss Axioskop LSM 510 meta confocal laser scanning microscopy (Carl Zeiss Ltd, UK) with a ×10 (EC Plan-Neofluar 10×/0.30) and ×5 (EC Plan-Neofluar 5×/0.16) dry objective lens was used to acquire optical sections (512 × 512 pixels). Argon and Helium-Neon laser multi-track protocol was used. This protocol allowed alternate excitation of each fluorophore (CMFDA (λex = 488nm, λem= 517nm) and PI (λex = 543nm, λem = 650nm) and thereby dual visualisation of live and dead cells within individual optical sections. The sensitivity and detector gain were manually adjusted before image acquire to avoid pixel saturation and optimal imaging of each optical sections. The diameter of the pinhole was set to 1 Airy Unit (diameter which allowed rejection of out-of-focus light) for detection of red and then manually adjusted for detection of green to get the same optical slices for both fluorophore. Using these parameters, the superficial zone (approx. 30 µm deep; z-axis) of the articular cartilage was imaged in multiple optical sections and the sections were reconstructed using an image analyser (LSM image browser) to produce CLSM projections.

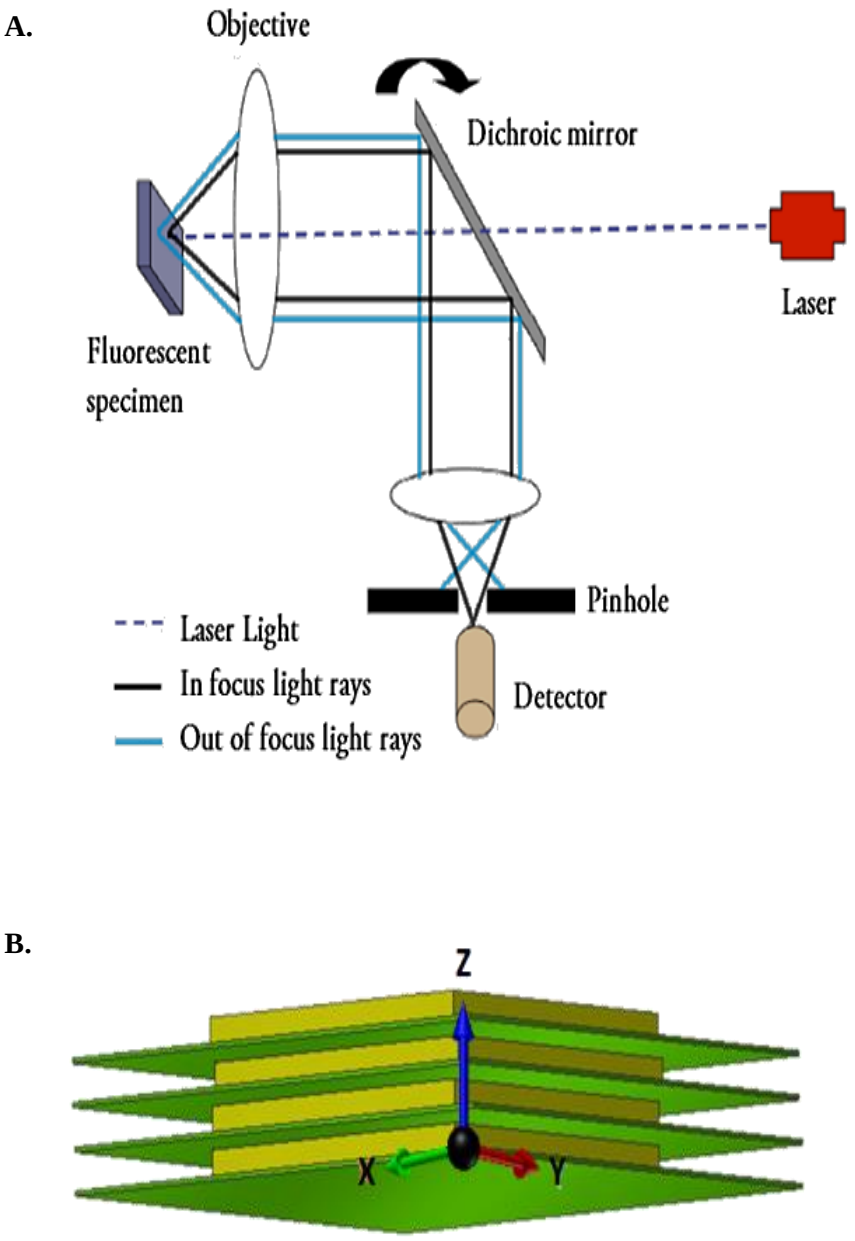


Figure 3.5: Confocal laser scanning microscopy. A. Exclusion of out-of-focus light by the pinhole allows detection of only in-focus light and imaging tissues at high-resolution. **B.** Optical sections of cartilage will be imaged top-down from the articular surface to a depth of ~ 30 μm into the tissue.

3.12 Isolation of chondrocytes

Chondral flakes with no attached subchondral bones were harvested from the bovine MCP using a no. 11 scalpel blade. Flakes were immediately placed in complete chondrocyte media until the tissue collection was completed. The media was then removed and 2.5% (w/v) trypsin was added and the cartilage pieces were incubated for 30 mins at 37°C. Following incubation cartilage pieces were washed twice with PBS and placed in complete chondrocyte media and 2mg/ml of collagenase. Chondral flakes were further incubated for 24 h on an orbital shaker at 37°C to allow tissue digestion. Following 24 h incubation, media containing digested cartilage was placed in centrifuge tubes and centrifuged at 1000 rpm (192 *g*) for 10 min to pellet the chondrocytes. After centrifugation, the supernatant containing the collagenase was removed and the cell pellet was re-suspended in PBS. Cell suspension in PBS was further centrifuged for 10 min at 1000 rpm and this whole washing process was repeated twice. The final chondrocyte pellet was re-suspended in chondrocyte complete media (2×10^6 cells/11 ml media) and plated in tissue culture flasks [86]. Only chondrocytes at P0 passage number were used for migration assays.

3.13 Passaging cells

Medium was removed from the tissue culture flasks and each flask was rinsed once with 5 ml of PBS. Diluted typsin/EDTA solution [Trypsin/EDTA (0.05% trypsin and 0.02% EDTA) was diluted with PBS at a 3:1 ratio] was added to the culture flask and the flasks were incubated at 37°C for 2 mins at which time 90% of the cells lift up from the flask. After the cells had detached, they were quickly transferred to a sterile 50 ml centrifuge tube and the trypsin/EDTA in the flask was neutralised using 1 ml serum. Cell specific serums were used in each case. The flask was then rinsed once with PBS to collect the residual cells, the rinse was added to the centrifuge tube and the whole solution was centrifuged for 5 min at 192 g. Cell pellets were suspended and cultured in their cell specific complete media in cell specific tissue culture flasks.

3.14 Collection of conditioned media from injured cartilage: Injury conditioned media (IJCM)

Cartilage pieces were finely cut using no.11 scalpel blades. Media were conditioned by placing the finely cut pieces of cartilage (approximately 3 mm × 1 mm; 600 mg/15 ml) in serum-free DMEM containing 1g/l glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acid and 0.1% bovine serum albumin (BSA) (DMEM+0.1% BSA) and incubating at 37° C in a humidified atmosphere at 5% CO_2 for 24 h. Media were collected as injury conditioned media (IJCM) for either immediate use or stored at -20° C (Figure 3.6).

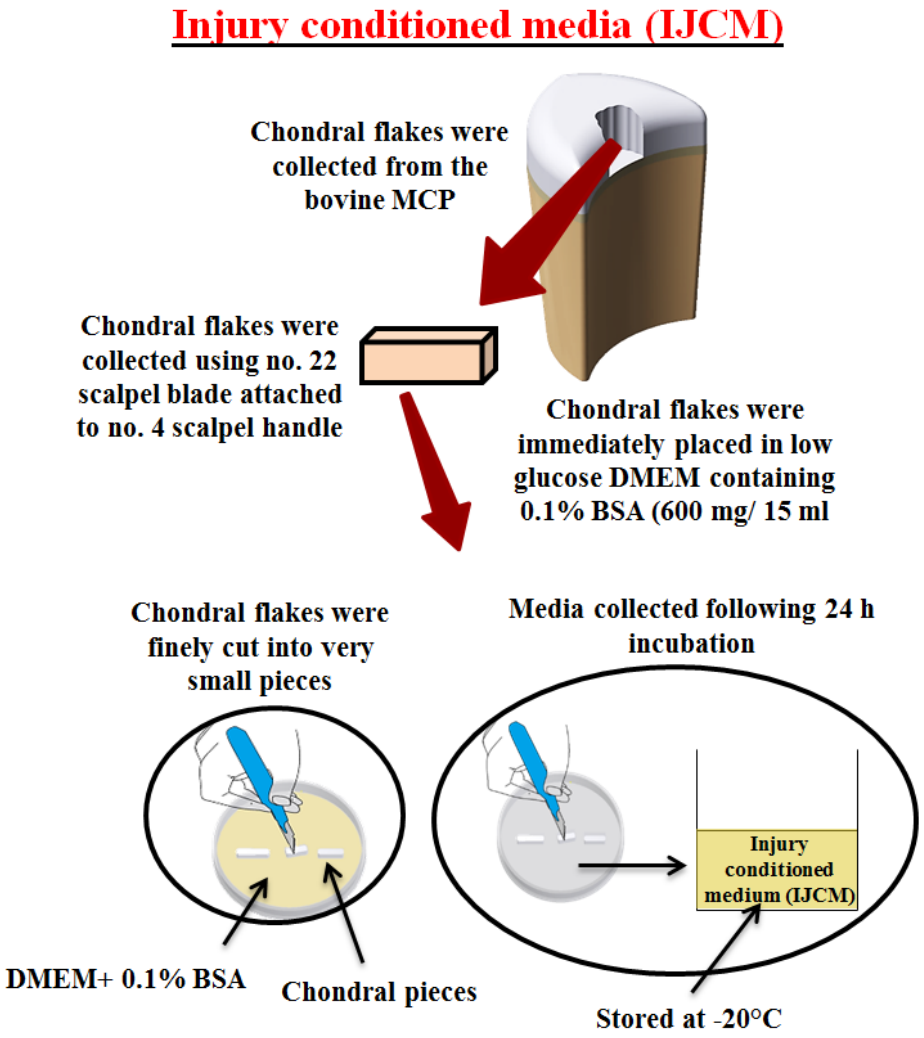


Figure 3.6: Collection of injury conditioned media (IJCM) Bovine metacarpophalangeal joints obtained from the local abattoir were dissected and opened under sterile conditions. Hyaline cartilage was harvested from the condyles using no.11 scalpel blade attached to no.3 scalpel holder. Cartilage was finely cut into pieces using no.22 scalpel blade attached to no.4 scalpel holder in the presence of serum free media supplemented with 0.1% bovine serum albumin (BSA). Cut pieces of cartilage were incubated in the same serum free media for 24 h at 37°C and 5% CO_2. Following 24 h incubation, cartilage pieces were discarded and media was collected as injury conditioned media (IJCM) for either immediate use or stored at -20°C for later use.

3.15 Heparin-treatment of injury conditioned medium (IJCM)

A wide variety of proteins such as growth factors, proteolytic enzymes and chemokines sequester in the cartilage matrix through their interactions with GAGs found ubiquitously on the cell surface and in the ECM. One typical GAG molecule that binds to chemokines and growth factors is heparin. Study of protein-binding properties of heparin sulphate (HS), heparin and other GAGs have been limited by the requirement for chemical modification prior to attachment to inert or derivatised surfaces and also by the lack of a simple method for analysis. 96-well heparin-binding plates offer a simple solution to this problem. Cold plasma polymerisation was used to coat the microtiter plates with allyl amine which generates a surface for non-covalent immobilisation of heparin in a functionally active state. These heparin-binding plates were then used to treat IJCM and remove any heparin-binding proteins present in the IJCM. To coat the plate with heparin, 200 µl of heparin solution (50 µg/ml in PBS) was added to each well and the plate incubated at 4°C overnight [87]. Following overnight incubation, the heparin solution was removed from each well and the wells were washed twice with PBS. The PBS was removed and 300 µl IJCM was then added to each well and the plate further incubated at 4°C for 24 h. Following incubation, the heparin-treated IJCM was collected for either immediate use or stored at -20°C.

3.16 Migration assays

3.16.1 Induction and quantitative assessment of mesenchymal stem cell migration in the 24-Well Transwell^{TM} assay

Due to insufficient reports in literature on assessment of SF MSC chemotactic migration using Transwell assays it was first important to establish the protocol and conditions for the analysis of MSC migration seeded in 24-Well Transwell chambers and reliably identify the translocated cells. Transwell inserts of dimension 12 mm in diameter and 1.12 cm^2 membrane growth area were used. The membranes contained 8-µm diameter pores. 650 µl of serum was added to the lower wells and 100 µl of cell suspension containing 2 $\times 10^6$ cells per ml of serum free media supplemented with 0.1% bovine serum albumin (BSA) was added in triplicate to the top wells (Figure 3.7). Plates were incubated at 37°C in a humidified atmosphere at 5% CO_2. Cells attached to the lower part of the

membrane were fixed with formaldehyde in combination with cell wipes to remove non-migrated cells from the upper side of the membranes and a crystal violet staining step to mark the migrated cells. 5 different microscopic fields were imaged per membrane at x20 magnification and number of cells in each image was counted using the Image-J software (Figure 3.8) [88].

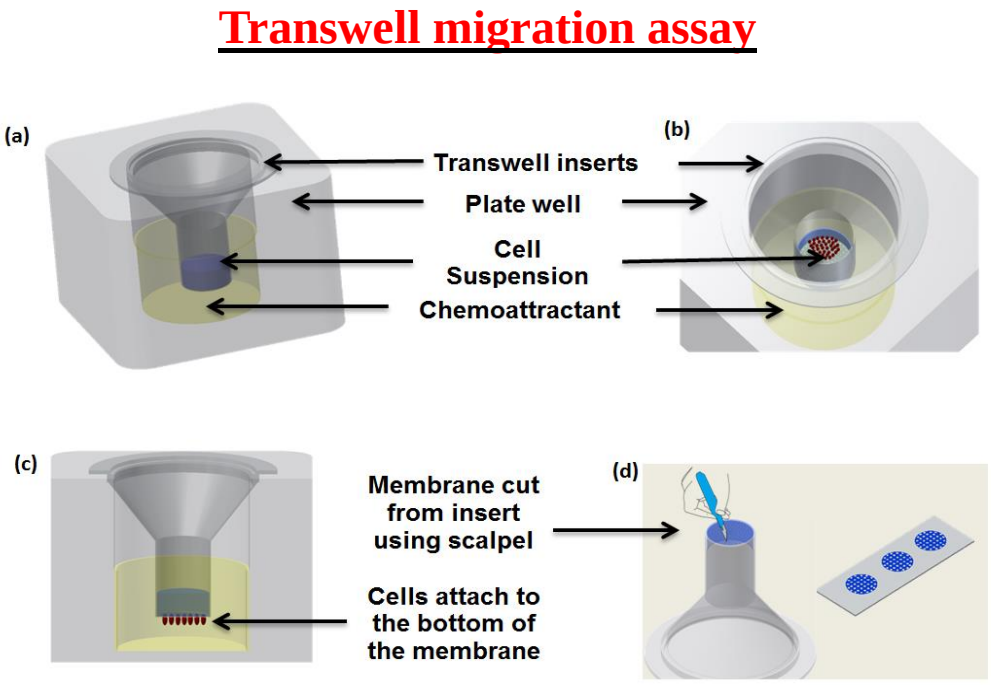


Figure 3.7*:* Side (a) and top (b) schematics of one transwell insert. The insert is 12 mm in diameter with membrane pore diameter of 8 µm. The insert sits on top of the well which keeps the insert membrane about 1 mm off the bottom of the well. This prevents membrane disturbance when added to the 24 well plates. (c).MSCs were seeded in DMEM+0.1% BSA in the upper well and the cells can migrate in vertical direction through the pores (8 µm) on the membrane in response to attractants on the lower well. Migrated cells attach to the bottom of the membrane. (d). Membranes from the upper wells were cut from the inserts, stained with crystal violet, mounted on glass microscope slides and imaged using light microscopy to count the number of cells.

5 different microscopic fields were imaged per membrane at x20 magnification and number of cells in each image was counted using the Image-J software.

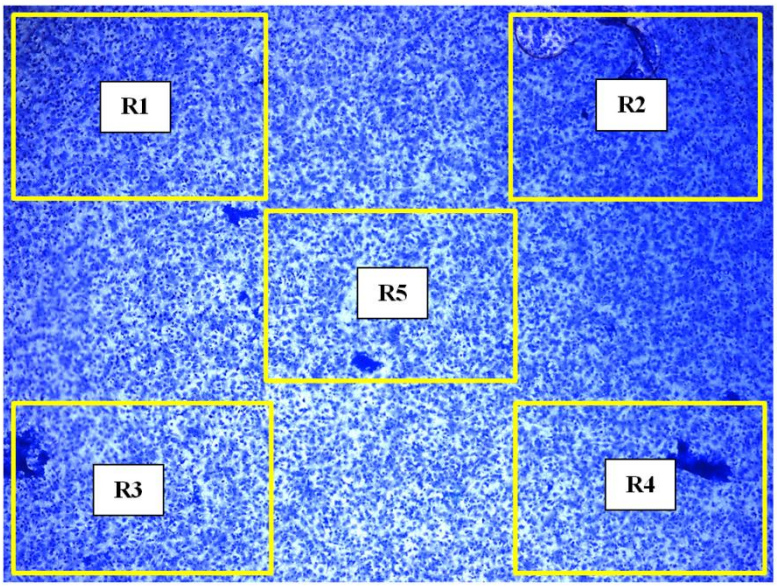


Figure 3.8: Digital image of a representative membrane imaged at x4 magnification. For cell counting, five different microscopic regions (R1-R5) as labelled were imaged at x20 magnification producing 5 images per membrane. Total number of migrated cells per membrane was quantified by addition of the number of migrated cells quantified per region (R1+R2+R3+R4+R5). Cells were counted using the Image-J software. *Please scan the QR code to watch a video of the analysis process using Image-J.*

3.16.2 Quantifying cell migration using calcien AM: comparison between calcein AM and crystal violet staining method

An alternative to crystal violet staining to quantify cell migration in transwell assays is to fluorescently label the cells and quantify fluorescence from the migrated cells using a microplate reader. We assessed and compared this method with the crystal violet staining method in order to optimise the quantification step of the assay. Cells were stained with calcein-AM which is the acetomethoxy derivative of calcein. It is a hydrophobic compound which can freely pass through the cell membrane. Once inside the cell, intracellular esterases hydrolyse calcein-AM to remove the acetomethoxy group. In this form calcein is hydrophilic, gets trapped inside the cells and gives out strong green fluorescence which can then be quantified using a plate reader (Figure 3.9). This simplifies the detection of the migrated cells by eliminating the need to remove the remaining cells from the top-side of the membrane and to manually count the number of cells in each membrane. Cells were stained by adding calcein-AM to the bottom well, thereby only immersing the migrated cells in the fluorescent label. Trypsin/EDTA was used to detach the migrated cells from the membrane and the eluted solution was read in a microplate reader at an excitation >485 nm and emission at 520 nm in 96-well plates.

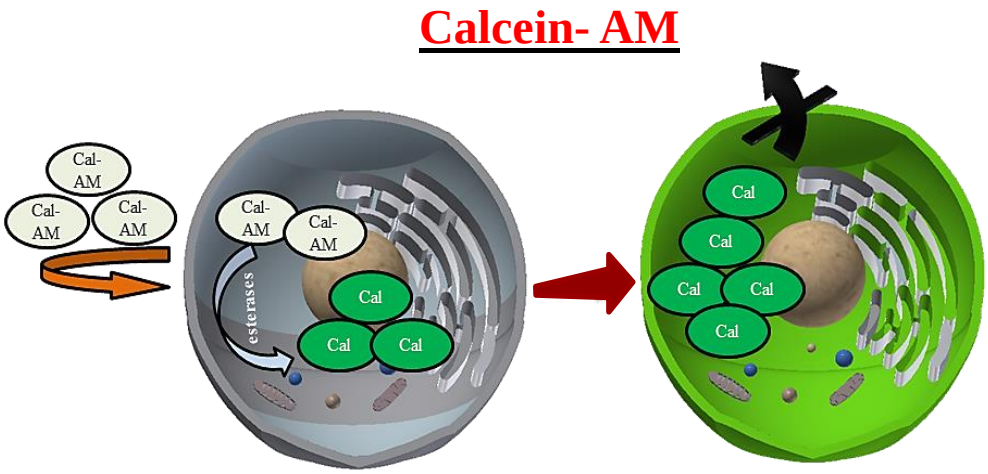


Figure 3.9: Use of calcein-AM to quantify number of migrated cells following Transwell assay. Calcein-AM is a hydrophobic compound which can pass through the cell membrane. Once inside the cell, esterases hydrolyse calcein-AM to produce calcein which is hydrophilic and cannot leave the cell. As calcein is fluorescent, when trapped inside the cell it makes the cell fluoresce which can be quantified using a plate reader.

3.16.3 Assessment of MSC migration using scratch assays

Even though the widely used transwell assay reliably detects differences in cell migration in response to varying stimulus, it offers less readout options because it is an end point assay. In comparison, wound healing assay also known as scratch assay is an easy and economical method to measure cell migration *in vitro*. The basic principle of this method is based on the observation that upon creation of an artificial gap (scratch) on a confluent cell monolayer, cells on the edge of the scratch will move towards the gap to close the scratch until new cell to cell contacts are established again (Figure 3.10).

Scratch assays were performed in 24 well plates and each well was seeded with 300,000 cells. This cell seeding density was chosen based on preliminary experiments looking at various cell densities (100,000 – 500,000 cells/ well). Plates were maintained at 37° and 5% CO_2 for 24 h to allow cell attachment and formation of a confluent monolayer. If confluent monolayer did not form within 24 h then the plates were maintained till 100% confluency was achieved.

Complete media was then removed and replaced with serum free media (DMEM+0.1% BSA). These confluent monolayers were then 'wounded' by scraping off an area of the monolayer using a 200 µl pipette tip to create a gap of approximately 500 µm in width. Wound closure was monitored microscopically and images were taken at x10 magnification at various time intervals until the wound was completely closed or no longer progressing. Images were analysed and the width of the wound was measured using Image-J (Figure 3.11). Data was presented as % reduction in wound width from the time of the creation of the wound width (T0) and final image taken (T duration of the experiment) [82].

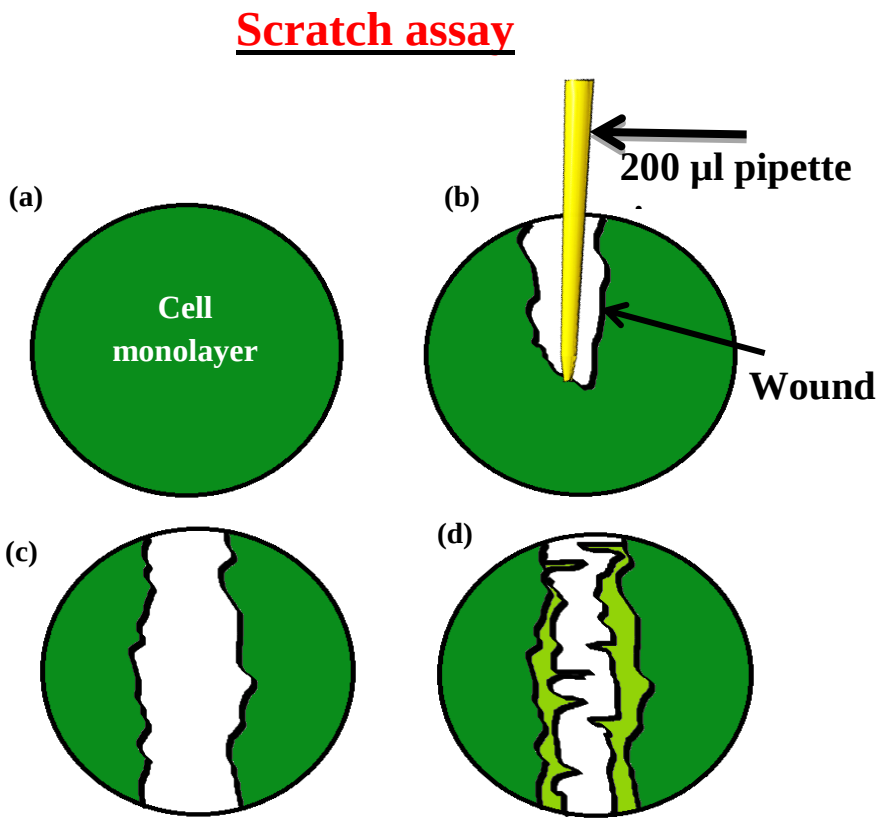


Figure 3.10: Principle of scratch assay. (a) Cells are grown to confluence in 24 well plates. **(b)** The monolayer is 'wounded' by scraping off an area of cells using a 200 µl pipette tip. **(c)** Monolayer is imaged immediately following creation of wound and **(d)** monitored microscopically and imaged at various time intervals to quantify cell migration as percentage reduction in wound width over time.

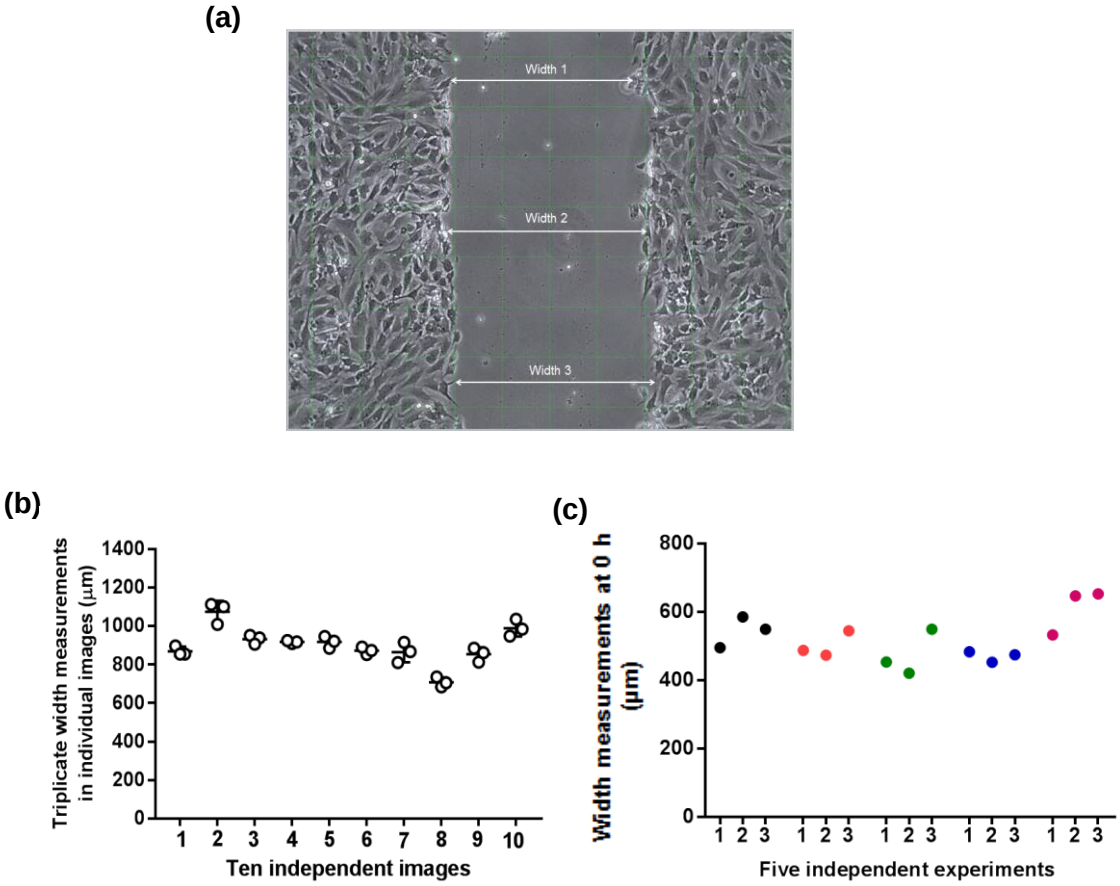


Figure 3.11: Wound width measurement and the variability between triplicate measurements at 0 h (immediately after creation of the wound). **(a)** A representative image of one scratch assay immediately after creation of the wound. In order to measure the width of the wound this image was imported into the ImageJ software. The image was then overlaid with a grid (60000 pixels^2) to divide the image uniformly (shown in green). This grid overlay creates 9 columns. Width measurements were taken in the middle of 2nd column (width 1), 5th column (width 2) and 8th column (width 3). The mean value of the three width measurements {(width 1 + width 2 + width 3)/3} was used as the width measurement for that particular image. **(b)** Graph showing the variability amongst the three width measurements (width 1, width 2 and width 3) in individual images. Mean variation amongst these three width measurements (standard deviation) amongst these 10 individual images was 40 µM. **(c)** Graph showing mean width measurements among triplicate assays. Variation in mean width between triplicate assays was on average 75 µM.

3.16.3 Assessment of MSC migration using cell exclusion zone assays

Cell exclusion zone assays were used to assess the requirement for cell activation or cell injury for MSC migration. This assay is based on the same principle as the scratch assay with the exception that the wound is created using silicon inserts instead of a pipette tip. Using these inserts also avoided problems such as unevenly thick gap created by cell scraping, and reattachment of floating cells in the wound area which then migrate towards the monolayer which have the risk of providing adulterated results. We used two different inserts available commercially from Ibidi (Figure 3.12) and Platypus Technologies Inc (Figure 3.13).

3.16.4 Ibidi inserts

With Ibidi inserts, two cell culture compartments separated by 500 µm thick wall were formed when the silicon insert is placed on the 24-well plate. 300,000 cells were seeded in both the compartments in complete MSC media and maintained at 37° and 5% CO_2 for 24 h to allow cell attachment and formation of a confluent monolayer. If confluent monolayer did not form within 24h then the plates were maintained till 100% confluency was achieved. Removal of the silicon insert from the surface of the culture plate resulted in two defined cell patches separated by a 500 µm cell free gap. Complete media was then removed and replaced with serum free media (DMEM+0.1% BSA). Migration into the cell exclusion zone was followed microscopically until the wound was completely closed or no longer progressing and images were analysed using Image-J with same protocol as used for scratch assays.

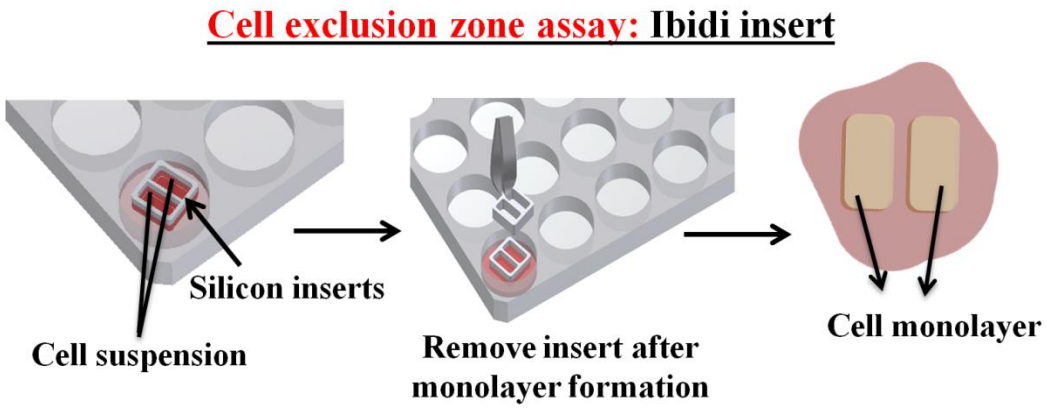


Figure 3.12: Cell exclusion zone assay using Ibidi inserts. (a) Single Ibidi culture insert in a well plate. **(b)** Principle of cell exclusion zone assay using Ibidi inserts. Cells are grown to confluence in Ibidi culture inserts over 24h to allow formation of two separate monolayers. Removal of the insert creates a cell-free gap between the two monolayers. Cell migration into the gap is monitored microscopically and quantified as percentage reduction in wound width similar to scratch assay.

3.16.5 Oris-FLEX inserts

Even though the cell exclusion zone assays with Ibidi inserts were well-standardised, easy to set up and does not require any special analysing equipment, care had to be taken that the stoppers tightly attached to the tissue culture plate. In our experience, silicon stoppers provided by Ibidi tend to lose their plate adhesive properties with multiple use and cells entered the exclusion zone beneath the stopper. To avoid this problem, we used small silicon stoppers designed by Platypus Technologies Inc. that fit into each well of a 96-well plate. The 96-well plates were also provided by Platypus Technologies. 100,000 cells were seeded per well and maintained for 24 h at 37° and 5% CO_2 for 24 h to allow cell attachment and formation of a confluent monolayer. This cell seeding density was chosen based on preliminary experiments looking at various cell densities (100,000 – 300,000 cells/ well). Complete media was then removed and replaced with serum free media (DMEM+0.1% BSA). Removal of the stoppers following cell adhesion created a 2 mm diameter circular cell free gap with sharp borders where the cells can then migrate to.

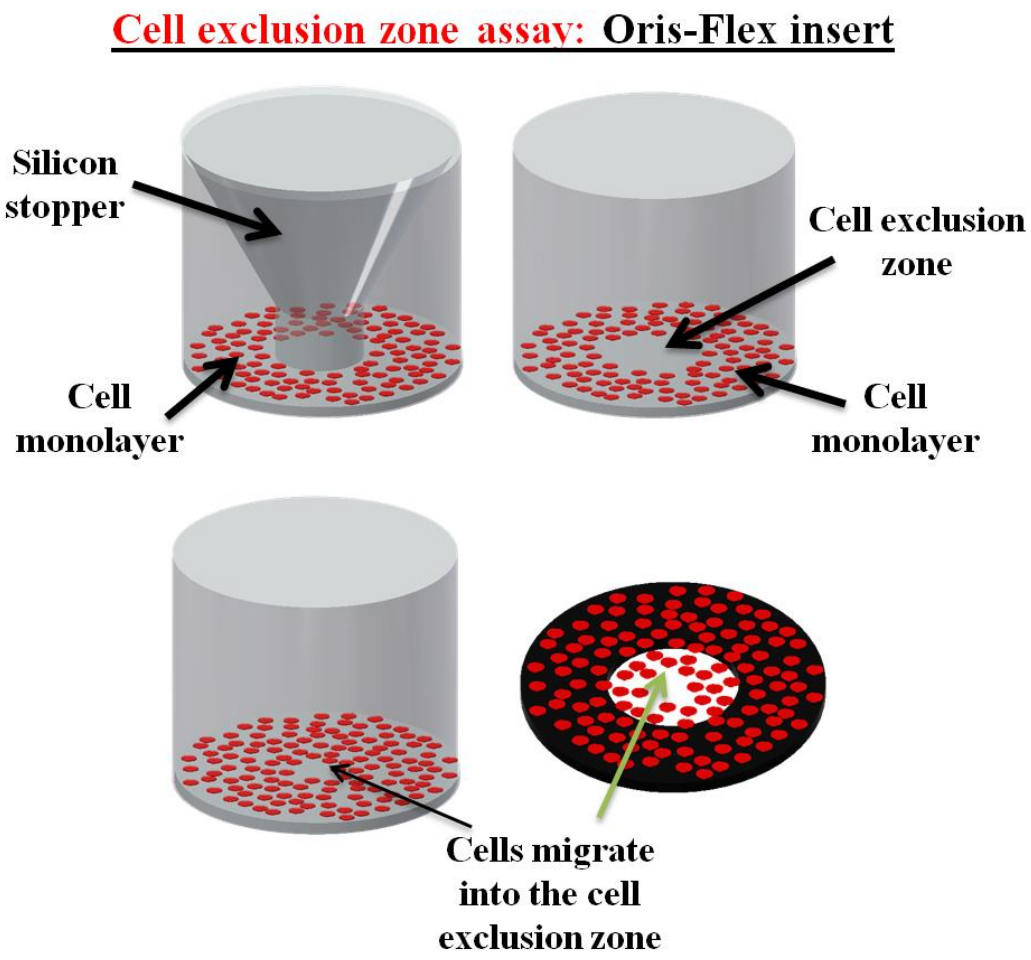


Figure 3.13: Principle of cell exclusion zone assay using Oris-Flex inserts. Cells are grown to confluence in Oris-Flex culture inserts in 96-well plates over 24 h to allow formation of monolayer. Removal of the insert creates a circular cell-free gap in the middle of the monolayer. Cell migration into the gap is monitored microscopically. Wound diameter is measured to calculate the wound area. Percentage reduction in wound area over time is used to quantify cell migration.

3.17 MSC migration in response to growth factors, cytokines, extracellular nucleotides

Cytokines and growth factors tested were: CXCL12 (400 ng/ml), TGFβ3 (10 ng/ml), FGF2 (5 ng/ml), CCL2 (30 ng/ml), CCL3 (50 ng/ml), CCL5 (50 ng/ml)

and TNFα (50 ng/ml). The concentrations were chosen based on the recommended concentration on the product data sheet.

Factors were supplemented to DMEM (1000 mg/ml glucose) with 0.1% BSA and the media was added directly to cell monolayers. The effect of the following purine compounds were also studied: ATP (250 μM) and UTP (250 μM) and stable purine analogues ATPγS (Adenosine-5'-(γ-thio)-triphosphate tetralithium salt; 250 μM) and UTPγS (Adenosine-5'-(γ-thio)-triphosphate tetralithium salt; 250 μM) on SF MSC migration. Nucleotides were either supplemented to DMEM (1000 mg/ml glucose) with 0.1% BSA or IJCM before being added to the cell monolayers.

3.18 Characterisation of IJCM using antibody and receptor antagonists against common heparin-binding chemokines

In order to characterise and identify the MSC migration stimulatory factors present in the IJCM, neutralising antibodies and receptor inhibitors were used against common heparin-binding chemokines previously reported to influence MSC migration [89]. The growth factors and cytokines tested were: FGF2 antibody (5 μg/ml), CXCL12 antibody (3.6 μg/ml), CXCR4 antagonist (AMD3100; 6.29 μM), TGFβ3 antibody (0.3 μg/ml), CX3CL1 antibody (1.5 μg/ml), CCR5 antagonist (7 μg/ml) and CCR3 antagonist (10 μg/ml). The concentrations were chosen based on (1) preliminary studies qualitatively assessing MSC migration in response to various concentrations of the cytokine inhibitors [used for antagonists against TGFβ3 (0.0003 – 0.3 μg/ml) and CXCL12 (0.36 – 30.6 μg/ml)] or (2) the suggested value on the antibody data sheet (used for all other antibodies) or based on values suggested in the literature (AMD3100) [90].

IJCM samples were pre-treated with the antibodies for 15 mins at ambient temperature prior to adding the IJCM to the scratch assays. Confluent monolayers of MSCs were pre-treated with the receptor antagonists for 30 mins at 37° C before creation of the wound and addition of IJCM. P2 purine receptors were blocked in scratch assays using P2 purine receptor antagonist suramin (1 mM; concentration chosen based on the suggestion in the data sheet). To assess if injury stimulates synthesis of the factors prior to release freshly harvested

cartilage was injured in serum free media in the presence of the protein synthesis inhibitor cycloheximide (10 µg/ml; Sigma-Aldrich, UK; concentration chosen based on qualitative preliminary studies) and injury conditioned media was collected following 24 h incubation as described above. To assess the requirement for matrix metalloproteinases (MMPs) for the release of factors following injury freshly harvested cartilage was injured in serum free media in the presence of broad spectrum metalloproteinase inhibitor marimastat (10 µg/ml; R&D Systems, UK; concentration chosen based on qualitative preliminary studies) and injury conditioned media was collected following 24 h incubation as described above.

3.19 Measurement of ATP content

ATP content was measured using ATP bioluminescence assay kit according to the manufacturer's instructions. Luciferase reagent was prepared by dissolving the whole content of one vial (provided in the kit) in 10 ml of dilution buffer (also provided in the kit) and incubating in ice without stirring or shaking for 5 mins. This luciferase reagent was added to the samples in the 96 well plates (50 µl reagent to each well) and the luminescence from the samples was measured by microplate reader.

ATP standard was prepared by dissolving the content of one vial (provided in the kit) in dilution buffer to give a final concentration of 10 mg/ml. This working solution was further serially diluted with the dilution buffer in the range of 10^{-6} M– 10^{-12} M ATP. Luciferae reagent was added to each dilution to produce a standard curve. The standard curve was used to quantify the concentration of ATP in the samples.

3.20 Induction of chondrogenesis using IJCM

For micromass cultures of MSCs, MSCs (5×10^5 cells/ 50 µl) were placed as a 50 µl droplet in the middle of the wells in 24 well culture plates and cells were allowed to attach over 24 h. Following incubation, the medium was replaced with either IJCM, IJCM with ATP (250 µM) and ascorbic acid (50 µg/ml) or ATP in serum free media (250 µM). Serum-free standard chondroinductive medium was used as positive control and 10% serum containing media was used as negative

control. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 10 days and respective media was changed every 2 days.

3.21 Statistical analysis

In this study there were two separate variables to consider as biological variable. Number one was the cells including both mesenchymal stem cells (MSCs) and chondrocytes which were harvested from different animals. Number two was the injury conditioned media (IJCM) which was produced from the cartilage harvested from different animals. However, in order to produce enough media for each experiment IJCM was collected from five separate animals. Therefore, the variability between animals for IJCM was not quantified. Hence, where independent experiment is mentioned it represents only the biological replicates in terms of cells that were harvested from different animals.

All experiments were performed in triplicate (technical replicate) and repeated three times (three independent experiments). Experiments using antibodies were repeated five times (five independent experiments). Where the data are represented as bar chart, graph shows the cumulative result from three independent experiments (n = 3). When the data are represented as scatter plot, graph shows individual results which include both technical replicate and biological replicate. Data were analysed using GraphPad Prism (version 6.0, Graphpad Software, Inc.,CA, USA). Data are presented as means ± SEM. SEM represents the spread among individual data points (including both technical and biological replicate) for individual conditions. One-way ANOVA followed by post-hoc test (Holm Sidak) was used to calculate the statistical significance of the difference in cell migration in response to different conditions. Differences were accepted as statistically significant at $P < 0.05$ and are indicated by either asterisks (*) or plus (+).

Chapter 4

Isolation and characterisation of mesenchymal stem cells

4.1 Introduction

The objective of this study was to isolate mesenchymal stem cells (MSCs) from the synovial fluid and bone marrow of the bovine metacarpophalangeal joint and investigate the multipotency of the cells by determining if the MSCs can differentiate into chondrogenic, osteogenic and adipogenic lineages. The differentiation potential of the MSCs for chondrogenic, osteogenic and adipogenic lineage was determined using by standard differentiation assays and qualitative analysis.

4.2 Results

4.2.1 Establishment of bone marrow and synovial fluid mesenchymal stem cell culture

Synovial fluid (SF) and bone marrow (BM) MSCs were isolated from the bovine metacarpophalangeal joints based on their capacity of plastic adherence under standard culture conditions using methodology described by Jones et al for SF MSCs [29]. BM MSCs appeared as adherent fibroblast-like cells with long projections and prominent nucleoli (Figure 4.1). SF MSCs were also adherent cells with similar fibroblast-like morphology but were smaller in size than the BM MSCs and were stellate shaped (Figure 4.2). Both MSCs have similar colony formation process, where they underwent several rounds of cell division following seeding into tissue culture flasks, with resulting fibroblastic colonies, each derived from a single cell (CFU-Fs) clearly detectable by day 7. Each colony, derived from a single cell, expanded in culture and merged to form monolayers of typical spindle-shaped fibroblasts (Figure 4.1 and 4.2).

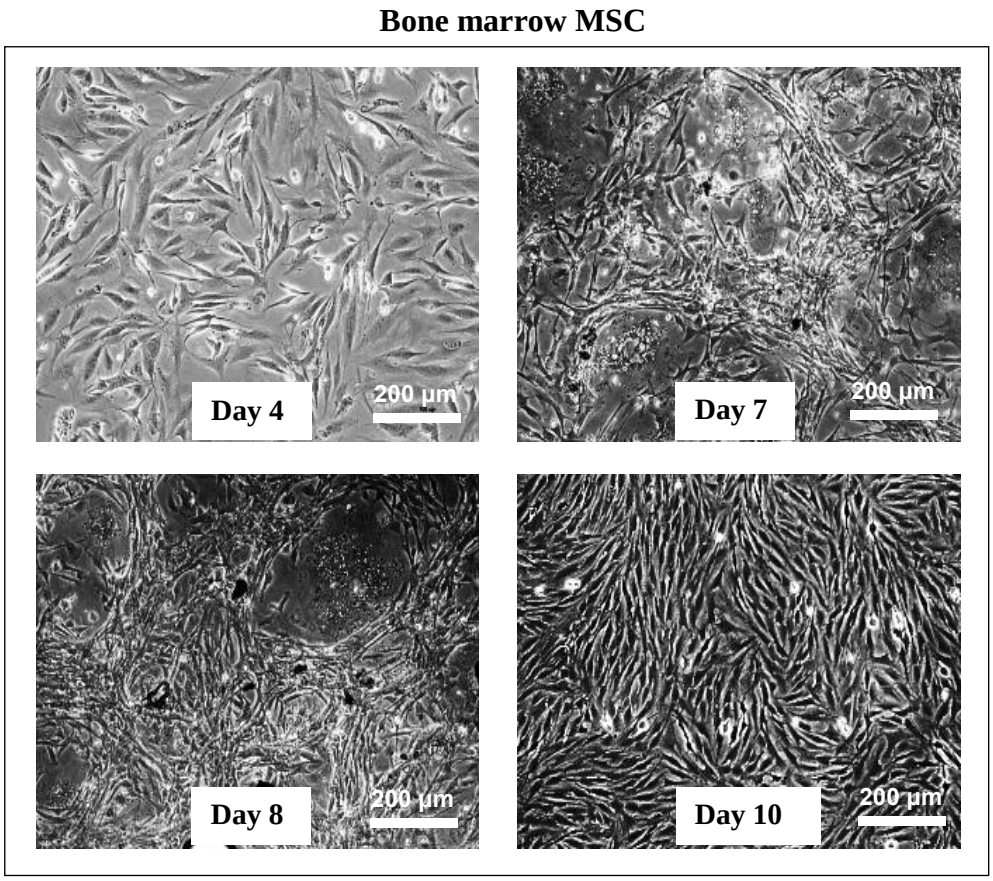


Figure 4.1: Bovine BM-MSC in 10 day culture. Cells were isolated based on their plastic adherence capacity. Following 4 days of culture of the isolated cells, small rounded cells formed long projections with adherent fibroblast-like appearance. At day 7, cells merged to form individual colonies called colony-forming units (CFU). These colonies then merged together to form a single monolayer. Scale bar represents 200 µm.

Synovial fluid MSC

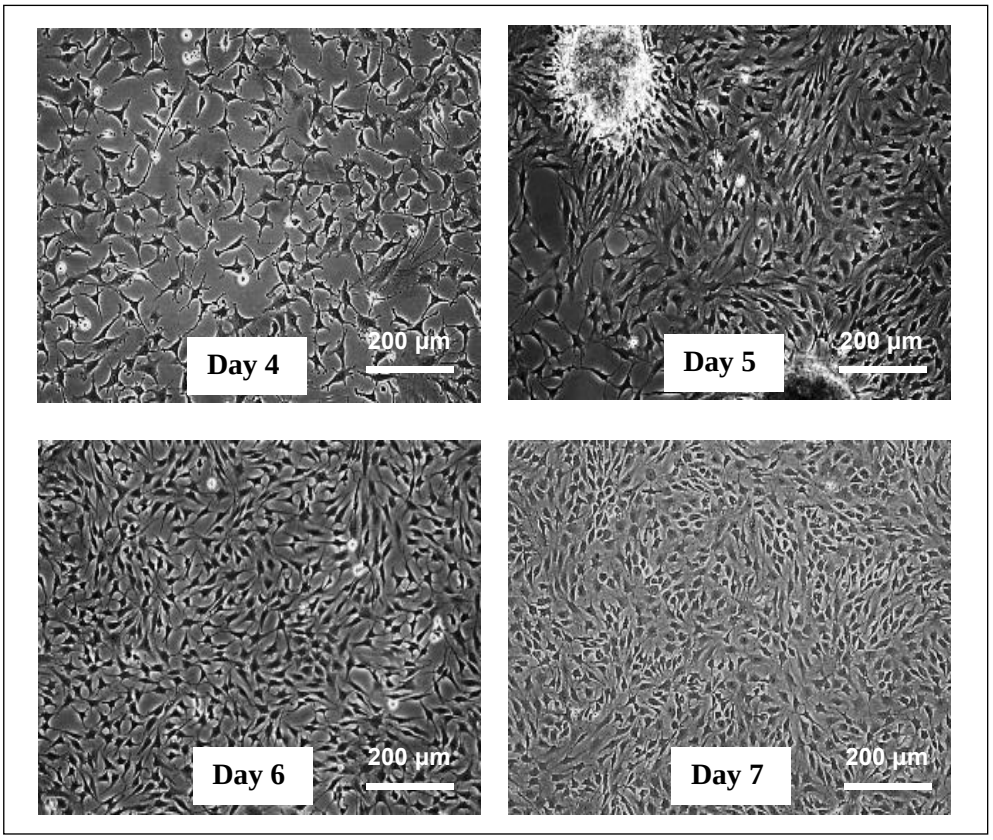


Figure 4.2: Bovine SF-MSC 7 day culture. Cells were isolated based on their plastic adherence capacity. Initially SF-MSCs appeared as small stellate shaped cells. Following 4 days of culture, these rounded cells formed small projections and appeared to have a polygonal morphology. By day 5, the single cells formed individual colonies which only lasted for one or two days. These colonies then merged together to form a single monolayer by day 7. Scale bar represents 200 µm.

4.2.2 Determination of multilineage differentiation capacity of *in vitro* cultured bone marrow and synovial fluid mesenchymal stem cells

BM MSCs and SF MSCs have been shown to differentiate into several types of mesenchymal lineages [29]. Monolayer cultures of BM and SF MSCs derived from the bovine joints were subjected to classic osteogenic, adipogenic and chondrogenic differentiation medium according to previously reported protocols. BM and SF MSCs cultured in complete MSC media were used as negative control.

BM and SF MSC micromass cultures were used to qualitatively assess chondrogenic differentiation. Micromass cultures were subjected to standard chondroinductive media or MSC complete media (control) for 21 days. Metachromatic staining with alcian blue, indicative of cartilage matrix was used to detect chondrogenesis. The periphery of the micromass cultures in chondroinductive media consisted of elongated cells, while the centre region contained rounded cells. Alcian blue staining was detected across the micromass but was stronger in the centre of the micromass (Figure 4.3). Moreover, stronger staining was observed for BM MSC micromass cultures than SF MSC cultures suggesting that BM MSCs may have higher chondrogenic potential than SF MSCs. Both the morphology of the cells and metachromatic staining were suggestive of cartilaginous matrix synthesis by both BM and SF MSCs. In control micromass cultures, lacking chondroinductive media, MSCs formed uniform cell layer, where BM MSCs appeared as spindle shaped fibroblastic cells and SF MSCs appeared as polyglonal fibroblastic cells. No alcian blue staining was observed in any of the control cultures for both BM and SF MSCs (Figure 4.3).

Micromass cultures of MSC were also assessed for immunohistochemical localisation of type II collagen. All micromass cultures stimulated with standard chondroinductive media showed positive staining with homogenous distribution of intense coloration indicative of high levels of collagen type II production. No staining was detected in negative controls where micromass cultures were maintained in low glucose DMEM supplemented with 10% MSC serum (Figure 4.3). Only SF MSC pellet cultures were used to quantitatively assess the success of chondrogenic differentiation. Cells from the first passage were used for pellet

preparation by centrifugation where a single compact pellet was formed per 50000 cells. Pellets were subjected to standard chondroinductive media for 21 days. SF MSC pellets cultured in complete MSC media were used as negative control. The extent of extracellular matrix accumulation was assessed by measuring the sulphated glycosaminoglycan (GAG) content of the pellets. Average sulphated GAG content was 14.75 ± 6.8 µg/pellet indicating chondrogenic differentiations. No sulphated GAG was produced in any pellets incubated in control media lacking chondrogenic factors. GAG content was not measured in BM MSC differentiation assays.

Intracellular alkaline phosphatase is a key marker of osteogenic differentiation. SIGMA *FAST*^{TM} BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) tablets were used as immunohistochemical stains for detection of alkaline phosphatase activity. Staining indicated that both BM MSCs and SF MSCs grown in osteogenic medium produced alkaline phosphatase activity 14 days postinduction, consistent with successful progression toward an osteogenic lineage (Figure 4.4).

Similar number of stained cells suggested that SF MSCs has an osteogenic differentiation capacity similar to that of cultured BM MSCs. In controls, lacking osteogenic supplements, no alkaline phosphatase stained cells were observed for both SF MSC and BM MSC cultures. Accumulation of lipid vacuoles following adipogenic induction was detected using 0.5% oil red O. Red fatty vacuoles indicating adipogenesis were detected in both SF MSC and BM MSC cultures grown in adipoinductive media 14 days post-induction (Figure 4.4). However, fewer numbers of SF MSCs stained with Oil red O compared to BM MSCs suggesting that SF-MSCs might have lower adipogenic capacity than BM MSCs. No red staining was detected in control MSC cultures lacking adipogenic supplements (Figure 4.4).

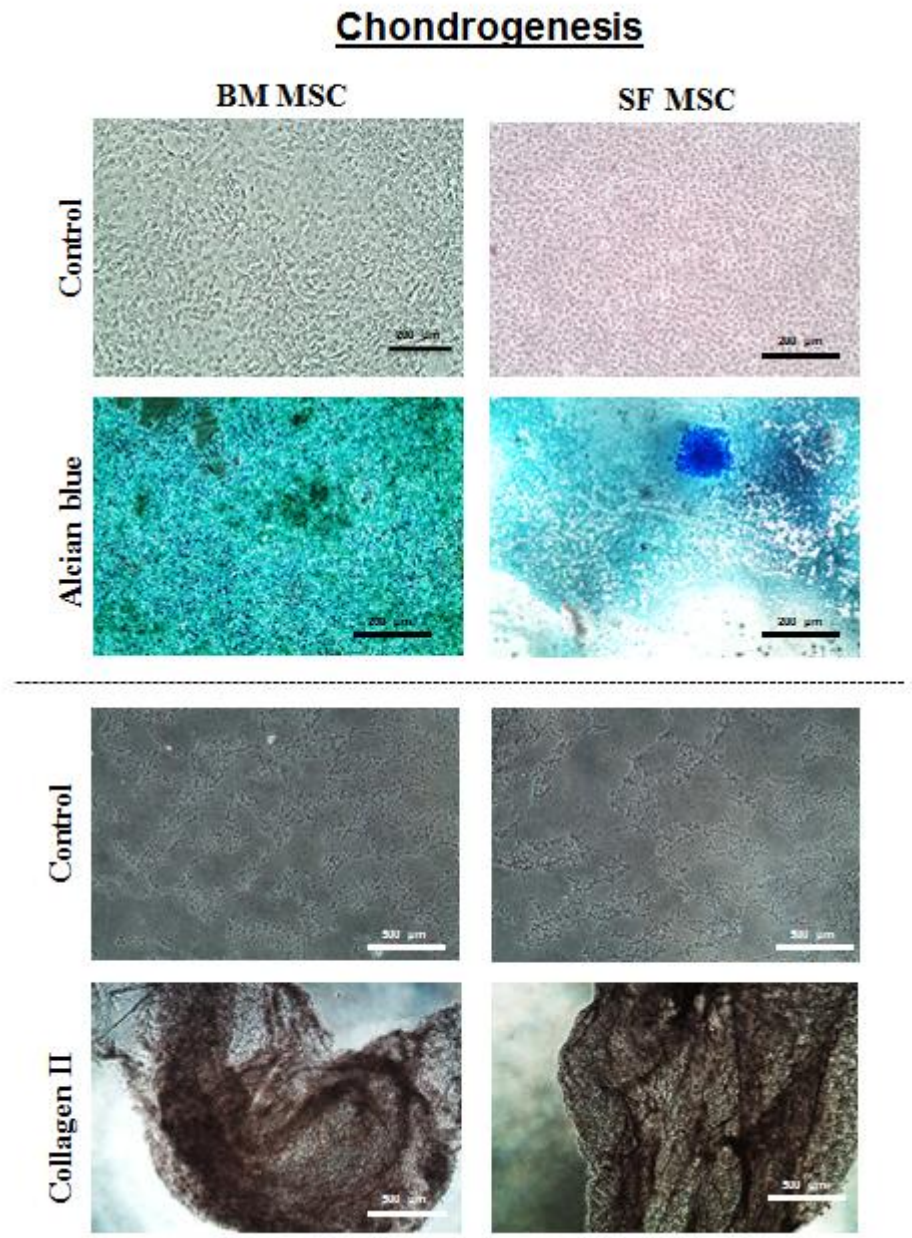


Figure 4.3: MSCs isolated from the BM and SF differentiated into chondrocytes. Control cultures were maintained in MSC complete media. Chondrogenesis was induced by stimulating 3D micromass cultures with standard chondroinductive media. Alcian blue was used to detect glycosaminoglycan deposition and collagen II immunohistochemistry was used for detecting collagen II formation in the micromass cultures. Images were taken at x10 magnification.

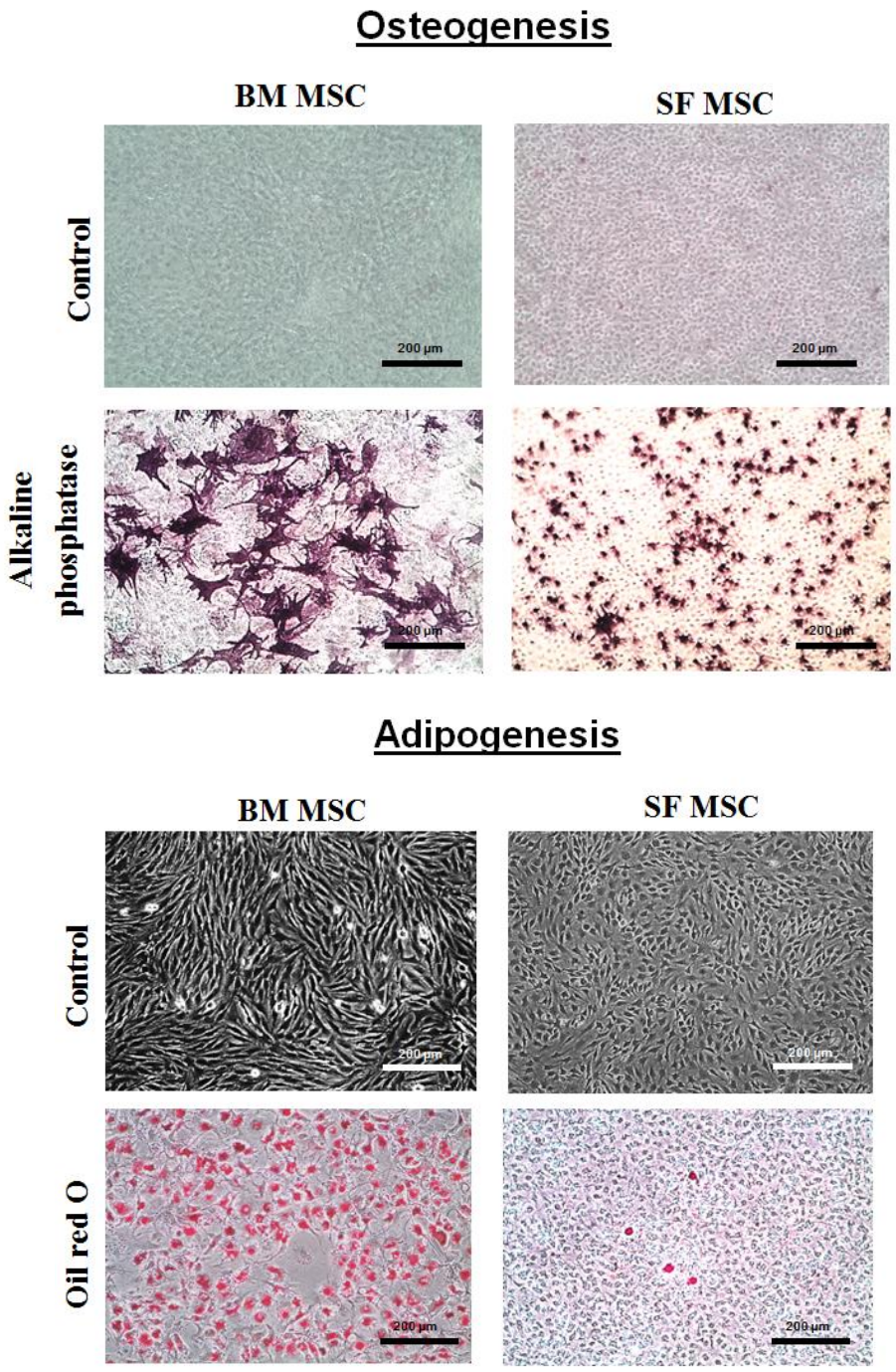


Figure 4.4: MSCs isolated from the BM and SF differentiated into osteoblasts and adipocytes. Control cultures were maintained in MSC complete media. Osteogenesis and adipogenesis was induced by stimulating monolayer MSC cultures with standard osteoinductive and adipoinductive media respectively. Differentiation was detected using, alkaline phosphatase for osteogenesis and oil red for Adipogenesis. Images were taken at x10 magnification.

4.4 Chapter discussion

MSCs were isolated from the SF and BM of the abattoir-derived bovine metacarpophalangeal joints based on their plastic adherence under monolayer culture conditions. Initially, until passage 2, MSC from each bovine joint were cultured in separate flasks to minimise the risk of infection. This also allowed observation of rate of SF MSC proliferation between different colonies (cells from different animals) and with their matched BM counterparts. Following plating both SF and BM MSC appeared as small round cells. SF MSCs consistently attached to the flask surface by day 2 whereas BM MSC attached between day 2 and day 4. SF and BM MSCs also differed in their morphology. For example, SF MSCs were small and less prominent nucleus whereas BM MSCs had long projections and prominent nucleoli (Figure 4.1 and 4.2). Longer proliferation time observed in our study is consistent with previous findings which showed that bovine SF MSC colonies had shorter population doubling times compared to BM MSC colonies [30].

Previous studies have reported that use of plastic adherence to isolate MSC from various animal models including rodents, pigs, cattle and sheep. In this study we used a skeletally matured bovine model due to its similarities in physiology and organ size with the humans [89]. Moreover, due to their longer life span compared to the laboratory animal models, bovine experimental model is considered to be good for long term experiments in regenerative medicine. It has been suggested that cells isolated by this method contains a heterogeneous population of progenitor cells that have various degrees of commitment. For example, at passage 0, we observed a mixture of elongated spindle-shaped fibroblast-like BM MSCs along with small round hematopoietic cells. This is similar to previous reports where flow cytometry analysis showed the presence of both CD271+ colonies which were multipotent and entirely mesenchymal along with CD45+ hematopoietic lineage cells [29]. However, in contrast to BM MSC cultures, we did not observe any phenotypic heterogeneity in SF MSC cultures at passage 0.

The source of the SF MSC is still unknown. A study by Jones *et al* suggested that SF MSC may be derived through break down of synovium [29]. Their suggestion is based on the finding that there was a positive correlation between the number of synovial microfragments and number of SF MSC. We have also observed

synovial microfragments in our undiluted synovial fluid samples from the bovine joint. However, since these fragments could also be a source for synoviocytes, they were removed from the diluted synovial fluid through filtration before centrifugation to isolate the MSCs. This reduced the number of MSCs isolated but allowed a homogenous population of SF MSC to be cultured.

Multipotent differentiation potential of both SF and BM MSC was evaluated using standard inductive media as described in the methods. Previous studies have suggested a dose-dependent effect of ascorbic acid and FCS on osteogenic differentiation of BM MSC [69, 73]. Using a bovine foetal model as a source of BM MSC, they showed that increasing concentration of ascorbic acid led to higher alkaline phosphatase activity and stronger mineralisation. Similarly, increasing FCS concentration also led to stronger mineralisation but did not affect the alkaline phosphatase activity. These results suggested that osteogenic differentiation could be enhanced by a higher ascorbic acid concentration and reduction of FCS to 2% or 5% may affect the differentiation process. However, since we only used differentiation as a measure for validating that our isolated cells were MSCs, we only analysed MSC differentiation under standard condition of 50 µg/ml ascorbic acid and 2% FCS. At these concentrations we observed osteogenic differentiation of both SF and BM MSC. Our chondroinductive media was supplemented with TGFβ1 which resulted in the production of GAG as confirmed both qualitatively using alcian blue staining and quantitatively using DMB assay. Similar results were previously reported by other studies using the same concentration of TGFβ. However, these studies also reported spontaneous MSC differentiation in the absence of TGFβ. However, results from the micromass assays for both SF and BM MSCs cultured in control media (high glucose DMEM supplemented with 10% FCS) where no GAG formation was observed suggested that supplementation of chondrogenic factors was required to stimulate MSC differentiation.

Using the standard inductive media, cultured SF and BM MSCs differentiated into chondrogenic, osteogenic and adipogenic lineages (Figure 4.3 and 4.4). However, SF MSC had lower adipogenic capacity than BM MSC. This is in agreement with results reported by Jones *et al* [29] which suggested that even though both SF and

BM MSCs had the potential to differentiate into bone, cartilage and fat, normal SF MSCs are considerably different in their phenotype from their BM counterparts.

Chapter 5

Methods for studying MSC migration

5.1 Objective

To assess MSC migration using the commonly used migration assays such as transwell assay, scratch assay and cell exclusion zone assay.

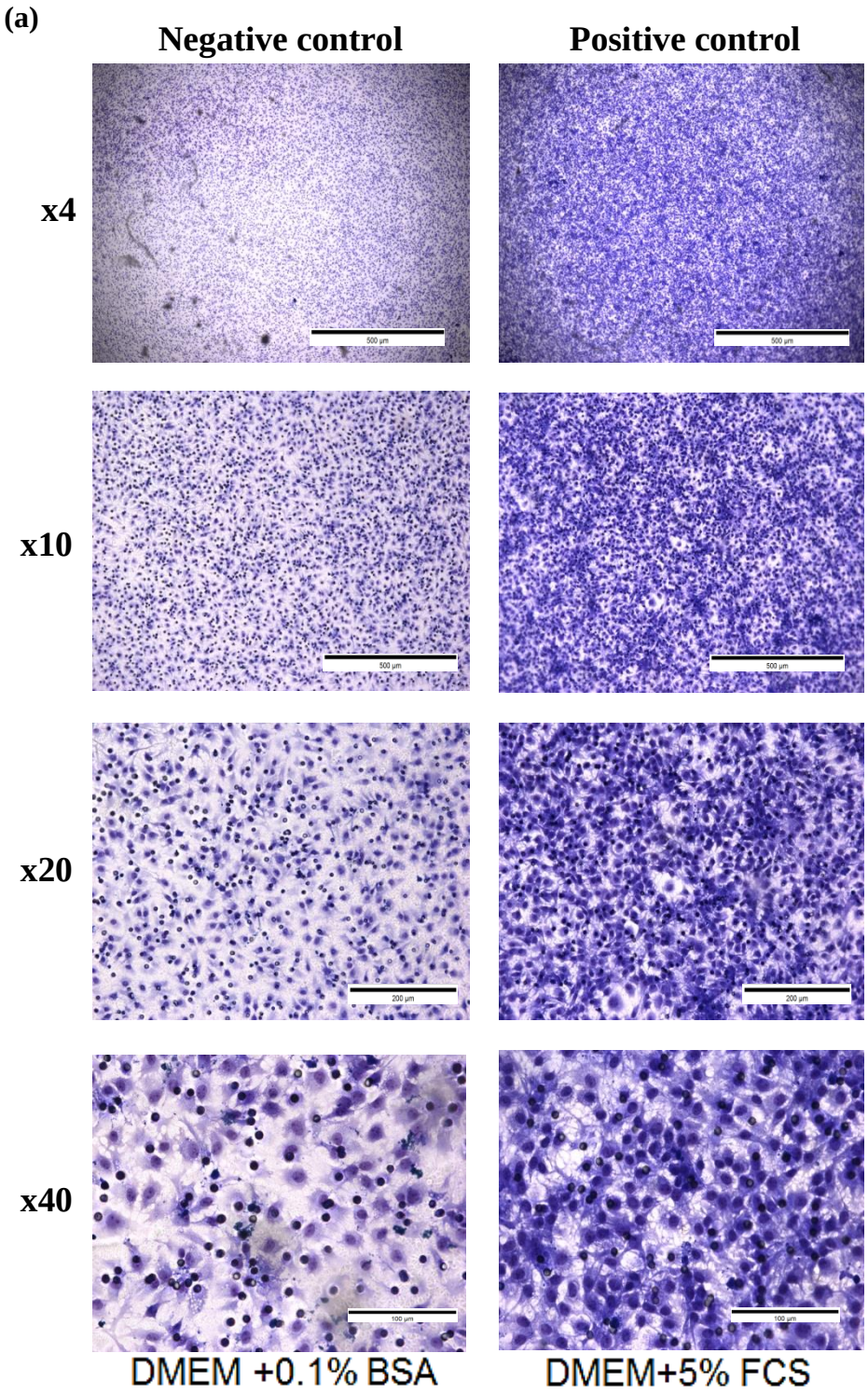
5.2 Chapter Introduction

Even though the commonly used migration assays are widely used and can reliably detect differences in cell migration in response to various stimuli, there are insufficient reports in the literature on assessment of bovine and human SF and BM MSC chemotactic migration using these techniques. The aim of this study was to optimise the conditions of the common migration assays to study bovine and human MSC migration in response to various stimuli. The three assays optimised in this study were Transwell assay, Scratch assay and Cell exclusion zone assay.

5.3 Results

5.3.1 Imaging membranes following Transwell migration assay

Transwell assays were carried out according to the protocol described in the methods (Section 3.16.1). Initially, fixed membranes were imaged under a light microscope using x4, x10, x20 and x40 lens (Figure 5.1a). Even though at x4 and x10 it was possible to identify the migrated cells, only using x20 and x40 it was possible to distinguish the lower openings of the membrane pores from the upper openings (Figure 5.1b). Between x20 and x40, x20 was chosen as the magnification for all subsequent experiments because it allowed imaging of greater membrane area. 5 different microscopic fields were imaged per membrane at x20 magnification for all subsequent experiments. Once, the membrane was cut out of the well, it's orientation with shiny side on the top also allowed to positively identify the location of the cells on the lower side of the membrane.


(a)
Negative control
Positive control
x4
x10
x20
x40
DMEM +0.1% BSA
DMEM+5% FCS

(b)

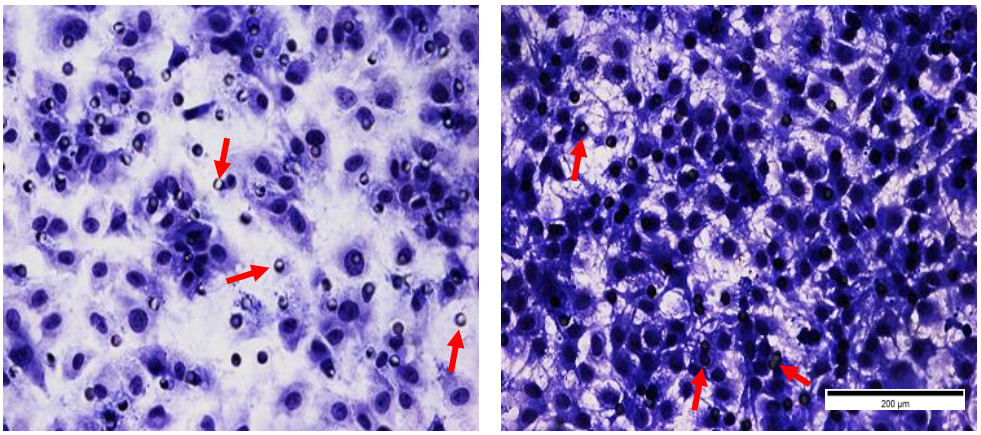


Figure 5.1: (a) Bovine SF-MSC migration in response to serum-free media and media supplemented with 5% serum. Same membrane was imaged at various magnifications to identify the optimal magnification for digital imaging which will then be used to quantify the number of migrated cells. x4 magnification allowed visualisation of greater membrane area however, it was more difficult to distinguish between cells which might lead to inaccurate quantification of the number of migrated cells. Only at x20 and x40 could individual cells be reliably identified. **(b) Migration of BM and SF MSC to 5% serum imaged at x20 magnification.** Cells were stained with crystal violet and the clear holes are the pores in the membrane through which the cells migrate (red arrows). BM MSCs had wider cytoplasmic projections compared to SF MSCs. The cell morphology of the migrating cells was similar for both MSCs.

5.3.2 Influence of collagen and fibronectin pre-coating on MSC migration in Transwell assay

Influence of pre-coating the lower side of the membrane on chemotactic migration was tested using SF MSCs [88]. Membrane was either pre-coated with collagen or fibronectin by incubating the lower side of the membrane with the ECM solution for an hour. ECM solution did not touch the upper side of the membrane. A significant increase in SF MSC migration was observed when the membrane was pre-coated with fibronectin compared to control with all serum concentrations. For example, 0.5 fold increase in migration ($p<0.05$) at 0% serum concentration, 0.9 fold increase ($p<0.05$) at 1% serum concentration and 1.4 fold increase at 10% serum concentration ($p<0.05$) was observed with fibronectin coating compared to collagen coating. Moreover, a serum concentration dependent significant increase in migration was observed with fibronectin coating which was not seen with collagen coating (Figure 5.2). Hence, lower side of the membrane was coated with fibronectin for all further experiments to increase the sensitivity of the migration assay.

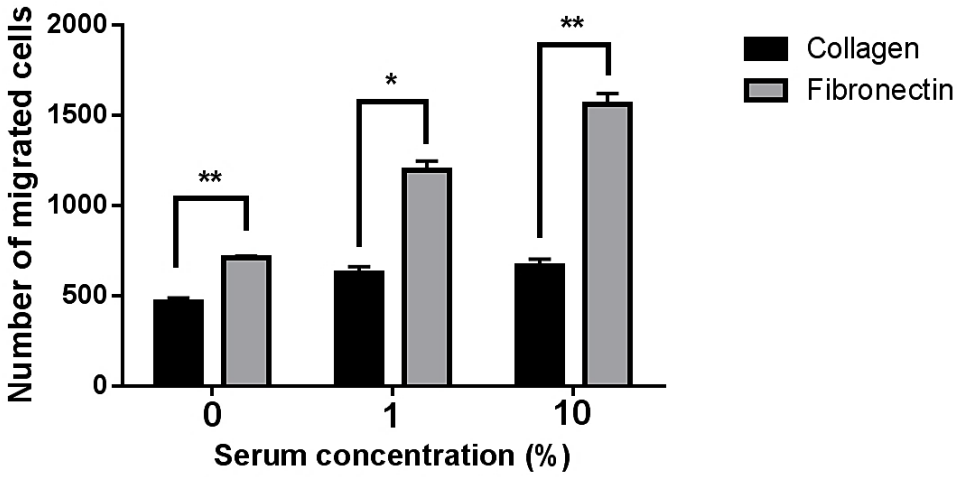


Figure 5.2: Influence of membrane pre-coating on number of migrated cells. Graphs showing mean number of migrated cells ± SEM. Membranes were either pre-coated with collagen or fibronectin and SF-MSC migration were assessed at various serum concentrations. Fibronectin pre-coating significantly increased MSC migration in response to all serum concentrations compared to fibronectin. * and ** $p<0.05$.

5.3.3 Assessment of MSC migration in response to varying concentration of serum in Transwell assay

Both SF and BM MSCs migrated in the absence of serum in the media (DMEM+0.1% BSA). Presence of serum significantly increased SF MSC migration such that at SF MSC demonstrated a 0.8 fold increase ($p<0.001$) in response to 1% serum and 0.5 fold ($p<0.0001$) to 5% serum compared to 0% serum (Figure 5.3 and 5.5). Significant increase in BM MSC migration was observed only in response to 5% serum (3 fold increase ($p<0.0001$) compared to 0% serum) (Figure 5.4 and 5.5). Since significant migration was observed in response to 5% serum with both SF and BM MSCs but not with 1% serum with BM MSCs, 0% serum concentration was chosen as negative control and 5% serum concentration was chosen as positive control for all subsequent transwell migration assays. These results also suggested that the optimised protocol allowed reliable induction and determination of MSC migration in response to a chemoattractant stimulus.

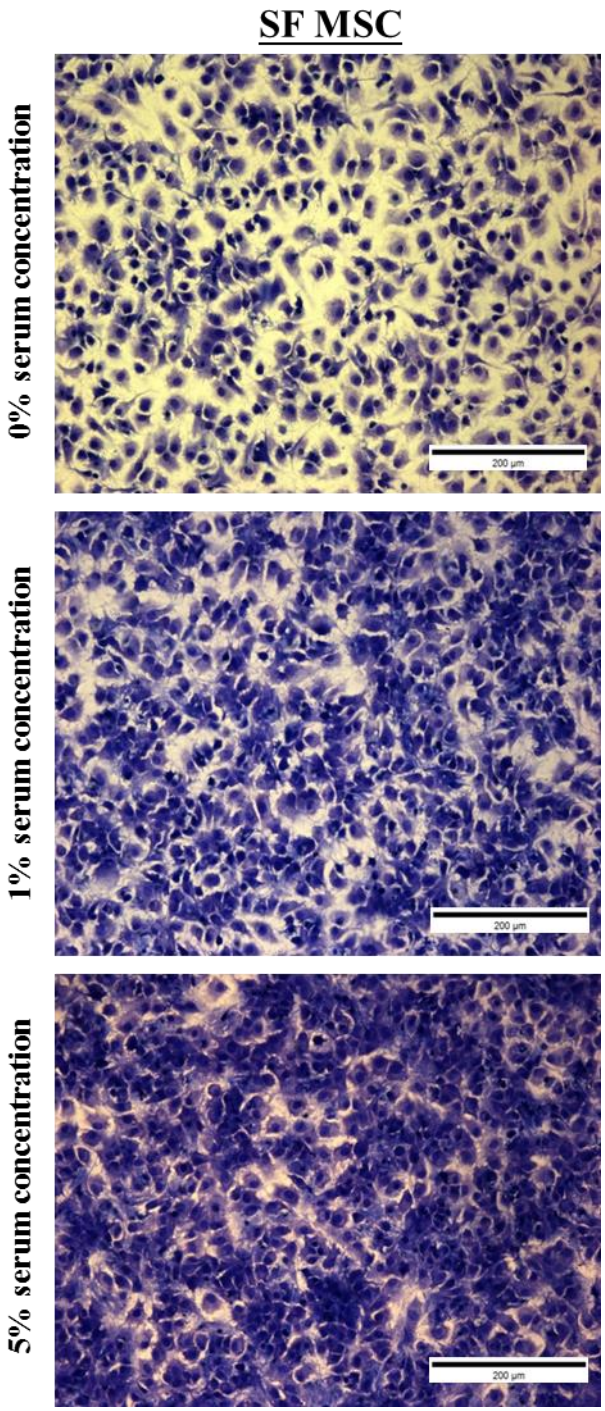


Figure 5.3: Migration of bovine SF MSCs over 24 h in response to various serum concentrations. Representative image of one microscopic field at x20 magnification is shown here. Membranes were stained with crystal violet. Visually, an increase in migration with increase in serum concentration was seen. However, no such linear relationship was observed when the number of migrated cells was quantified.

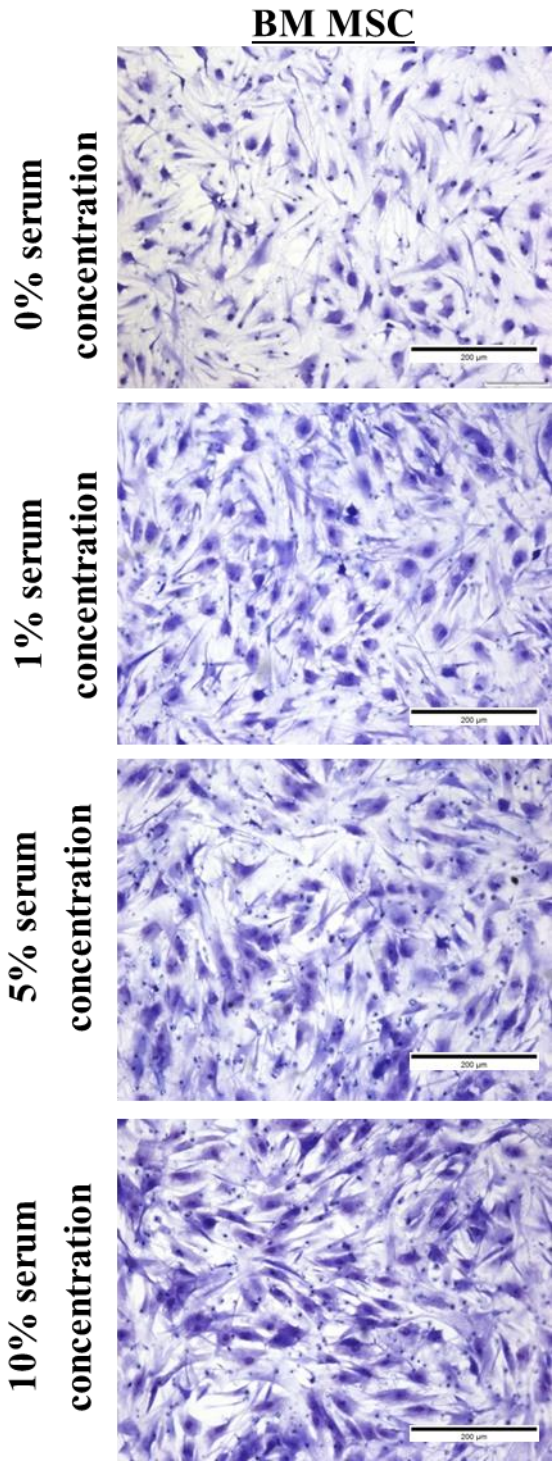


Figure 5.4: Migration of bovine BM MSCs over 24h in response to various serum concentrations. Representative image of one microscopic field at x20 magnification is shown here. Membranes were stained with crystal violet. Visually, an increase in migration with increase in serum concentration was seen. However, no such linear relationship was observed when the number of migrated cells was quantified.

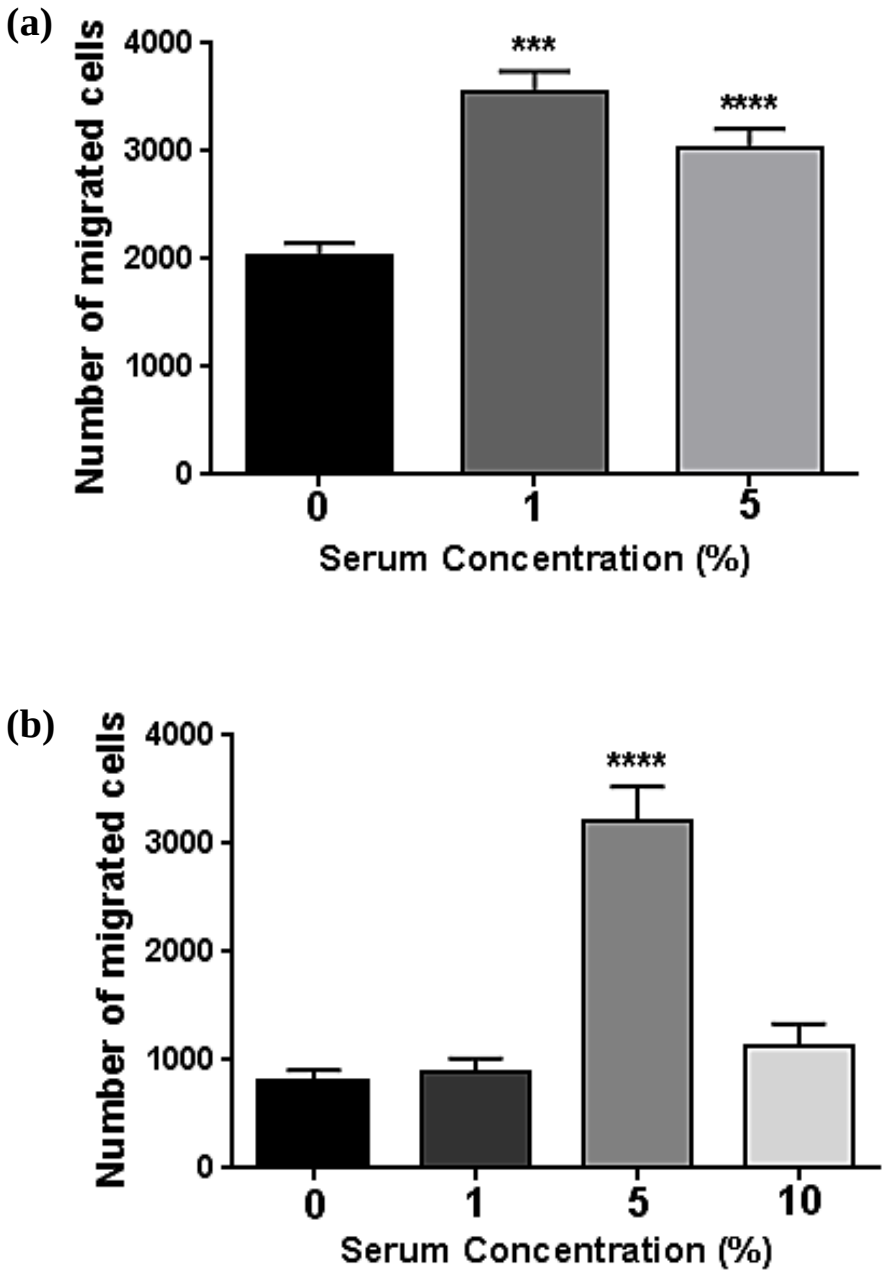


Figure 5.5: Migration of bovine (a) SF and (b) BM MSCs over 24h in response to various serum concentrations. Graphs showing mean number of migrated cells ± SEM. (a) Presence of serum significantly increased the number of migrated SF MSCs. However, no significant increase was observed when the serum concentration was increased from 1% to 5%. (b) In contrast, no significant increase in migration was observed in response to 1% serum compared to serum free media with BM MSCs. Significant increase was only observed with 5% serum. *** $p<0.001$ and **** $p<0.0001$ compared to 0% serum concentration.

5.3.4 Assessment of MSC migration at multiple time points in Transwell assay

Migratory potential of MSCs was evaluated at multiple time points to optimise the time of the migration assay. Migration assay was terminated at 4 h, 8 h and 24 h. Our MSC cultures suggested that neither SF- nor BM MSCs proliferated within any of these time points which are in agreement with previous findings [91, 92]. Number of migrated cells was quantified following termination of assay at each time point. Significant increase in SF MSC migration in response to 5% serum concentration (positive control) compared to 0% serum concentration (negative control) was only observed following 8 h migration ($p<0.05$) and maintained at 24 h migration ($p<0.05$) but not after 4 h migration (Figure 5.6 and 5.8). In contrast, 5% serum concentration significantly increased BM MSC migration compared to 0% serum concentration at all three time points ($p<0.05$) (Figure 5.7 and 5.8). Since, significant increase in migration was observed at both 8 h and 24 h for SF and BM MSCs, 24 h was chosen as the duration of the assay in order to allow maximum time for MSC migration.

Post-hoc (Holm Sidak) analysis followed by one-way ANOVA suggested a significant increase in SF MSC migration with time in response to 5% serum concentration ($p<0.05$). For example, 2.1 fold increase ($p<0.05$) in migration at 8 h and 1.9 fold increase ($p<0.05$) in migration at 24 h was observed compared to 4 h. Significant increase in migration in response to 0% serum concentration was only observed following 24 h migration. 0.6 fold increase in migration at 8 h and 1.6 fold increase ($p<0.05$) in migration at 24 h was observed compared to 4 h. In contrast, significant increase in migration was observed with time for both 0% and 5% serum concentration with BM MSC. For example, 2 fold increase ($p<0.01$) and 2.4 fold increase in migration ($p<0.05$) was observed compared to 4h for 0% serum concentration. Similarly, 2.8 fold increase in migration ($p<0.05$) was observed at both 8 h and 24 h compared to 4 h with 5% serum concentration.

Pearson product-moment correlation suggested that there was a strong correlation between number of migrated cells over time for SF MSC with 0% serum concentration ($R^2 = 0.98$) and for BM MSC with 5% serum concentration ($R^2 = 0.87$). Only a weak correlation was observed for SF MSC with 5% serum

concentration (R^2 = 0.36) and for BM MSC with 0% serum concentration (R^2 = 0.58). ANOVA analysis of the correlations suggested that the regressions were not statistically significant indicating that migration assay time cannot be used to predict the number of migrated cells.

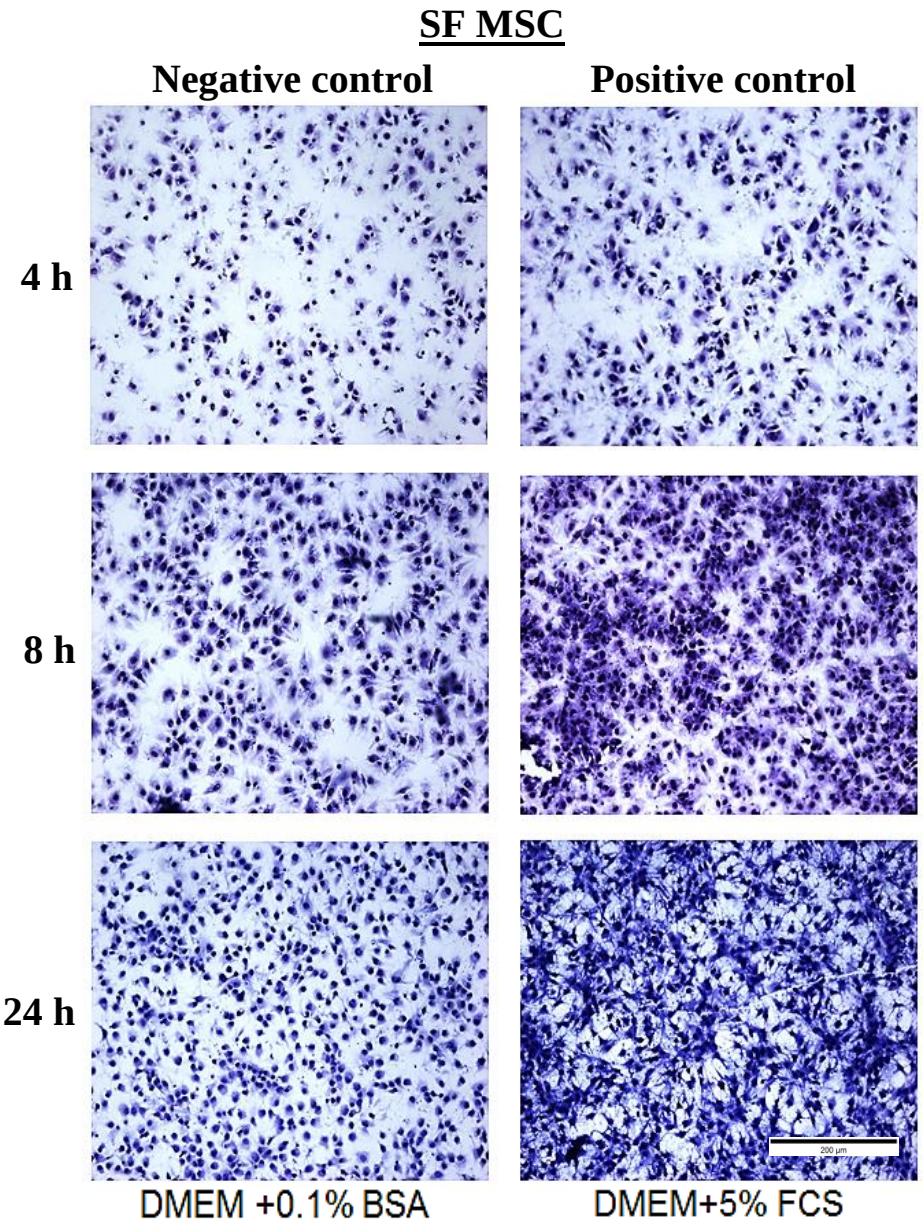


Figure 5.6: Bovine SF-MSC migration in response to serum free media and media containing 5% serum over time. Membranes were stained with crystal violet. Representative image of one microscopic field at x20 magnification is shown here.

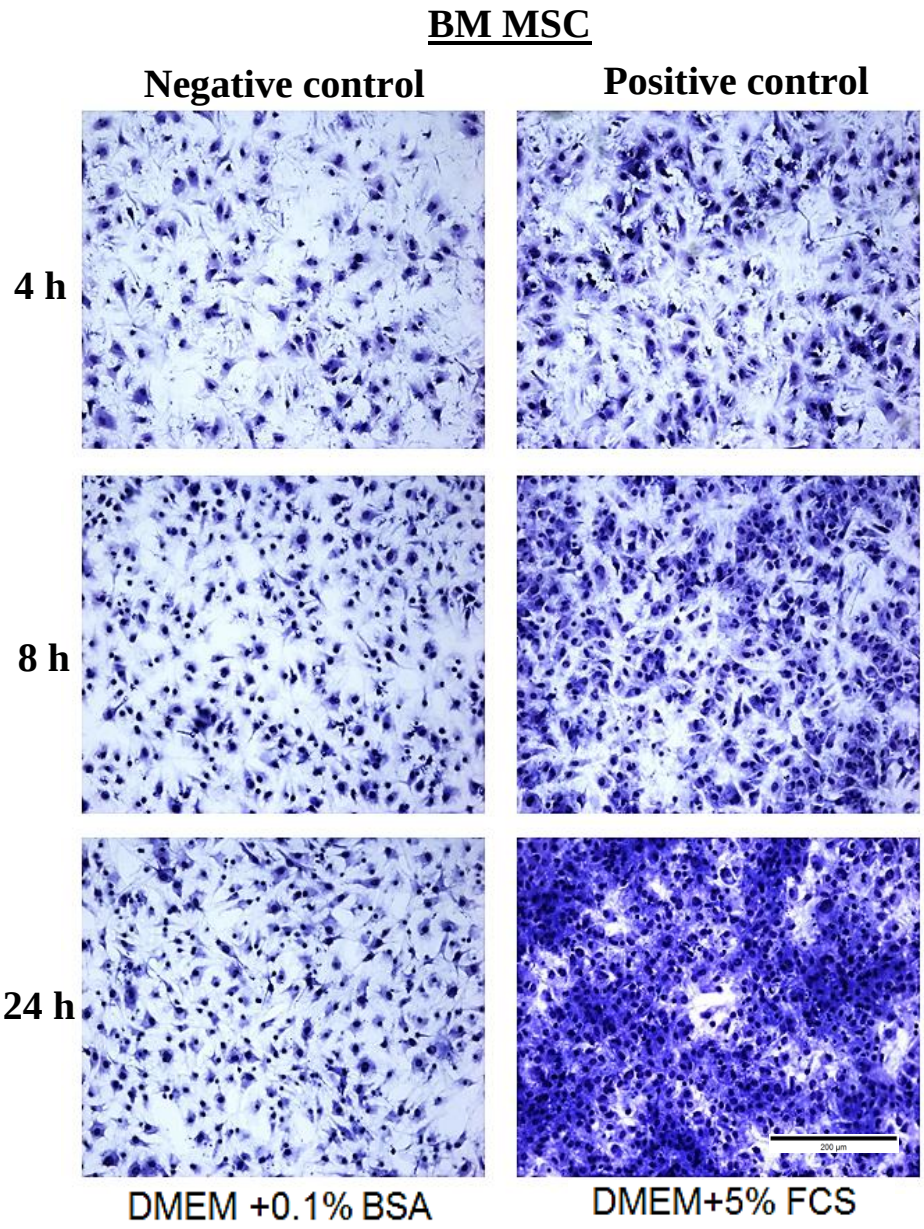


Figure 5.7: Bovine BM-MSC migration in response to serum free media and media containing 5% serum over time.
Membranes were stained with crystal violet. Representative image of one microscopic field at x20 magnification is shown here.

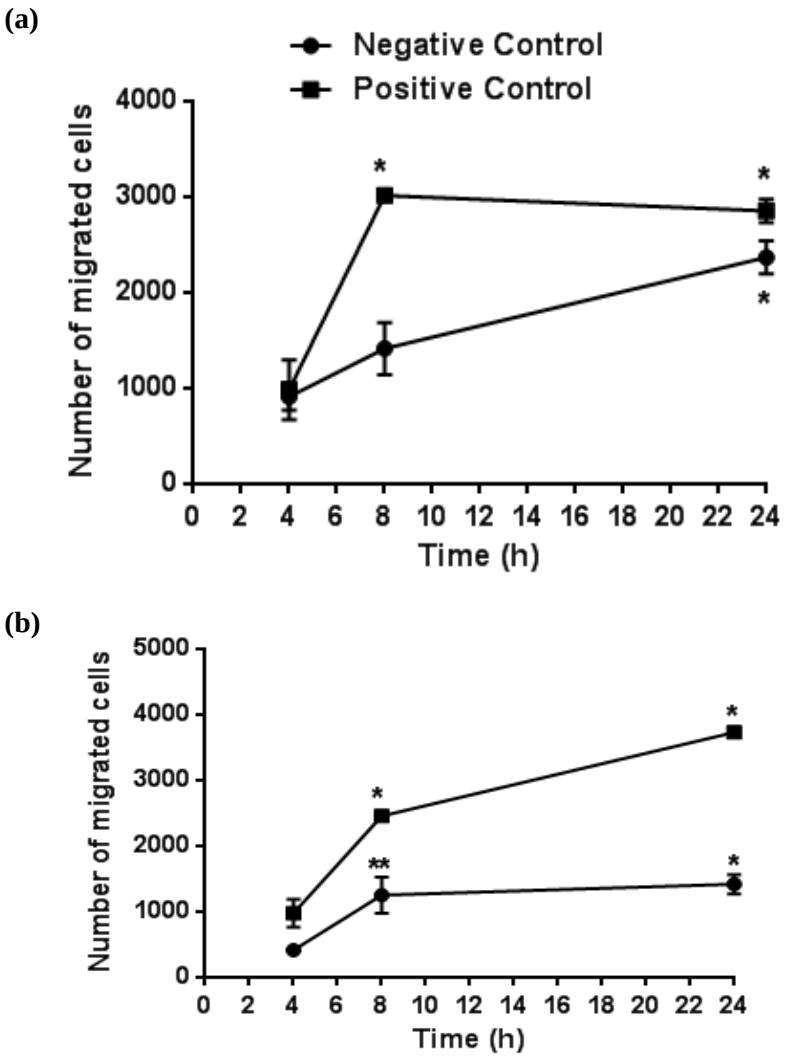


Figure 5.8: Graph showing the mean number of migrated cells ± SEM over time. Assays were terminated at 4 h, 8 h and 24 h and number of cells was quantified at each time point. * $p<0.05$ and ** $p<0.01$ compared to 4 h for respective controls.

5.3.5 Assessment of human bone marrow mesenchymal stem cell migration in Transwell assay

Following establishing that chemotactic migration of bovine MSCs can be studied using the Transwell assay system, we assessed human bone marrow mesenchymal stem cell (hBM MSC) migration in the Transwell assay. The lower side of the membrane was coated with fibronectin and the assay was set up as described previously for bovine MSCs. Following assay termination, cells were stained with crystal violet and imaged using light microscopy. Migrated cells could be reliably identified at x20 magnification. Number of migrated cell was counted in 5 different microscopic fields as described before for bovine MSCs [93] (Figure 5.9).

Similarly to bovine BM MSCs, hBM MSCs migrated in the absence of serum. No significant difference in migration was observed in the presence of 0.1% serum in the media. However, a serum concentration dependent significant increase in migration was observed between 1% serum concentration and 10% serum concentration. For example, 0.6 fold increase ($p<0.001$) in migration was observed in response to 1% serum compared to 0% serum and 0.4 fold increase ($p<0.001$) in migration was observed at 10% serum compared to 1% serum (Figure 5.10). These results suggested that Transwell assays could be used to study MSC migration in response to a chemoattract.

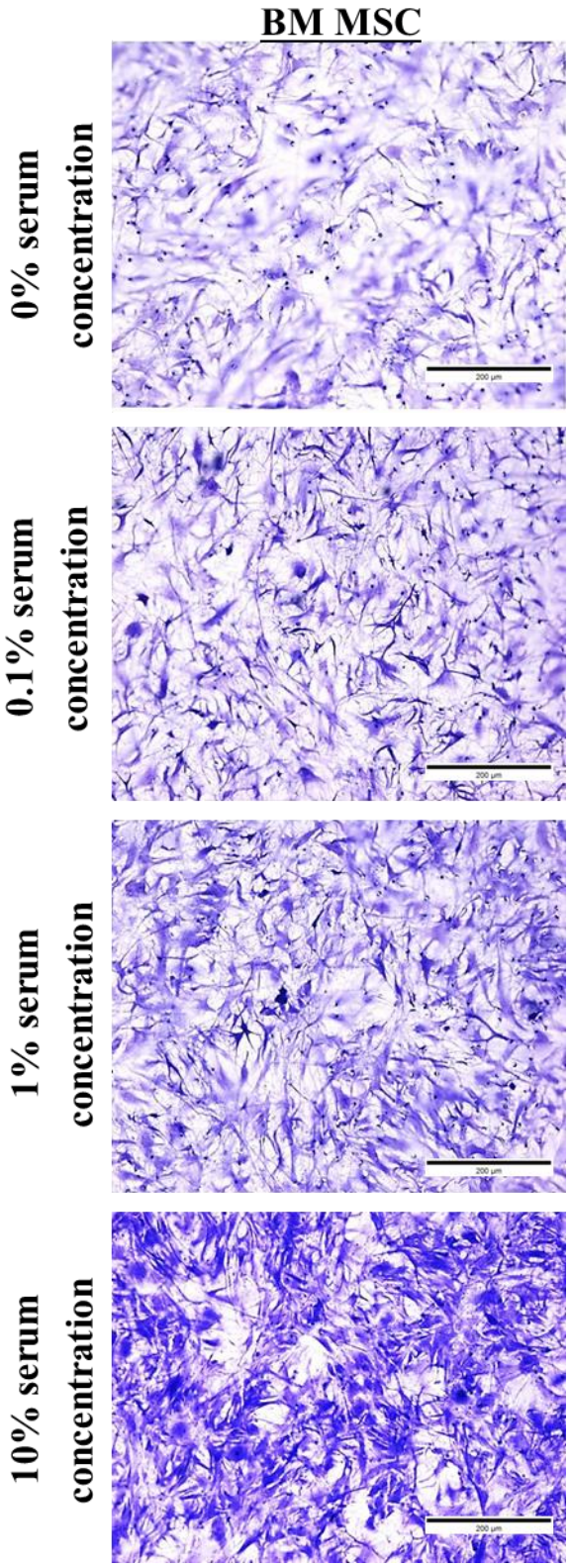


Figure 5.9: Human BM MSC migration over 24 h in response to various serum concentrations. Membranes were stained with crystal violet. Representative image of one microscopic field at x20 magnification is shown here.

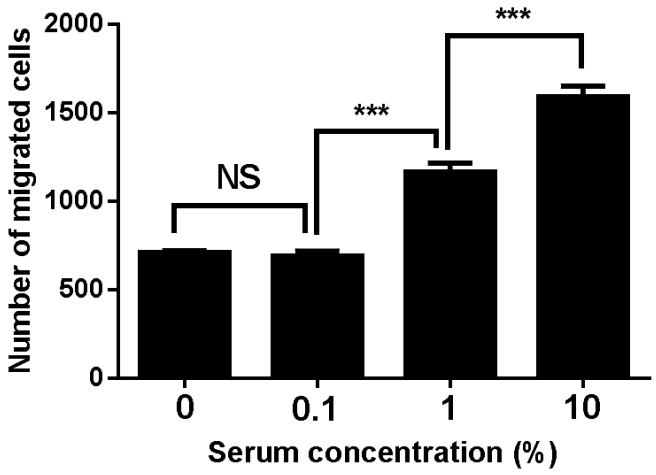


Figure 5.10: Migration human BM MSCs over 24 h in response to various serum concentrations. Graphs showing mean number of migrated cells ± SEM. Presence of 0.1% serum did not significantly increase MSC migration compared to serum free media. However, 1% serum significantly increased migration compared to both serum free media ($p<0.001$) and 0.1% serum ($p<0.001$). Similarly, 10% serum significantly increased migration compared to serum free media ($p<0.0001$), 0.1% serum ($p<0.0001$) and 1% serum ($p<0.001$). *** $p<0.001$ and **** $p<0.0001$

5.3.6 Quantifying cell migration using calcien AM: comparison between calcein AM and crystal violet staining method

Two different cell types were used to compare calcein-AM and crystal violet staining as methods for quantifying the number of migrated cells. With crystal violet staining, cells need to be manually counted which is time consuming. In contrast, calcein-AM allows rapid analysis of cell migration as the fluorescence can be read immediately using the microplate reader within 7 mins. However, crystal violet allowed direct measurement of the number of migrated cells whereas calcein-AM provides a comparative value in relative fluorescence unit (RFU) among different stimuli. The RFU can be converted to number of migrated cells,

but this requires a standard curve. Moreover, the results suggested that there was a difference in the fluorescence reading depending on if the plate was read from the top or from the bottom on the plate reader.

Using top reading, no significant difference in migration with increasing serum concentration was observed with bovine SF MSCs (bSF MSCs) and only a significant increase was observed at 10% serum concentration with human BM MSCs (hBM MSCs) (Figure 5.11). In contrast, when the same reading was taken from the bottom a significant increase was observed with 10% serum concentration in bSF MSCs and with 1% and 10% serum concentration in hBM MSCs. This suggested that bottom reading was more reliable at discriminating between the numbers of migrated cells at different serum concentrations and caused by the migrated cells settling on the bottom of the lower collecting well.

When crystal violet was used to quantify the number of migrated cells in the same experiments, a serum concentrated dependent increase in migration was observed with hBM MSC over a range of 1-10% FCS. Although this serum concentration dependence was not observed with bSF MSC 1 and 10% FCS, a significant difference in migration was observed between presence and addition of 1% serum in the media (Figure 5.11). This suggested that even though the crystal violet method was more time consuming, it was more sensitive and reliable in detecting a difference in migration between different chemoattractants. Hence, we used crystal violet as out method of staining to quantify the number of migrated cells unless a rapid analysis was required.

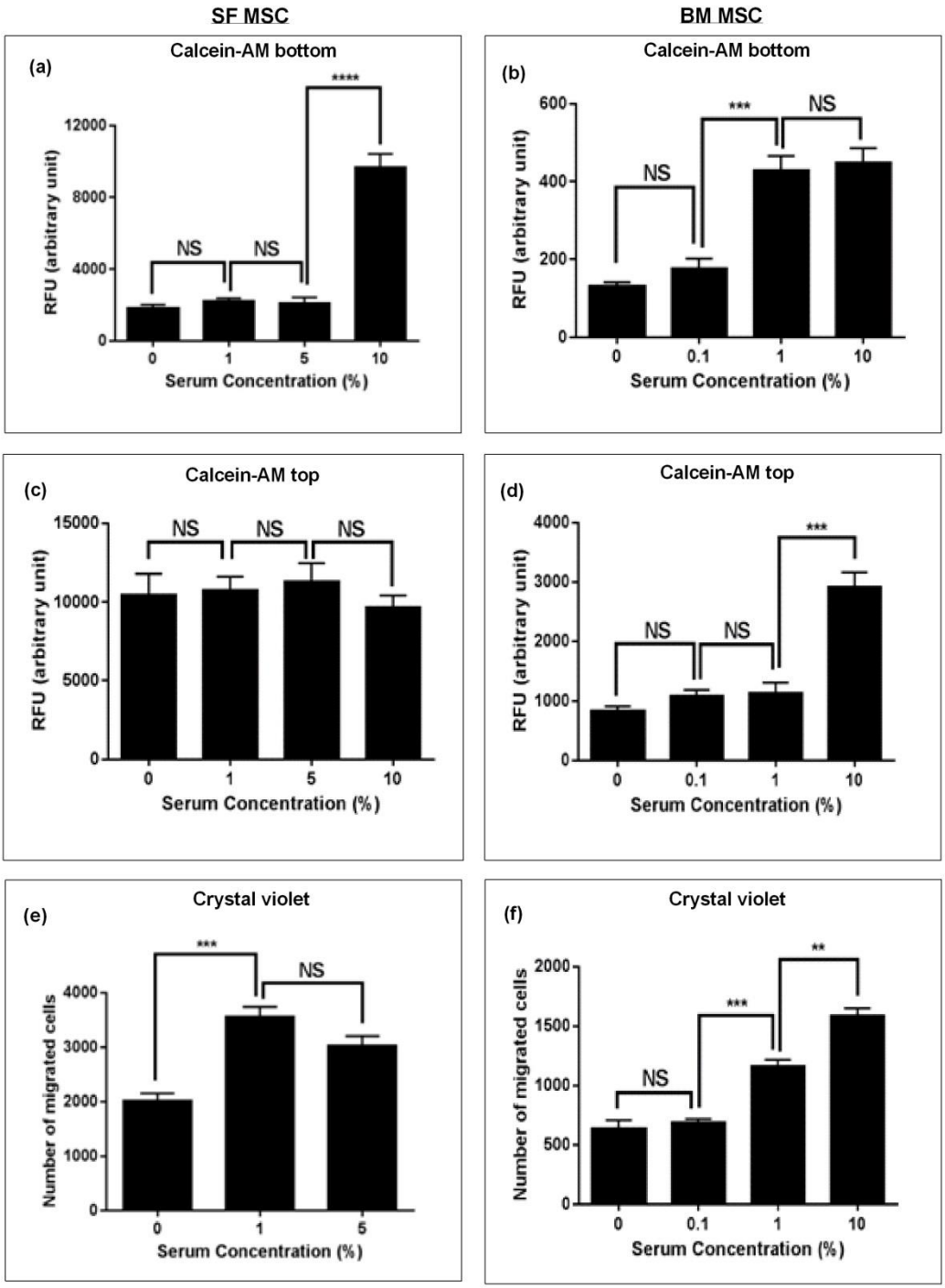


Figure 5.11: Migration of bovine SF MSC (left column) and human BM MSC (right column) in response to various serum concentrations quantified either using calcein-AM (a-d) or crystal violet (e-f).
Fluorescence reading from the bottom of the plate was more sensitive at detecting differences in migration in response to different serum concentrations compared to reading taken from the top of the plate. Further, crystal violet was more sensitive at detecting differences in migration in response to different serum concentrations compared to calcein-AM.

5.3.7 Assessment of MSC migration using scratch assays

Scratch assays were carried out according to the protocol described in the methods (Section 3.16.3) in response to serum free media to establish cell seeding density and duration of migration assay. Since little migration was observed over 30 h (Figure 5.12), and the Transwell assays were carried out for 24 h, 24 h was chosen to be the duration of the SF MSC scratch assay. All further experiments involving SF MSCs were terminated following 24 h and serum free media was used as negative control. Reduction in wound width over 24 h was calculated using the formula below to quantify MSC migration in scratch assays (Figure 5.13).

$$\%\,\boldsymbol{reduction\ in\ wound\ width} = \frac{(width\ (0\ h) - width\ (24\ h)}{width\ (0\ h)} \times 100$$

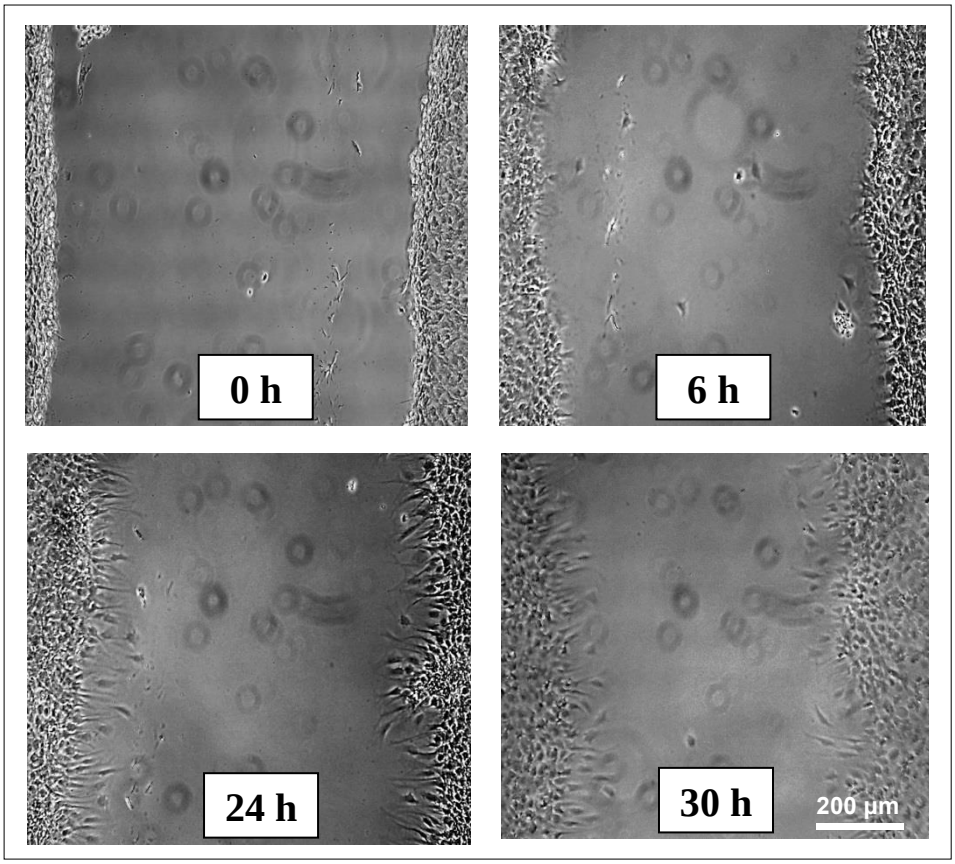


Figure 5.12: Migration of bovine SF MSCs in response to serum free media (DMEM+0.1% BSA) was evaluated using the monolayer scratch assay. Migration was monitored microscopically at a regular interval and imaged at 0 h (immediately following creation of the wound), 6 h, 24 h and 30 h. *Please scan the QR code to watch the time-lapse video of SF MSC migration in scratch assay.*

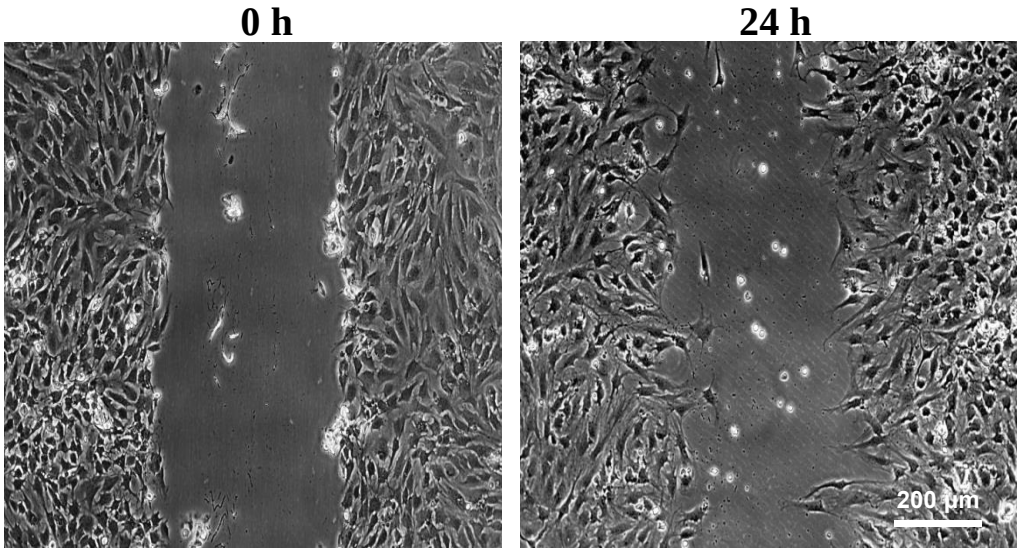


Figure 5.13: Reduction in wound width was measured to quantify MSC migration over 24 h. Width was measured as described in the methods and averaged to get the wound width at 0 h (immediately following creation of the wound) and 24 h following creation of the wound. Reduction in wound width was measured by subtracting wound width at 0h from wound width at 24 h. Migration was quantified as percentage reduction in wound width using the formula: $\frac{(\text{width (0h)} - \text{width (24h)}}{\text{width (0h)}} \times 100$. *Please scan the QR code to watch how this analysis was done used Image-j.*

When the same protocol was followed to carry out scratch assays using bovine BM MSCs, using serum free media as negative control, cells detached from the plate over 24 h (Figure 5.14).

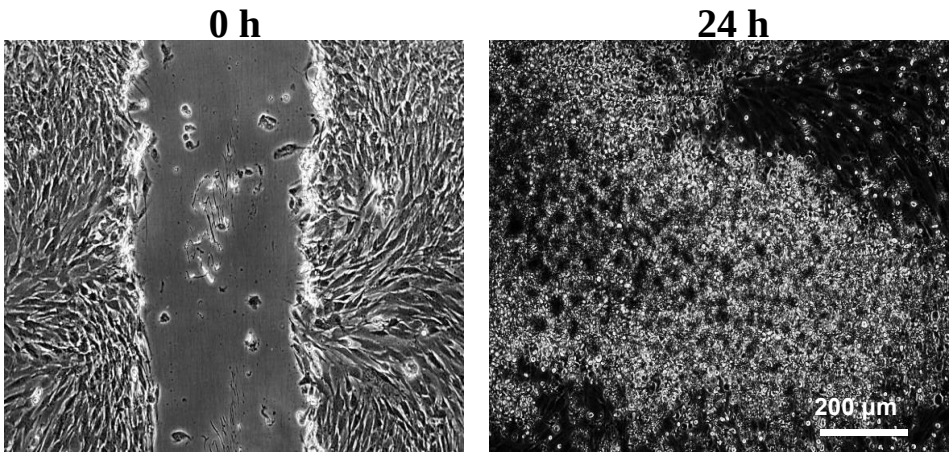


Figure 5.14: Migration of bovine BM MSCs was evaluated using the monolayer scratch assay. BM MSCs lost their plastic adherence following 24 h migration in serum free media (DMEM+0.1% BSA**).**

In order to avoid this problem, plates were fibronectin-coated by incubation in 6.5 µg/ml of fibronectin for 1 h at 37°C. Migration was followed over 24 h with images taken at T0 (time of creation of wound), T6 (6 h after creation of wound), T17 (17 h after creation of wound) and T 24 (24 h after creation of wound). MSCs maintained their plastic adherence till T17 but lost this attachment at T 24 (Figure 5.15). Hence, all further scratch assays involving BM MSCs were carried out in fibronectin pre-coated plates and terminated after 17 h (T17) from the creation of the wound.

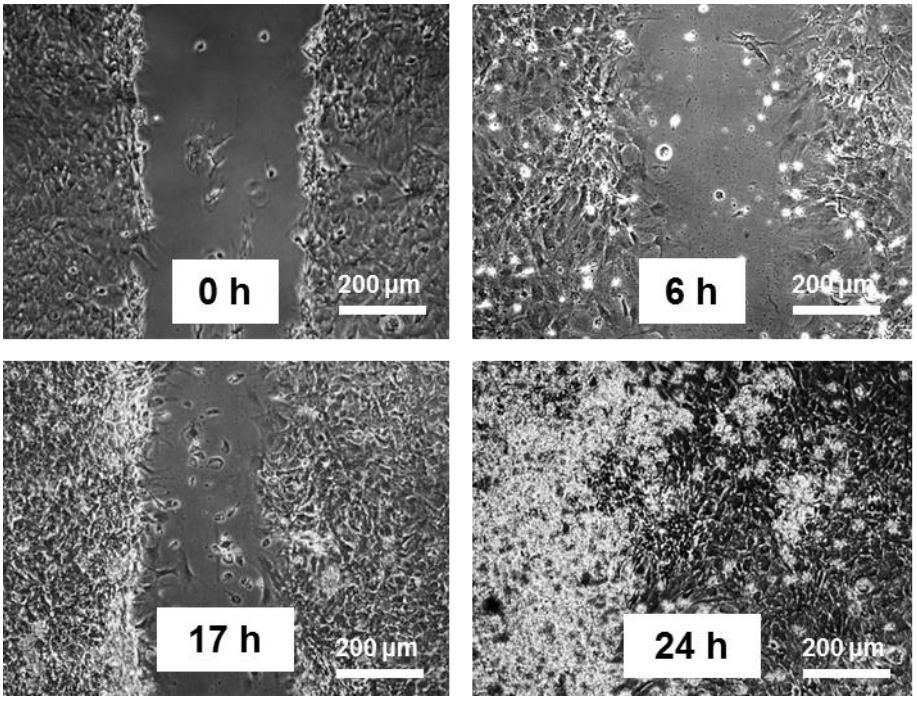


Figure 5.15: Migration of bovine BM MSCs evaluated in monolayer scratch assays. Plate wells were pre-coated with fibronectin prior to cell seeding. BM MSCs retained their plastic adherence in fibronectin coated plates for 17 h but this adherence following 24 h migration. Hence, all scratch assays were performed over 17 h in fibronectin pre-coated plates.

In the scratch assays, migrating SF MSCs formed elongated spindle-like cell bodies, displaying two directional-polarities, with two elongated leading edge one in the direction of the gap and the second to maintain contact with the cells in the monolayer. Migrating cells make multiple contacts either with the same cells or with several different cells in the monolayer to preserve the functional cell-cell junctions (Figure 5.16 a). BM MSCs exhibit a different mode of migration compared to SF MSCs and migrate collectively as sheets of cells (Figure 5.16b).

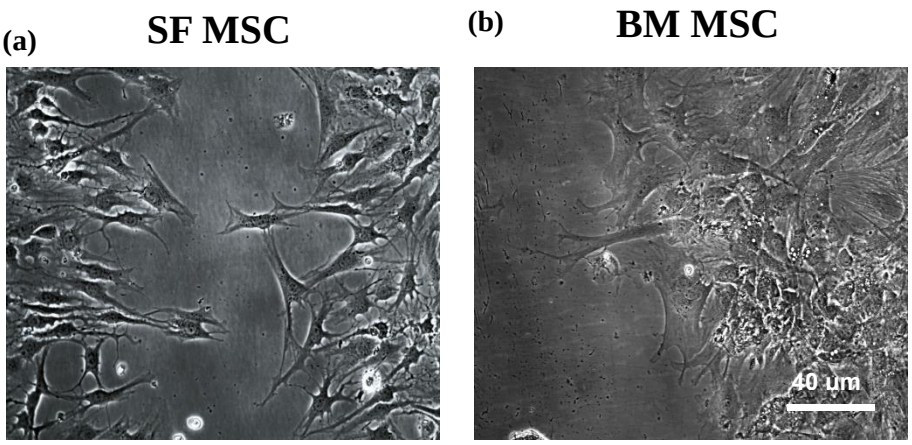


Figure 5.16: Mode of bovine SF MSC (a) and BM MSC (b) migration in scratch assays. SF MSCs form elongated spindle-like projections with bi-directional polarities. BM MSCs in contrast migrate collectively as sheets of cells.

5.3.8 Assessment of MSC migration using cell exclusion zone assays

Cell exclusion zone assays were used to assess the requirement for cell activation or cell injury for MSC migration. We tested two different inserts available commercially from Ibidi (Figure 5.17) and Platypus Technologies Inc (Oris-FLEX inserts) (Figure 5.18).

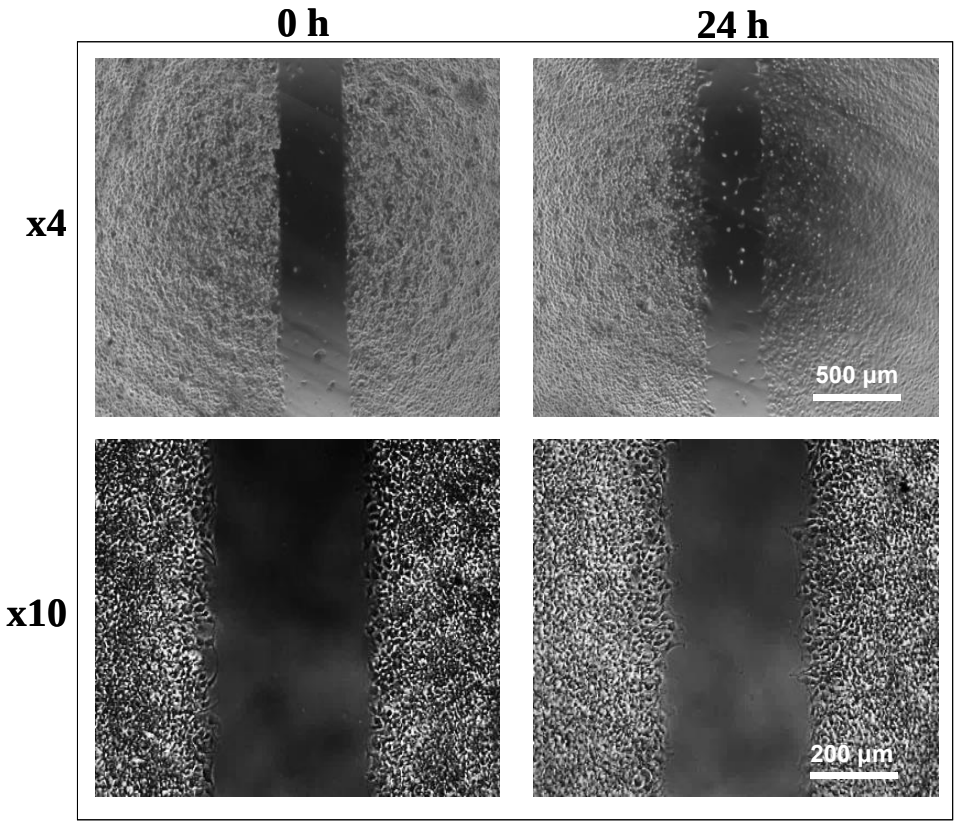


Figure 5.17: Bovine SF MSC migration was evaluated in cell exclusion zone assay using Ibidi inserts. Images were taken at x4 magnification and x10 magnification at 0 h (immediately following creation of wound) and 24 h after creation of wound. Image taken at x10 magnification was optimal for image analysis and measurement of wound width.

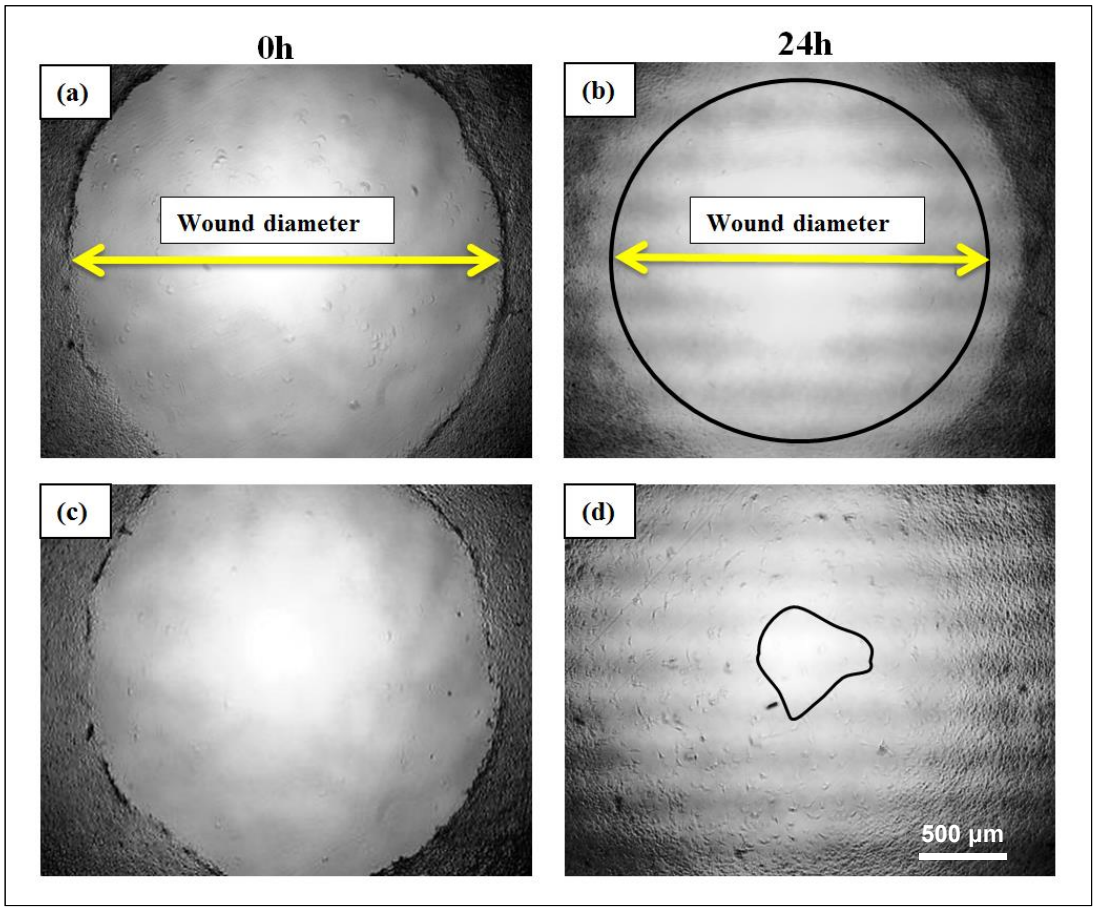


Figure 5.18: Bovine SF MSC migration was evaluated cell exclusion zone assay using Oris-FLEX inserts. Images were taken at x4 magnification at **(a)** 0 h immediately following creation of wound) and **(b)** 24 h after creation of wound. Only at this magnification it was possible to visualise the entire wound area. Wound area was calculated from the diameter of the wound. Reduction in wound area was used to quantify cell migration. **(c, d)** However, problems involved calculating wound closure using this method since not all wounds closed in perfect circles.

5.4 Chapter Discussion

In this study the conditions were optimised for three different assays to assess *in vitro* migration of bovine and human MSC. Three different tissue sources of MSCs were tested. These were were bovine SF MSC, bovine BM MSC and human bone marrow MSC (hBM MSC). Previous studies have reported the use of Transwell assays to investigate hBM MSC [88]. However, it was still important to establish the protocols and conditions for the analysis of bovine MSC migration in response to the serum stimulus and reliably detect the migrated cells for this study. Five conditions were investigated to optimise the Transwell assays. These included identifying the positive and negative controls, point of assay termination, influence of pre-coating the membrane with ECM protein, magnification at which to image the membrane and method for quantifying the number of migrated cells.

Serum has previously been reported to be a potent inducer of MSC migration [88]. In this study, it was found that both bovine and human MSCs can migrate in the absence of serum but presence of serum significantly enhanced this migration. A serum concentration-dependent increase in migration with bovine MSCs was not observed in that a higher migration was observed with 1% serum compared to 5% with bovine SF MSCs (Figure 5.5a), suggesting that 1% serum gave a maximal response. Similarly, a higher migration was observed in response to 5% serum compared to 10% serum concentration with bovine BM MSCs which suggested that 5% FCS may have been optimal for these MSCs (Figure 5.5b). In contrast, a serum concentration dependent level of migration was observed with hBM MSCs in (serum concentrations ranging from 1-10%) (Figure 5.10).

It is possible that the difference observed between human and bovine MSCs was most likely due to a species difference. Alternatively, there are reports [69] of rapid decrease in MSC migratory capacity following brief period of *in vitro* culture. One *in vivo* study observed a 10% reduction in migration of culture-expanded murine BM MSCs to the BM compared to freshly isolated BM MSCs. They suggested that the most likely reason for this loss of migration capacity was the alteration of the migration molecules present on the surface of the cells during their *in vitro* incubation. These include cytokine receptors and adhesion molecules and indeed loss of integrin β1, SH3 and ICAM-1 and reduction in production of extracellular molecules have been previously reported following *in vitro* culture of

human MSCs [94]. It may be possible that the human MSCs were more sensitive to these effects of *in vitro* culture than the bovine MSCs and this accounted for the difference between the cell types.

Moreover, since extended period of culture and repeated passaging affects the MSC phenotype, the bovine MSCs were used within passage 1-3 for these experiments. In combination with avoiding cross contamination between animals and using the MSCs at low passage number, these experiments were performed such that each serum concentration was tested on MSCs from different animals. Therefore, it was also possible that the apparent lack of serum concentration dependent migration for the bovine MSCs was due to intra-animal variation in migration capacity. Further, it is also possible that the expression of receptors on the surface of the MSCs isolated from bovine and human sources are different by their natural physiology. Hence, the differences observed were simply due to differential expression of surface molecules between the two species.

In order to minimise the variabilities in the succeeding migration experiments, bovine SF and BM MSCs were isolated from several animals and they were cultured separately until P1 (passage one) to avoid cross contamination if one sample became infected with microbial or fungal contamination. From P2-P3 MSCs from the different animals were pooled for each type of MSCs. Samples of the pooled MSCs at P1 were stored in liquid nitrogen so that all repeats of the experiment were carried out using the same cells to ensure consistency in the results. Further, all MSCs were cultured in serum which had been specially tested and validated to support the clonal enumeration and expansion of MSCs by the manufacturer. All repeats of the same experiment or comparative experiments were performed using the same lot of serum to avoid variations in culture that may arise from the lot to lot variation of the serum.

These experiments also demonstrated the importance of fibronectin in MSC survival. For example, it was observed that pre-coating the Transwell membranes with fibronectin induced higher MSC migration compared to collagen coating. Furthermore, the BM MSCs did not survive in 24-well tissue culture plates in the absence of serum and cell survival in serum-free medium was only promoted following pre-coating of the well plates with fibronectin. Fibronectin is a dimeric

glycoprotein organised into fibrils to form the insoluble ECM. Many studies have reported the importance of fibronectin interaction with the cell surface receptors in regulating various cell functions such as growth, adhesion, differentiation and migration. One study has suggested that fibronectin regulates MSC migration through α5β1-integrin mediated activation of platelet-derived growth factor receptor (PDGFR)-β [95]. The report showed that binding of integrin present on the MSC surface to fibronectin promoted PDGFR- β receptor clustering and association which in turn led to autophosphorylation of the PDGFR-β and provided the docking sites for the downstream signal transduction molecules. These molecules included phosphoinositide 3-kinase (PI3K) which stimulated actin reorganisation and mediated MSC migration, phospholipase Cγ (PLCγ) which promoted cell motility and growth and Src family tyrosine kinase which affected cell proliferation. They also found that PDGFR-β mediated cell migration was specifically regulated by binding to specific ECM ligands such that binding to fibronectin and not to laminin induced autophosphorylation of the receptor. Therefore, it was possible that the increased migration observed with fibronectin pre-coating of the Transwell membranes compared to collagen pre-coating; might be regulated in the same manner such that fibronectin and not collagen was more potent at inducing receptor autophosphorylation. Also, since the autophosphorylation of the receptor occurred in the absence of growth factors, it was possible that fibronectin pre-coating of the tissue culture plates promoted BM MSC survival in the similar manner in the absence of serum in the media.

In the experiments presented in this thesis we used both Transwell and scratch assays to validate MSC migration in response to various stimuli. Using two different assays allowed validation of MSC migration both at the single cell level in the absence of cell-cell contact Transwell assay) and as sheet of cells with intact cell-cell contact (scratch assay). However, compared to Transwell assays, scratch assays were easier to set up and maintain to monitor MSC migration for a longer period of time. Moreover, the ability to monitor cell migration at a regular interval without needing to terminate the assay meant that cultures could be monitored until migration was complete or no longer progressing.

Cell exclusion zone -based migration assays using two different commercially available silicon inserts were also investigated for the bovine SF and BM MSCs.

The rationale for using this approach is that in the migration assay no stimulation of the MSCs occurs immediately prior to the addition of media containing migratory factors). Both in the Transwell system and scratch assay the cells are subjected to physical and or chemical stimulation of the MSCs immediately prior to addition of the media samples. i.e. in the Transwell system the MSCs are subjected to trypsinisation and centrifugal forces and in the scratch assay the cell layer was wounded .

In the exclusion zone assay it was observed that Oris-FLEX stoppers provided by Platypus Technology were more durable such that it retained its adhesion capacity with multiple uses. In contrast, Ibidi inserts lost their plastic adherence following one-time use. This meant new inserts needed to be purchased frequently which was not economical. However, Ibidi inserts allowed easier comparison of data between scratch assays and cell exclusion zone assays since both allowed measured of % reduction in wound width compared to % reduction in wound area (Oris-FLEX).

Chapter 6

Migration of mesenchymal stem cells to injured cartilage

6.1 Objective

The objective of the following experiments were 1) to determine the level of cell death after cartilage injury by sharp and blunt trauma and 2) to determine if Mesenchymal stem cells (MSCs) isolated from the SF and BM of the bovine metacarpophalangeal joint migrate towards injured cartilage in response to the injury.

6.2 Chapter introduction

It is essential to understand the natural healing mechanism following cartilage damage to develop treatments designed to augment that repair. Increasing evidence suggests the importance of MSCs as repair cells in wound healing and inflammatory diseases. Microfracture technique where the repair is mediated completely by the proliferation and differentiation of BM MSCs [27] demonstrates the importance of BM MSCs as a source of repair cells for cartilage injury. More recently, MSCs have been identified in synovial fluid (SF) [28-30] which increased 7-fold in arthritic patients compared to normal patients. This suggested that SF MSCs may also play a role in homeostatic repair during cartilage diseases.

Previous studies have also reported the migration capacity of MSCs to the injury site in other tissues such as fractured bone, traumatic brain injury, lung injury, liver injury, acute renal failure, acute burns and radiation-induced injuries [89]. For example, MSC recruitment to the site of the fracture occurred within 1 day of the injury although the exact mechanism and source of MSC is still unknown [96]. Studies have suggested a combined source of BM, adjacent soft tissues, periosteum, peripheral blood vessel walls and peripheral blood [89]. An increase in circulating MSC-like cells in peripheral blood of acute burn patients have also been reported, with higher number of cells found in young patients with more extensive burns [97]. The study suggested that MSCs were mobilised into the bloodstream through acute burn signals although this hypothesis has not yet been confirmed. Bone sarcoma and obstructive apnoea studies have also suggested a similar MSC mobilisation where an increase in circulating MSC was reported compared to healthy individuals [89]. Therefore, MSC recruitment to the injured tissue and their consequent participation in the reparative process may be part of the natural healing process.

The aim of this study was to develop an *in vitro* model to investigate SF and BM MSCs migration in response to injured cartilage. We also compared the migration of MSCs towards injured cartilage with non-injured and dead cartilage.

6.3 Results

6.3.1 Cartilage injury assessed through live/dead staining

Prior to mechanically injuring the cartilage it was important to establish that non injured cartilage i.e. freshly isolated cartilages had recovered from any injury caused by harvesting using a scalpel. Hence, freshly isolated cartilage pieces were rested in media (as described in section 3.4.1) with 10^{-7}M dexamethasone. The rested cartilages were stained (CMFDA and PI), fixed and imaged using confocal laser scanning microscopy. No obvious region of cartilage damage was observed. However, some dead chondrocytes could still be seen (Figure 6.1b). The rested discs were then injured using a no.11 scalpel blade with a single, linear cut, caused by passing the blade once through the middle of the discs in a straight line (Figure 6.1a). Sharp injury led to splitting of the matrix and chondrocyte death along the edges of the injury (Figure 6.1c). Even though the number of dead cells was not quantified, a greater area of chondrocyte death was observed following injury compared to the rested cartilage control.

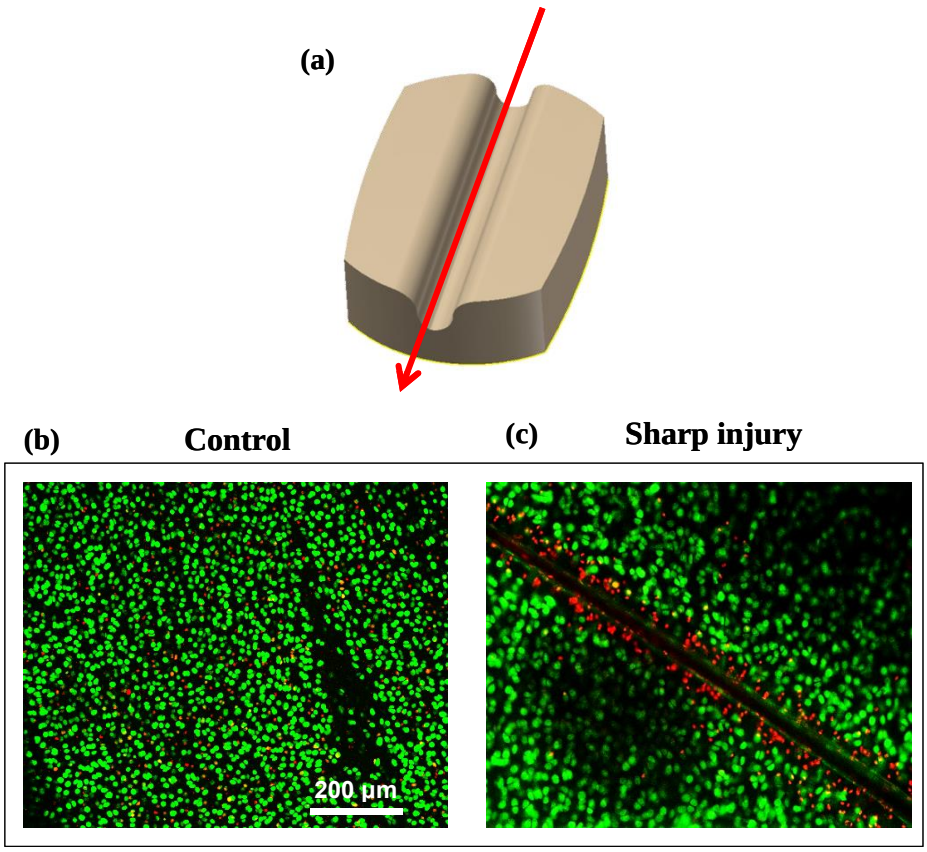


Figure 6.1: Assessment of cartilage injury. (a) In order to obtain injured cartilage, each cartilage disk was subjected to several cuts using a no.11 scalpel blade. Red arrow showing the direction of the cuts, with the cartilage cut in push-through mode passing the scalpel blade perpendicular to the long axis of the discs in straight lines. Each scalpel injury was made in the same direction and next to each other for standardisation of the injury. Cartilage remained submerged in media during creation of injury to prevent cartilage drying. Cartilage disks (imaged at ×10 magnification). Green cells (cells stained with CMFDA) represent live cells and red cells (cells stained with PI) represent dead cells. Cartilage disks were placed in DMEM supplemented with 10^{-7} M dexamethasone immediately following collection and maintained in the media for 72h. **(b)** Control disks with no protocol based cuts had no obvious region of injury but few cell deaths were still observed. (c) Following creation of sharp injury, localised chondrocyte death was observed along the edges of the injury.

6.3.2 Assessment of cartilage damage following sharp and blunt injury

Chondrocyte viability following sharp and blunt injury was assessed using CMFDA and PI and imaged using CLSM. Sharp injury was induced by No. 22 scalpel blade attached to a No. 4 scalpel handle and a metal biopsy punch. The cut was made by passing the blade once through the cartilage surface from the top of the cartilage to the bottom of the cartilage in a straight line. Blunt injury was produced using a Swiss style hammer. Injury was induced once with the specified tool with no additional sliding or rotational movement. Following injury, each explant was immediately stained with CMFDA and PI and fixed for microscopy.

Both No. 22 scalpel blade and the metal punch produced a fine linear injury with chondrocyte death confined to the edges of the injury (Figure 6.2a and b). Chondrocyte death from the hammer impact was more diffused and spread out from the region of impact (Figure 6.2c).

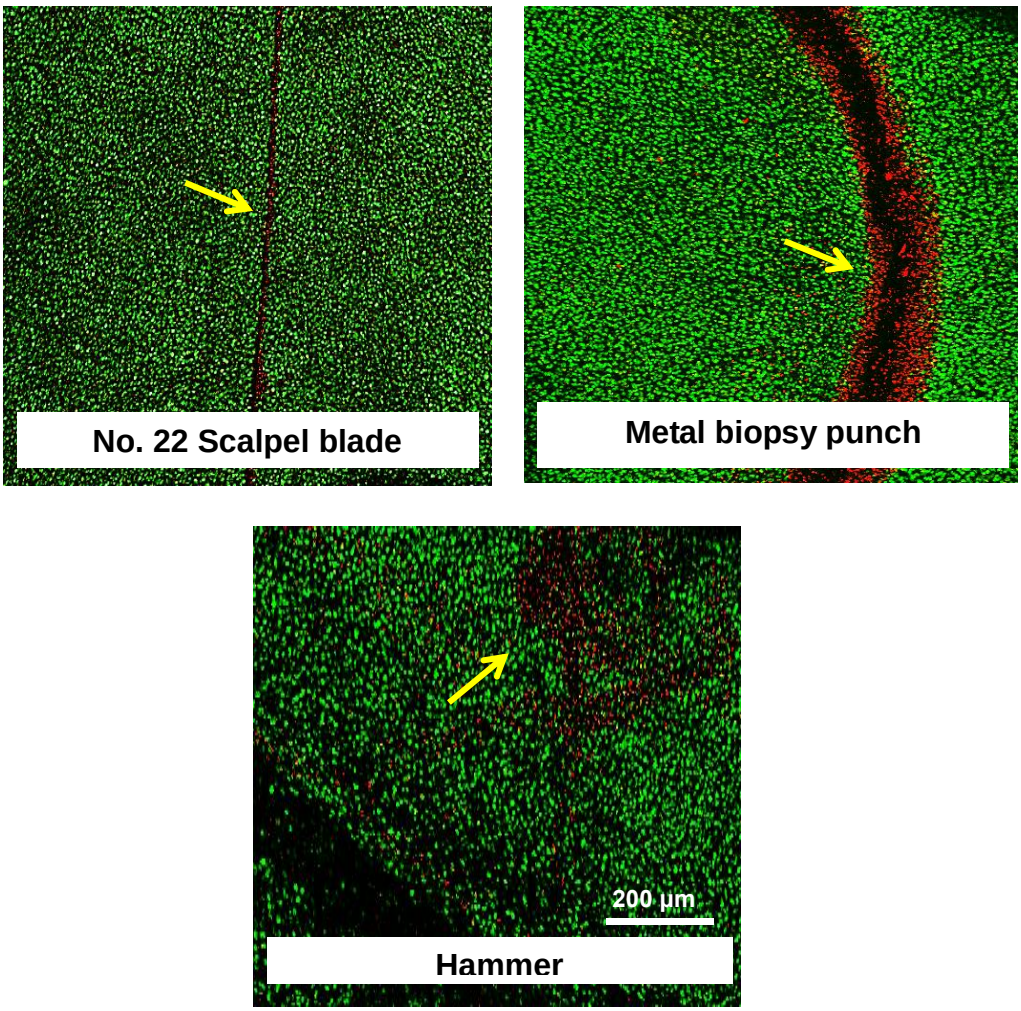


Figure 6.2: Both sharp and blunt injury causes chondrocyte death around the edges of the injury. Yellow arrow points to the region of injury. All images were taken at ×5 magnification. **(a)** No.22 scalpel blade (sharp injury) **(b)** biopsy punch (sharp injury) **(c)** hammer (blunt injury). Immediately following injury, chondrocyte death was localised around the cut or region of impact.

6.3.3. Assessment of chondrocyte viability 2 h following sharp and blunt injury

Chondrocyte viability was also assessed 2 h after mechanical injury of the cartilage discs. Following injury explants were incubated in high glucose DMEM for 2 h before being processed for CLSM imaging. There was increase in chondrocyte death for both blunt and sharp injury. However, chondrocyte death seemed to spread at a higher rate from the region of impact with blunt injury compared to sharp injury.

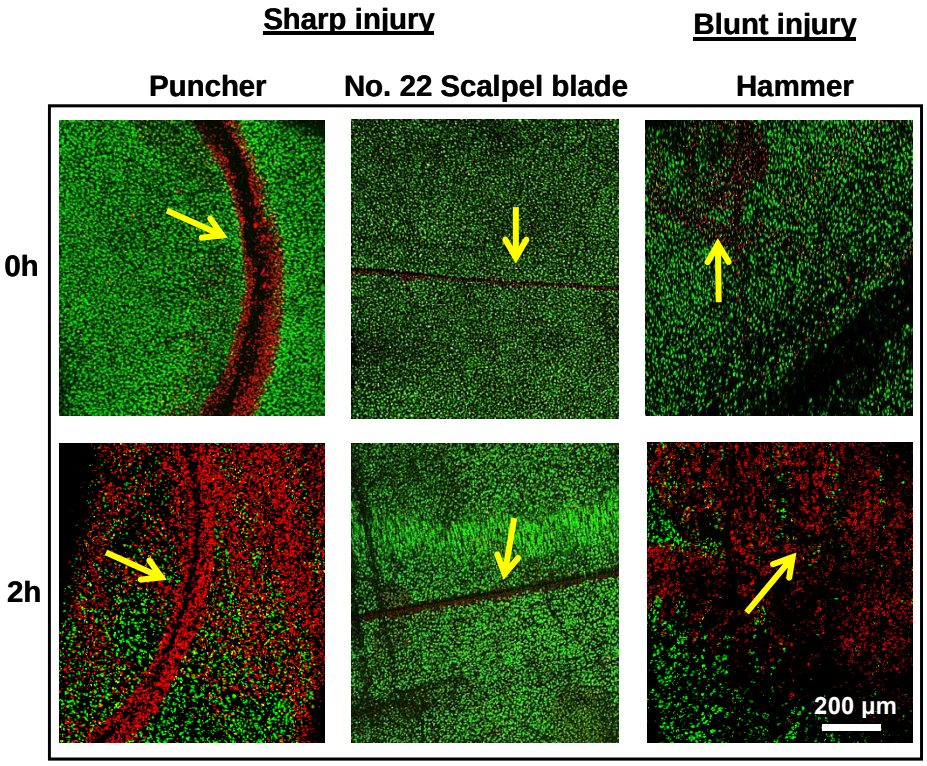


Figure 6.3: Following injury chondrocyte death increases with time. This increase is greatly enhanced in blunt injuries compared to sharp injuries. Arrows point to the region of wound creation and impact. All images were taken at ×5 magnification. Note: The 0 h images shown here are same as those shown above in figure 6.2.

6.3.4 MSCs migrate specifically to the site of the cartilage injury

To investigate if the MSCs can respond to injured cartilage, SF MSCs were labelled with PKH67 and incubated with injured cartilage for 24 h (Figure 6.4a). Cartilage injury was created by cutting the cartilage surface multiple times with a scalpel blade (Figure 6.4b, c and d). Following 24 h, injured cartilage explants were imaged using fluorescence microscopy. SF MSCs specifically aligned along the edges of the injury (Figure 6.4e, f and g) suggesting that MSCs can respond to cartilage injury and can migrate specifically to the injury site where they might be involved in repairing the injury.

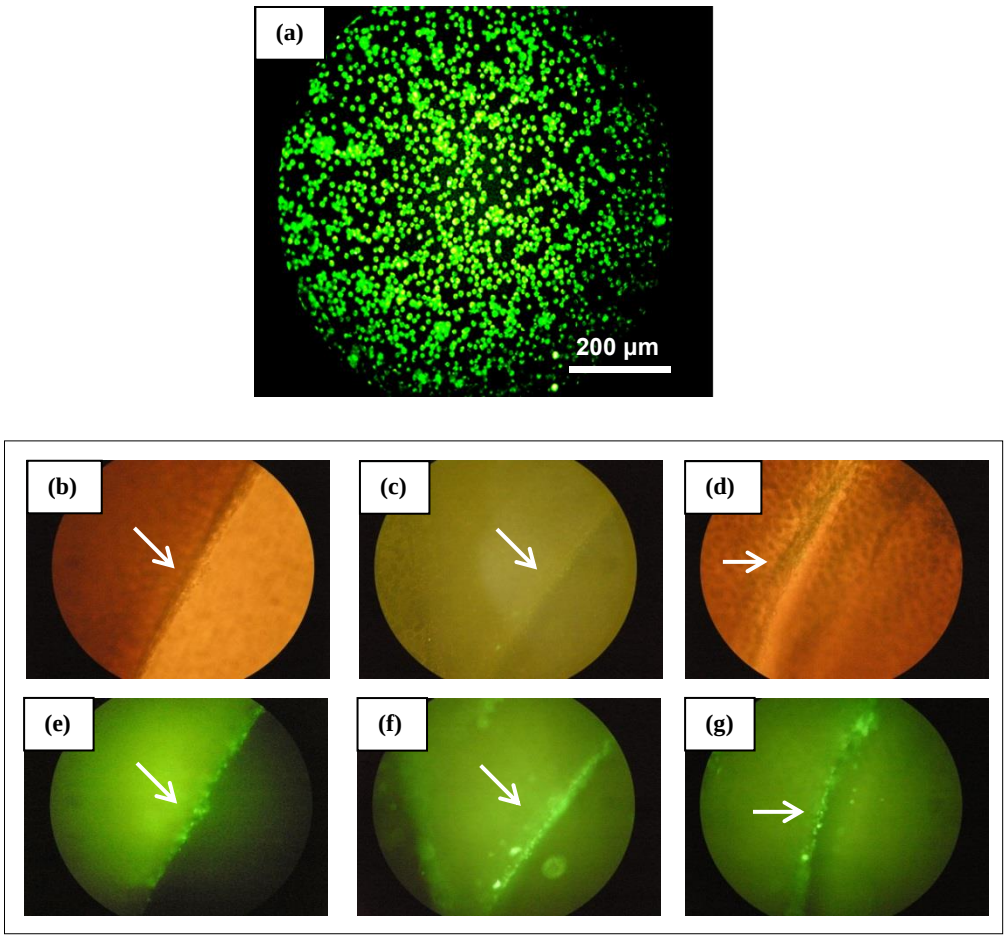


Figure 6.4: Migration of fluorescently labelled SF MSCs in response to injured cartilage. Freshly harvested articular cartilages were 'rested' by incubating in chondrocyte culture media for 72 h. cartilages were then washed with PBS and injured by cutting the surface several times with no.11 scalpel blades. Injured cartilage was incubated for 4 h with the PKH67 labelled SF MSCs. **(a)** Fluorescently labelled SF MSCs prepared by incubating the cells with PKH67. **(b) (c) (d)** Phase contrast microscopy showing the edges of the scalpel cut injury indicated by the white arrows. **(e) (f) (g)** Fluorescent microscopy showing SF MSCs aligned along the edge of the injury.

6.3.5 Migration potential of SF MSC to injured cartilage

Migration potential of SF MSC to injured cartilage was evaluated using the Transwell migration assay. Serum free media supplemented with 0.1% BSA was used as negative control and media with 0.1% BSA and 5% serum was used as positive migration control. Devitalised cartilage was produced by the freeze-thawing technique described in section 3.4 and non injured cartilage produced by resting in medium for 72 h. On completion of the assay, a significant 0.6 fold increases ($P<0.05$) in migration was observed with MSCs inresponse to injured cartilage compared to control. Even though no increase in migration was observed in response to dead cartilage compared to control, 1.8 fold increases ($P<0.05$) in SF-MSC migration was observed in response to non injured cartilage (Figure 6.5). Following termination of the migration assay, the viability of each cartilage disk was assessed using PrestoBlue assay. All non injured cartilages and injured cartilages were found to be viable and dead cartilages were non-viable at the time of the experiment.

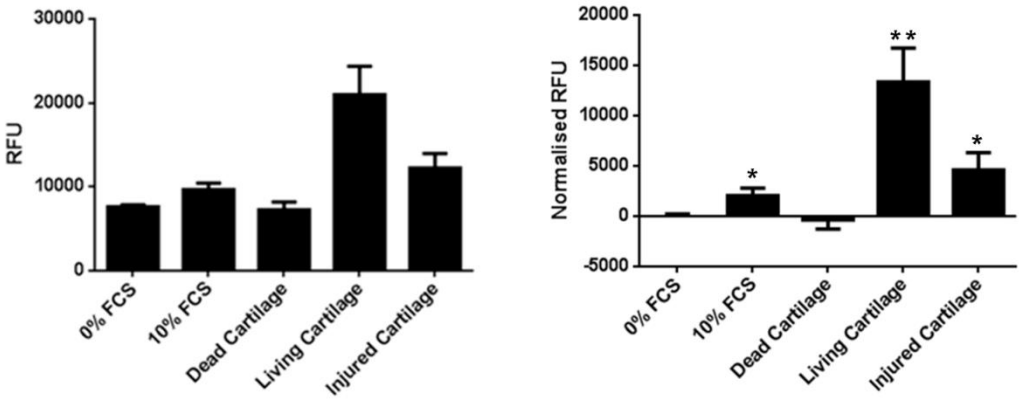


Figure 6.5: Migration of SF-MSCs in response to injured, non-injured and dead cartilage. Cells were stained with calcein-AM. Preliminary data suggested that SF-MSCs have the capacity to migrate in response to both injured and non-injured cartilage but not dead cartilage. Following staining with calcein-AM, cells where detached from the insert membranes and the fluorescence was read at an excitation >485 nm and emission at 520 nm. **P*<0.05.

6.3.6 Migration potential of BM MSC to injured cartilage

The experiment was repeated using BM MSCs and the number of migrated cells was quantified using crystal violet. DMEM/0.1% BSA was used as negative control and DMEM/0.1% BSA+ 5% FCS were used as positive control. Again devitalisation of the dead cartilage was achieved using freeze-thawing and the cartilage was rested for 72 h in culture medium to give non injured cartilage. Following 24 h migration assay BM MSCs showed an increase in migratory capacity to the positive control (1.2 fold) (*P*<0.05), injured cartilage (1 fold) (*P*<0.05), dead cartilage (1.4 fold) (*P*<0.05) and living cartilage (0.9 fold) (*P*<0.05) compared to control (Figure 6.6). Therefore, in these experiments MSCs not only migrated to living cartilage but they also migrated to dead cartilage.

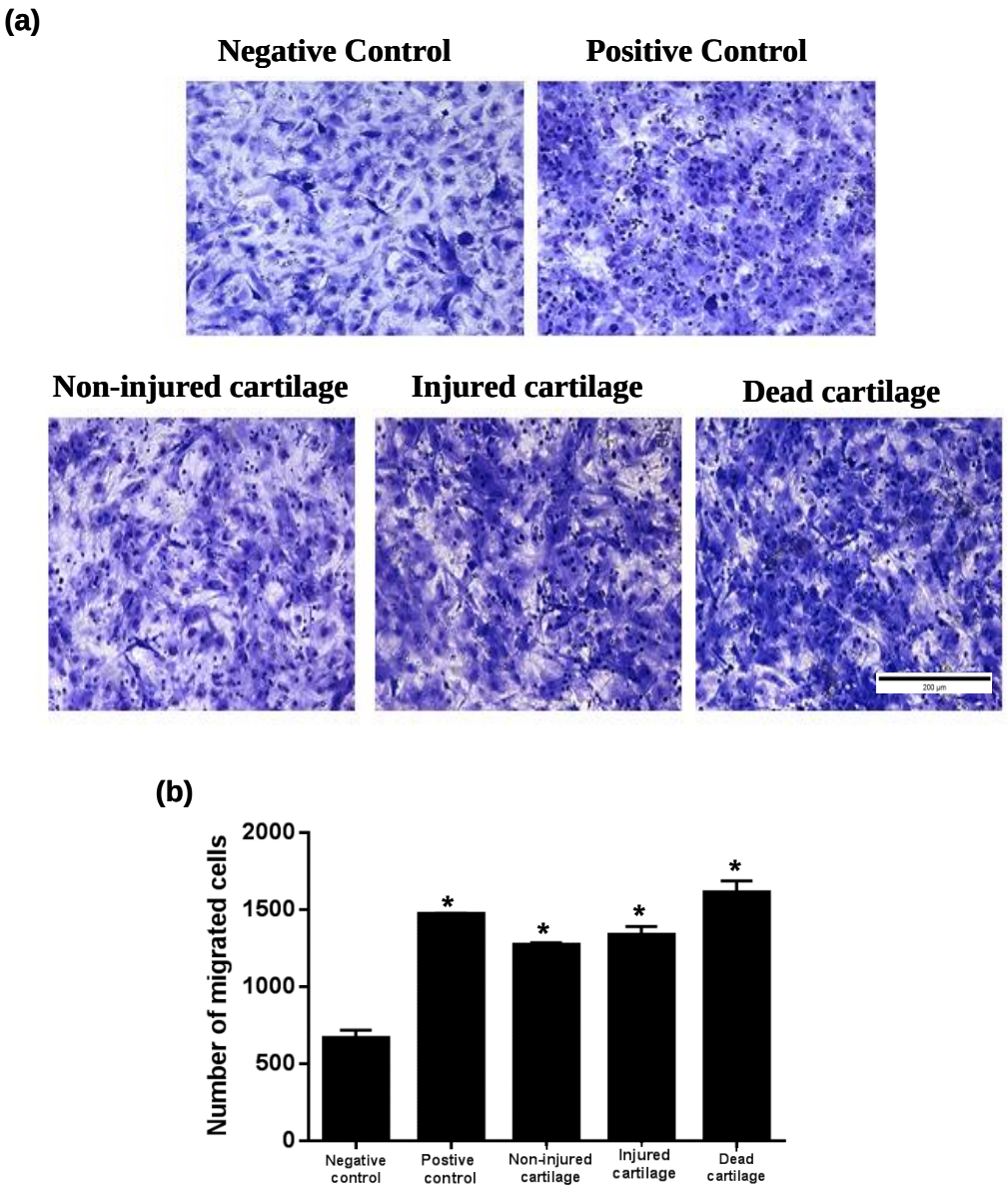


Figure 6.6: Migration of BM MSCs in response to injured, non-injured and dead cartilage. (a). Membranes were stained with crystal violet. **(b).** Number of migrated cells in 5 microscopic fields were counted and data expressed as mean number of migrated cells per 5 fields in 3 membranes ± SEM. (c). Normalised mean was calculated by subtracting the mean of each variable from the mean of DMEM+0.1% BSA (negative control). Data suggests that MSCs can migrate in response to injured, non-injured and dead cartilage.* $P<0.05$.

6.3.7 Assessment of cartilage discs viability following optimisation of protocol to produce non injured and dead cartilage

In these experiments, non-injured cartilage was produced by incubating the freshly isolated cartilage pieces in culture medium containing 10^{-7}M dexamethasone (section 3.4.2). The dead cartilage was devitalised by microwave irradiation as described in section 3.4.3. The PrestoBlue assay was used to assess cartilage viablity following treatment of cartilage with microwave irradiation and compared with the viablity of non injured and injured cartilage. Following 2 h incubation of cartilage discs with PrestoBlue no significant difference in fluorescence (RFU) was observed between control media (DMEM+10% serum+10% PrestoBlue, in absence of cartilage) and cartilage discs killed by microwave irradiation. In contrast, significant difference in fluorescence was observed between living discs (non injured cartilage discs and injured cartilage discs) and control media (Figure 6.7).

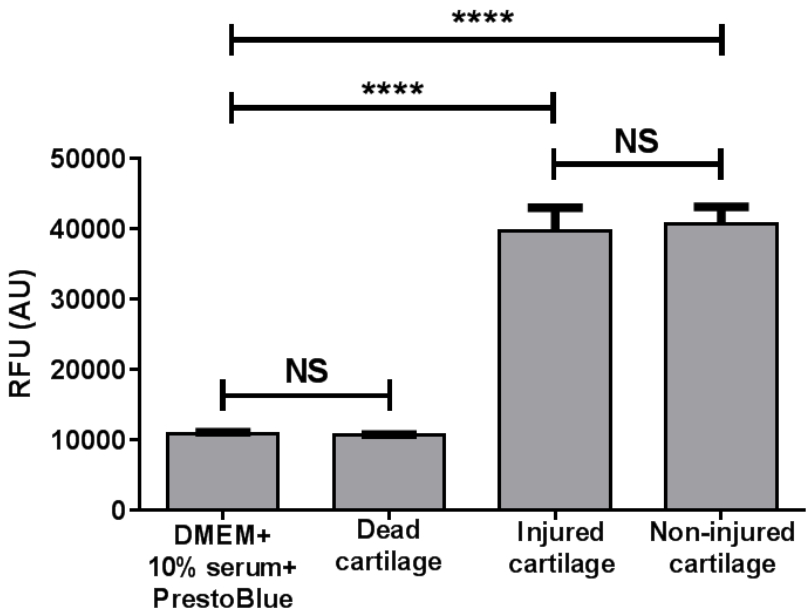


Figure 6.7: Assessment of cartilage viability using PrestoBlue assay. Cartilage viability was tested using PrestoBlue assay. All uninjured living and injured cartilages were viable and dead cartilages were non-viable at the time of the experiment.

6.3.8 Migration potential of SF and BM MSCs to injured, non-injured and dead cartilage following optimisation of protocol to produce non injured and dead cartilage

To test if there was higher MSC migration towards injured cartilage compared to non injured and dead cartilage, migration potential of SF MSCs to injured cartilage was evaluated using cartilage discs in transwell migration assay. Following 24 h migration assay, significant migration were observed with both SF and BM MSCs in response to injured cartilage compared to non-injured and dead cartilage. In response to injured cartilage following 24 h, SF MSC migration was 35% higher ($p<0.05$) compared to non-injured cartilage and 52% higher ($p<0.05$) compared to dead cartilage. In response to injured cartilage following 24 h, BM MSC migration was 62% higher ($p<0.01$) compared to non-injured cartilage and 48% higher ($p<0.01$) compared to dead cartilage (Figure 6.8 and 6.9).

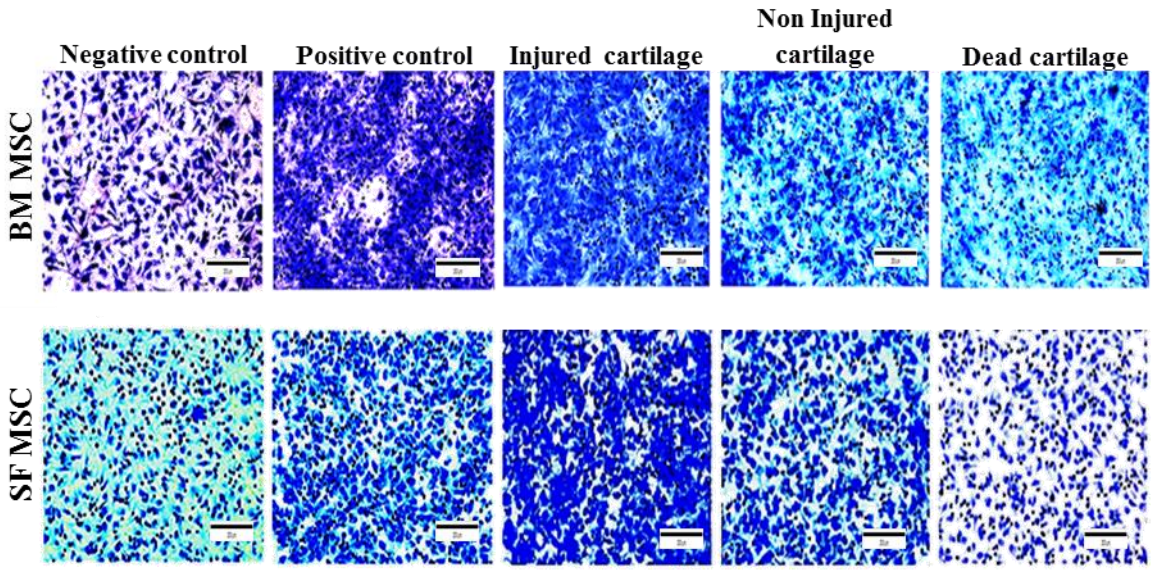


Figure 6.8: SF and BM MSC migration (24 h) in response to injured, non-injured and dead cartilage. Membranes stained with crystal violet and imaged at x20 magnification.

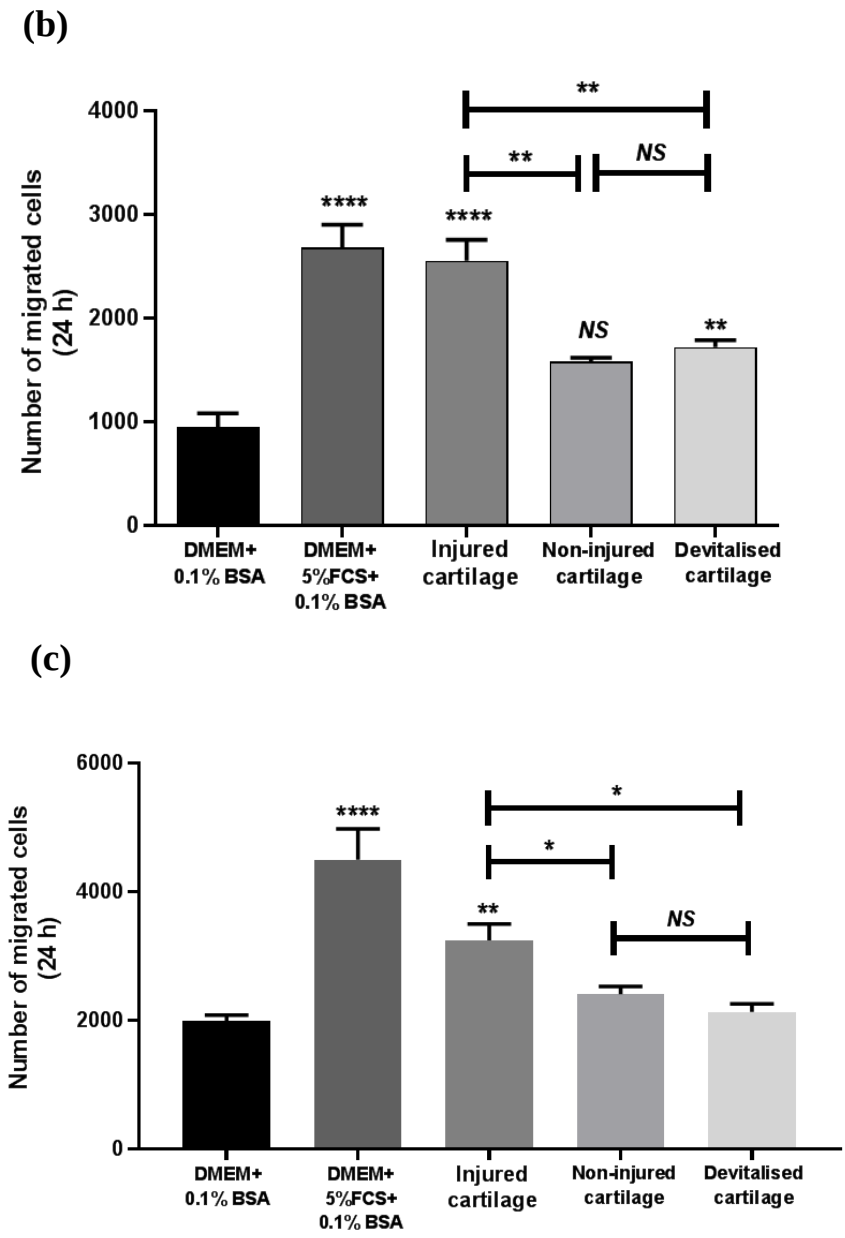


Figure 6.9: SF and BM MSC migration (24 h) in response to injured, non-injured and dead cartilage.(a) and (b): Graph showing mean number of migrated cells per 5 microscopic fields in 3 membranes (mean ± SEM). *p<0.01 **p<0.001 ****p<0.0001 compared to negative control and as indicated by bars.

6.4 Chapter Discussion

Using an *in vitro* model of cartilage injury and Transwell migration assay it was demonstrated that both BM and SF MSC migrate significantly more in response to injured cartilage compared to non injured cartilage. In our preliminary experiments we began by developing a chondral model to study the effect of blunt and sharp injury on cartilage. Using a hammer and chisel produced inconsistent chondral blocks and this issue was overcome by using a dremel. However, this had two main disadvantages: it required prolong exposure of cartilage to airflow and it produced an increased temperature at the cutting edge. Previous experiments using the bovine metatarsophalangeal joint have reported that airflow caused altered cartilage appearance, enhanced cartilage drying and increased chondrocyte death [98]. Moreover, even though rehydrating the cartilage using a saline-soaked gauge reversed the macroscopic appearance of the cartilage related to dehydration, it did not decrease the level of chondrocyte death associated with cartilage drying. The same group have also reported chondrocyte death associated with increased temperature due to drilling [99]. Hence, production of chondral explants using a dremel was not an effective method the method was further modified to use a 6 mm biopsy punch to collect 1.5 mm thick and 6 mm diameter cartilage discs.

Using these discs, the effect of sharp and blunt injury on chondrocyte survival in the injury areas immediately following injury and 2 h after injury was studied. Immediately following sharp injury, chondrocyte death was observed around the edges of the injury which remained confined to the edges of the injury at 2 h after injury. In contrast, with blunt injury, localised cell death spread outwards from the point of impact and increased chondrocyte death was observed at 2 h after impact compared to that seen immediately after impact. We also observed the spread of cell death outward from the cut cartilage surface following 2 h incubation when the injury was created using biopsy punch. This could be due to the pressure applied to the cartilage with the biopsy punch during creation of the injury.

These results were in agreement with previous reports comparing blunt injury with sharp injury where they found that injury by sharp scalpel caused less chondrocyte death compared to blunt impact produced by a trephine [47, 100]. Since blunt injury affected greater area of the cartilage matrix, time dependent increase in cell death with blunt injury compared to sharp injury is suggestive of a

relationship between matrix disruption and cell viability. This view is also supported by previous findings where localised cell death was observed around the impact-induced cracks but not around the impacted region without cracks [101].

Matrix disruption- induced cell death may occur by various mechanisms including cell death due to changes in cell morphology and interruption of cell-matrix interaction. For example, using integrin antibody it was found that collagen-chondrocyte interaction was crucial for chondrocyte survival where chondrocytes deprived of matrix molecules underwent apoptosis [102]. It is also important to note that some cell death may have been through direct injury without matrix damage simply by cutting through the cells.

Due to its ease of reproducibility and induction of a highly localised region of damage and we chose to investigate the model of using sharp injury on articular cartilage in which to study MSC migration towards injured cartilage. Using this model and fluorescently labelled SF MSC we found that, following injury, MSCs specifically migrated to the site of the injury where they aligned at the edges of the cut. The Transwell assay was the used to compare and quantify the number of MSCs that migrated towards the injured cartilage compared to migration to non injured and dead cartilage. Several difficulties were faced carrying out the experiments because it is difficult to obtain cartilage which has not already been injured due to the collection process.

One way to minimise the cartilage injury was to use osteochondral explants however it is difficult to make firm conclusions about the effect of MSC migration in response to injured cartilage. This was because there will be no clear indication whether MSC migration was in response to injured cartilage or the injured underlying subchondral bone or both. Hence, it was important to excise the cartilage from the osteochondral explants. Using this process, it was difficult to reproducibly collect cartilage explants of the same depth and further injury was exerted on the cartilage during the processing. Using a 6 mm biopsy punch to collect 1.5 mm × 6 mm (depth × diameter) discs overcame these issues of inconsistency, reproducibility and also minimised the amount of injury exerted through the collection process. During the initial preliminary experiments significant MSC migration to both non-injured and injured cartilage was observed

Migration to non-injured cartilage could either be in response to cartilage itself or as a result of the existing injury exerted on the cartilage during the collection process. To investigate the second possibility, media was supplemented with 100 nM dexamethasone. Dexamethasone is a potent synthetic glucocorticoid with a high receptor binding affinity. It is a common reagent used in standard chondroinductive and osteoinductive media and is also known to potentiate cartilage proteoglycan synthesis. One study investigating the effect of dexamethasone on cartilage matrix degradation reported that administration of dexamethasone could restore proteoglycan biosynthesis and loss of GAG in TNFα treated mechanically injured cartilage [103]. It was suggested that the inhibition of matrix degradation by dexamethasone may involve modulation of proteolytic activity of aggrecanases. Therefore, following collection, we treated the cartilage discs with 10^{-7} M dexamethasone for 72 h to reduce the cartilage damage caused by the collection process. CLSM images of the 'rested' discs showed no obvious region of injury as indicated by the lack of split matrix but a very low level of chondrocyte death was still observed. When the migration assay was repeated using these discs, no significant MSC migration was observed in response to the 'rested' cartilage compared to negative control (serum free media). This suggested that MSC migration observed in previous experiments to the 'non-injured control cartilage' was due to prior injury of the cartilage exerted from the collection process. Further, the low level of migration observed in response to the 'rested' cartilage could be due to the little remaining injury as indicated by the chondrocyte death.

Further investigation into the migration of MSC to devitalised cartilage was carried out to investigate whether migration of MSCs to the dead cartilage was a true response or due to leakage of cell contents during the freeze thawing process, causing release of intracellular proteolytic enzymes which in turn stimulated release of matrix proteins and promoted MSC migration. No significant SF MSC migration was observed when the dead cartilage was irradiated using microwave to kill the cartilage almost instantaneously by denaturing and precipitating out cartilage proteins. However, still significant migration of BM MSC to dead cartilage was observed suggesting that unlike SF MSC which migrated only to injured cartilage, BM MSC may migrate in response to both dead and injured cartilage. Such differential regulation of SF and BM MSC migration is suggestive

that during the natural healing process SF MSC might be the first to respond to the cartilage injury whereas BM MSC only migrate following severe damage. This is a possibility since SF MSC have direct access to the cartilage whereas BM MSC can only detect the injury if the injury extends to the underlying subchondral bone.

6.5 Chapter summary

In this chapter an *in vitro* model of cartilage injury was developed and using Transwell migration assay the migration potential of bovine BM and SF MSCs to injured cartilage was assessed. It was found that both BM and SF MSCs migrated significantly more towards injured cartilage compared to non injured cartilage. Furthermore, in contrast to SF MSCs, BM MSCs also migrated significantly more towards dead cartilage compared to non injured cartilage.

Chapter 7

Assessment of MSC migration and characterisation of injury conditioned medium (IJCM)

7.1 Objective

The objectives of these experiments were to 1) investigate whether *in vitro* injured articular cartilage released agents ('injury factors') which can promote migration of SF and BM MSCs and 2) characterise any 'injury factors' which are released by the articular cartilage injured *in vitro*.

7.2 Chapter Introduction

The ability of the MSCs to migrate specifically to the site of the cartilage injury suggested that injured cartilage may release stress and biochemical signals which can stimulate distant stem cells to migrate to the site of the injury. To our knowledge two other groups are investigating the signalling response of the articular cartilage to traumatic injury. The Vincent laboratory has shown that damaged articular cartilage releases a number of regulatory factors from heparan sulphate-bound stores within the pericellular matrix [104] They have shown that FGF2 [46], hepatoma-derived growth factor (HGF) and CTGF [105] were released following cartilage injury. FGF2 was shown to be associated with chondroprotective in vivo such that mice deficient of FGF2 developed significantly accelerated OA spontaneously and upon surgical joint destabilisation [106]. The role of HGF is still unknown. Similarly, Dell'Accio's group have shown that the WNT signalling and bone morphogenetic protein (BMP) pathway is activated following mechanical injury of cartilage [107]. They are currently investigating if these pathways play a role in stimulating mesenchymal stem cell involvement in the cartilage repair.

Several studies have also reported the importance of chemokines and cytokines as regulators of MSC migration. Chemokines are molecules that can induce directional cell migration by establishing chemical gradients along which cells can migrate. They are small molecules, 8-14 kDa in size containing four conserved residues of cysteine and subdivided into four families based on the two cysteine residues at the N terminal. The subfamilies are CXC, CC, (X)C and CX3C. The chemokine receptors are G-protein coupled receptors which are subdivided into the same four families as their ligands [108].

Therefore, the objective of these experiments was to investigate whether *in vitro* injured articular cartilage released agents ('injury factors') which can promote

migration of SF and BM MSCs and characterise 'injury factors' which are released by the articular cartilage injured *in vitro*.

7.3 Results

7.3.1 Assessment of mesenchymal stem cell migration in response to IJCM in Transwell assays

To investigate the possibility that migratory factor(s) released from the cartilage upon sharp injury stimulated MSC migration towards injured cartilage, culture medium was conditioned by incubating finely cut cartilage pieces for 24 h (refer section 3.14). The injury conditioned medium (IJCM) samples were put in the lower well of the Transwell chambers and assayed for stimulation of MSC migration. Serum free media (DMEM + 0.1% BSA) was used as negative control and media supplemented with 5% serum (DMEM+5% serum+0.1% BSA) was used as positive control. IJCM induced a high degree of MSC chemotactic migration such that the number of migrated cells was significantly higher when IJCM was present on the lower wells compared to serum-free control media (Figure 7.1). SF-MSCs demonstrated a 0.6 fold increase ($p<0.001$) (Figure 7.2a) and BM MSCs a 1.5 fold increase ($p<0.01$) (Figure 7.2b) in migration to IJCM compared to control. In comparison, SF-MSCs demonstrated a 0.5 fold increase ($p<0.01$) and BM MSCs a 3 fold increase in migration ($p<0.01$) in response to 5% serum compared to control. These results suggested that MSC migration stimulating soluble factors present in the cartilage were released upon cutting injury.

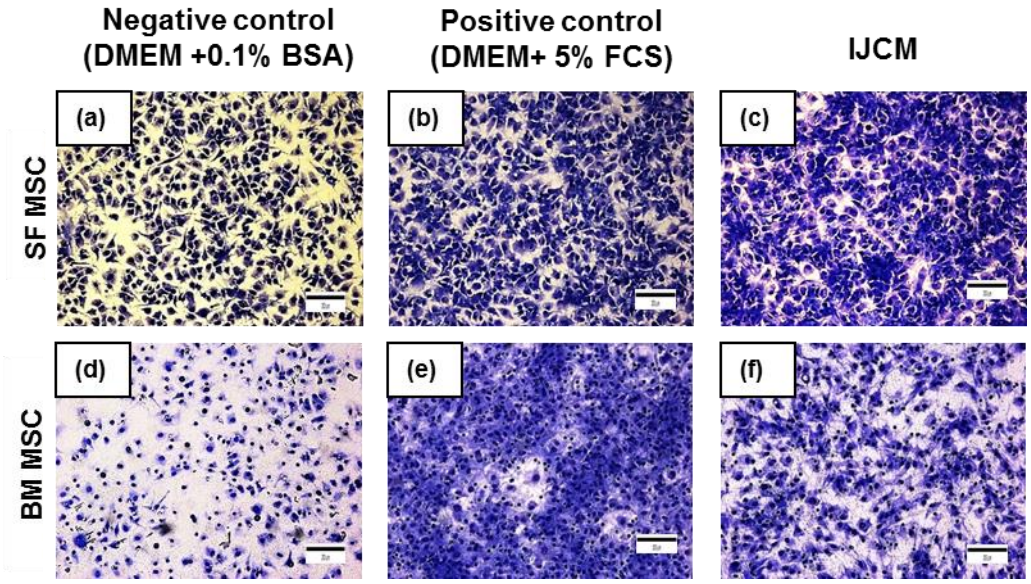


Figure 7.1: Migration of bovine SF-MSCs (a, b and c) and BM MSCs (d, e and f) in response to IJCM were studied using transwell assays over 24 h. Membranes were stained with crystal violet. Representative image of one microscopic field at x20 magnification is shown here. Presence of serum (5% serum; positive control) increased the number of migrated cells compared to serum free media (negative control) for both SF and BM MSCs. Similarly, IJCM also induced greater MSC migration for both MSCs.

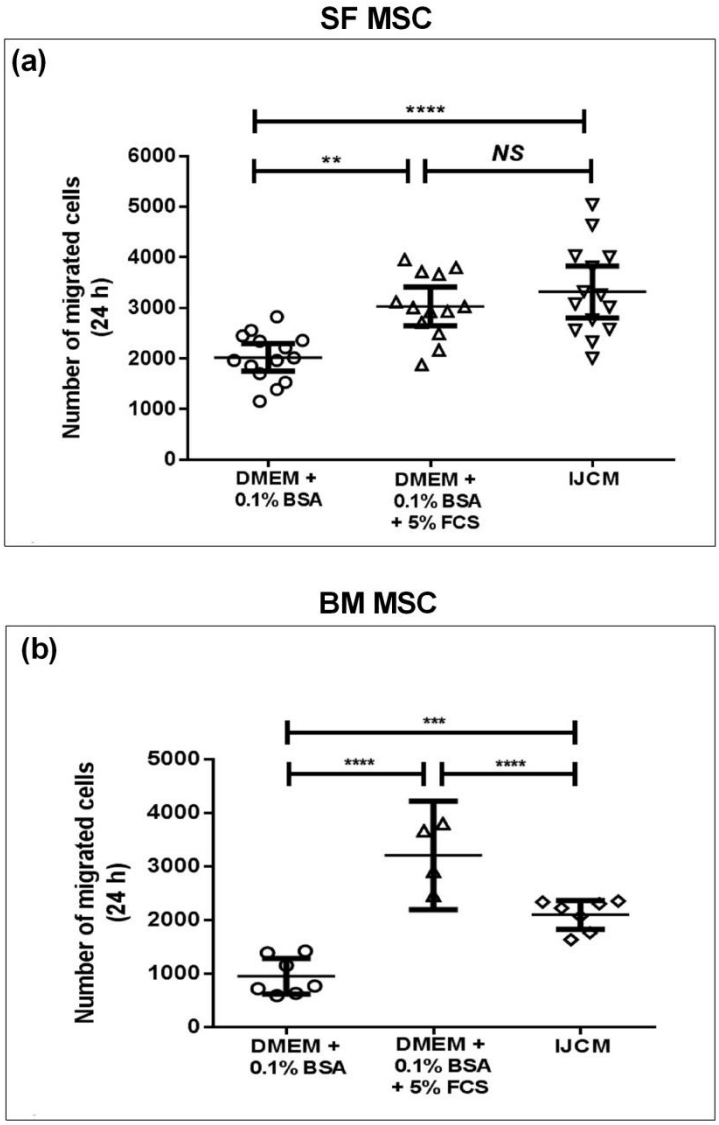


Figure 7.2: Migration of bovine (a) SF MSC and (b) BM MSC in response to IJCM over 24h assessed using transwell assays. Graph showing mean number of migrated cells ± SEM. **(a)** Both presence of serum and IJCM significantly increased the number of migrated SF MSCs compared to serum free media (DMEM+0.1% BSA). No significant difference in migration was observed between 5% serum and IJCM. **(b)** Both presence of serum and IJCM significantly increased the number of migrated BM MSC compared to serum free media. Further, 5% serum induced significantly higher number of migrated cells compared to IJCM. ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

7.3.2 Assessment of mesenchymal stem cell migration in response to IJCM in scratch assays

The effect of IJCM was validated using scratch assays. IJCM induced rapid wound closure of SF MSC scratch assays such that complete wound closure was observed consistently between 24 h and 36 h (Figure 7.3b). In contrast, slow wound closure was observed with serum free media and completion of wound closure was not achieved within 36 h (Figure 7.3a).

Wound closure was quantified for both SF MSCs (over 24 h) and BM MSCs (over 17 h) in response to IJCM by measuring the percentage reduction in wound width. In SF MSC scratch assays, IJCM which was generated under serum free conditions, affected wound closure more rapidly than serum free control (Figure 7.4a). At 24 h, when the extent of wound closure was approximately 75% ± (6%) (p<0.0001) for IJCM stimulated assays, the extent of closure for serum free control assays was only 14% ± (1%) (Figure 7.5a). Similarly, IJCM also induced significant BM MSC migration (approximately 87% ± (4%))(p<0.0001) into the wound compared to serum free negative control (48% ± (7%)) (Figure 7.4b and 7.5b).

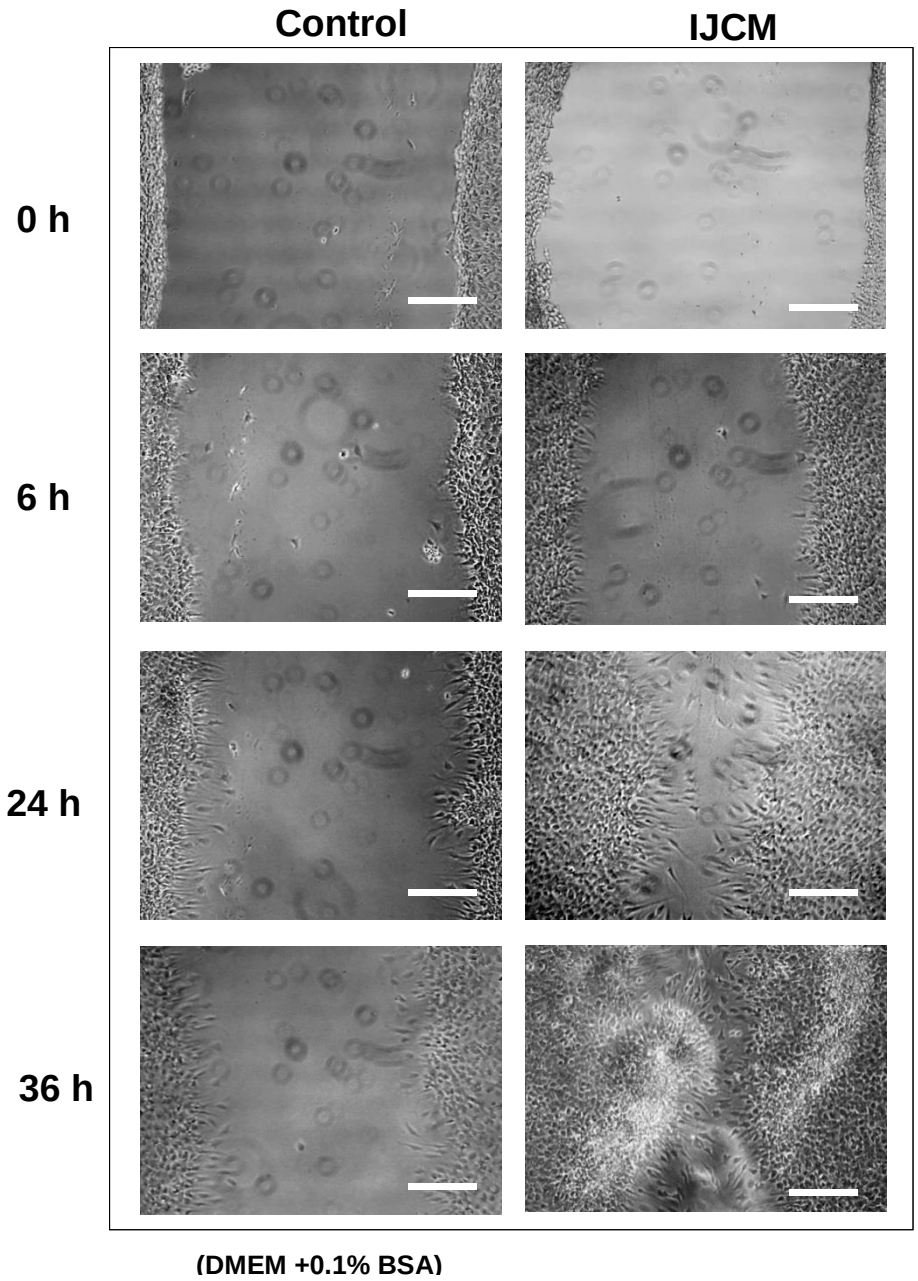


Figure 7.3: Migration of bovine SF MSCs in response to IJCM and serum free media (DMEM+0.1% BSA) was evaluated using the monolayer scratch assay. Migration was monitored microscopically at a regular interval and imaged at 0 h (immediately following creation of the wound), 6 h, 24 h and 36 h. IJCM induced rapid MSC migration into the wound compared to control. Images were taken at x10 magnification. Complete wound closure was observed consistently between 24 h and 36 h with IJCM whereas no wound closure was observed with control even after 36 h. Bar represents 200 μm. ***Please scan this image to watch the time-lapse video of SF MSC migration in scratch***

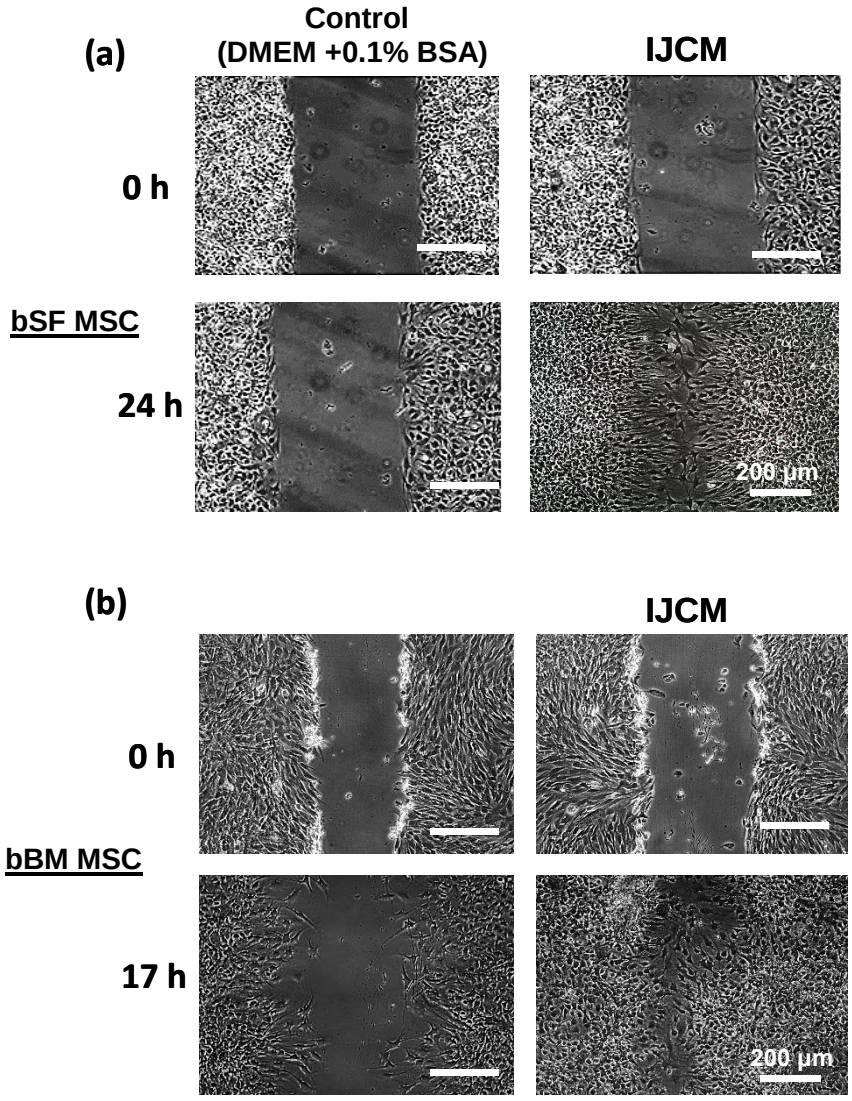


Figure 7.4: **Assessment of MSC migration in response to injury conditioned media (IJCM) using scratch assay.** Images were taken at x10 magnification. IJCM induced rapid wound closure in both **(a)** SF MSC and **(b)** BM MSC scratch assays compared to control.

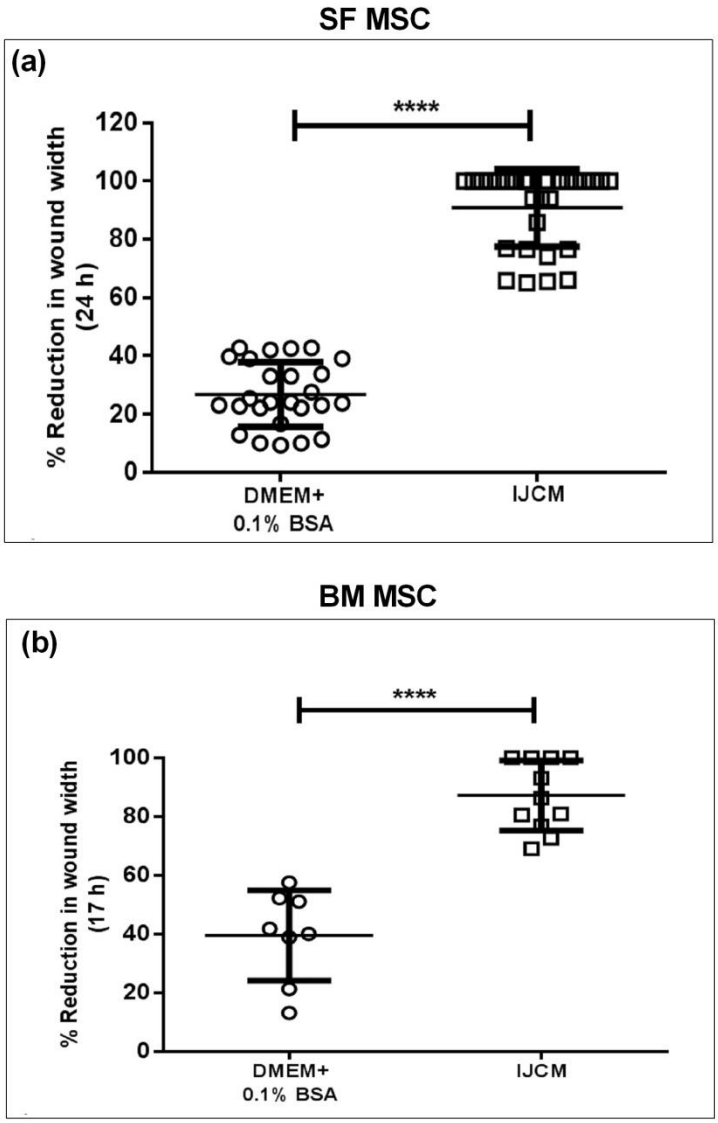


Figure 7.5: Assessment of MSC migration in response to injury conditioned media (IJCM) using scratch assay. Graph showing percentage reduction in wound width over 24 h (%) ± SEM for SF MSCs and over 17 h (%) ± SEM for BM MSCs. Significant reduction in wound width was observed with IJCM compared to control for both **(c)** SF MSC and **(d)** BM MSC. For SF MSCs, 75% ± (6%) reduction in wound width was observed with IJCM (p<0.0001) compared to only 14% ± (1%) with serum free media. Similarly, for BM MSC, 87 % ± (4%) reduction in wound width was observed with IJCM (p<0.0001) compared to 48% ± (7%) with control. Further, BM MSCs were more migratory than SF MSCs such that higher degree of wound closure was observed with serum free media for BM MSCs compared to control.

7.3.3 Assessment of migration of other skeletal cell types in scratch assays in response to IJCM

The specificity of IJCM to induce MSC migration was investigated using articular chondrocytes and the MG-63 (osteoblastic cell line) using scratch assay methodology and adding IJCM to the cell layers. Chondrocytes and MG-63 cells were grown to confluence and 'scratch' wounds were created using the same method used for MSCs (section 3.16.3). Serum free media (DMEM+0.1% BSA) was used as negative control and assay was monitored microscopically over 24 h. In chondrocyte scratch assays, serum free media did not induce rapid wound closure similar to MSCs (Figure 7.6a). However, in contrast to MSCs, IJCM also did not induce significant wound closure compared to the control (Figure 7.6b). For example, when approximately 31% ± (3%) reduction in wound width was observed with serum free media, 31% ± (3%) reduction in wound width was observed with IJCM (Figure 7.8a). Similarly, IJCM did not induce significant wound closure in MG-63 scratch assays compared to control (Figure 7.7). For example, when 85% ± (4%) wound closure was observed with serum free media, 77% ± (7%) wound closure was observed with IJCM (Figure 7.8b). These results suggested that IJCM may specifically induce migration in MSCs and may not stimulate migration of other skeletal cell types.

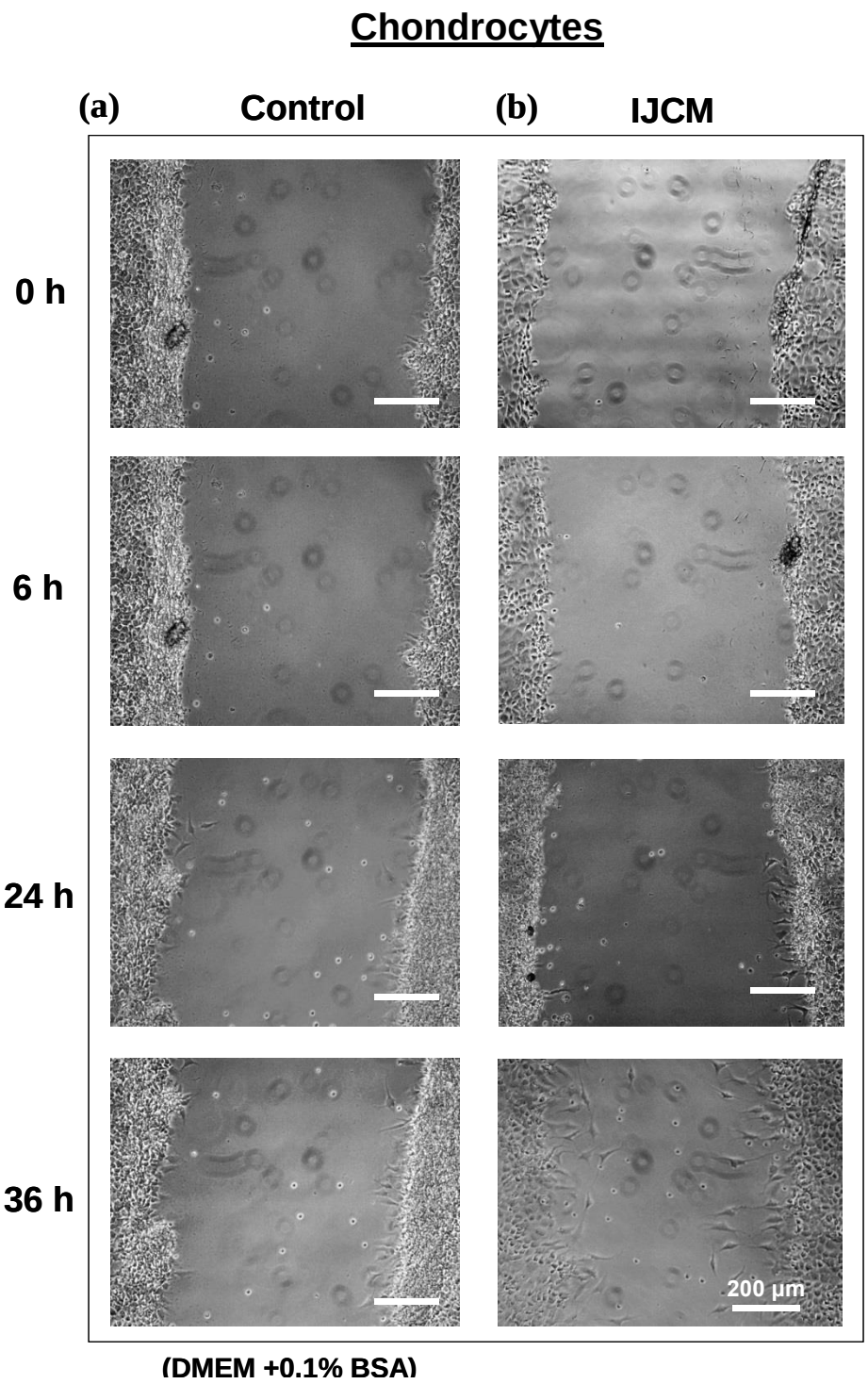


Figure 7.6: Migration of bovine articular chondrocytes in response to IJCM and serum free media (DMEM+0.1% BSA) was evaluated using the monolayer scratch assay. Migration was monitored microscopically at a regular interval and imaged at 0 h (immediately following creation of the wound), 6 h, 24 h and 36 h. IJCM did not induce rapid MSC migration into the wound compared to control. Complete wound closure was not observed for either negative control or IJCM following 36 h of wound creation.

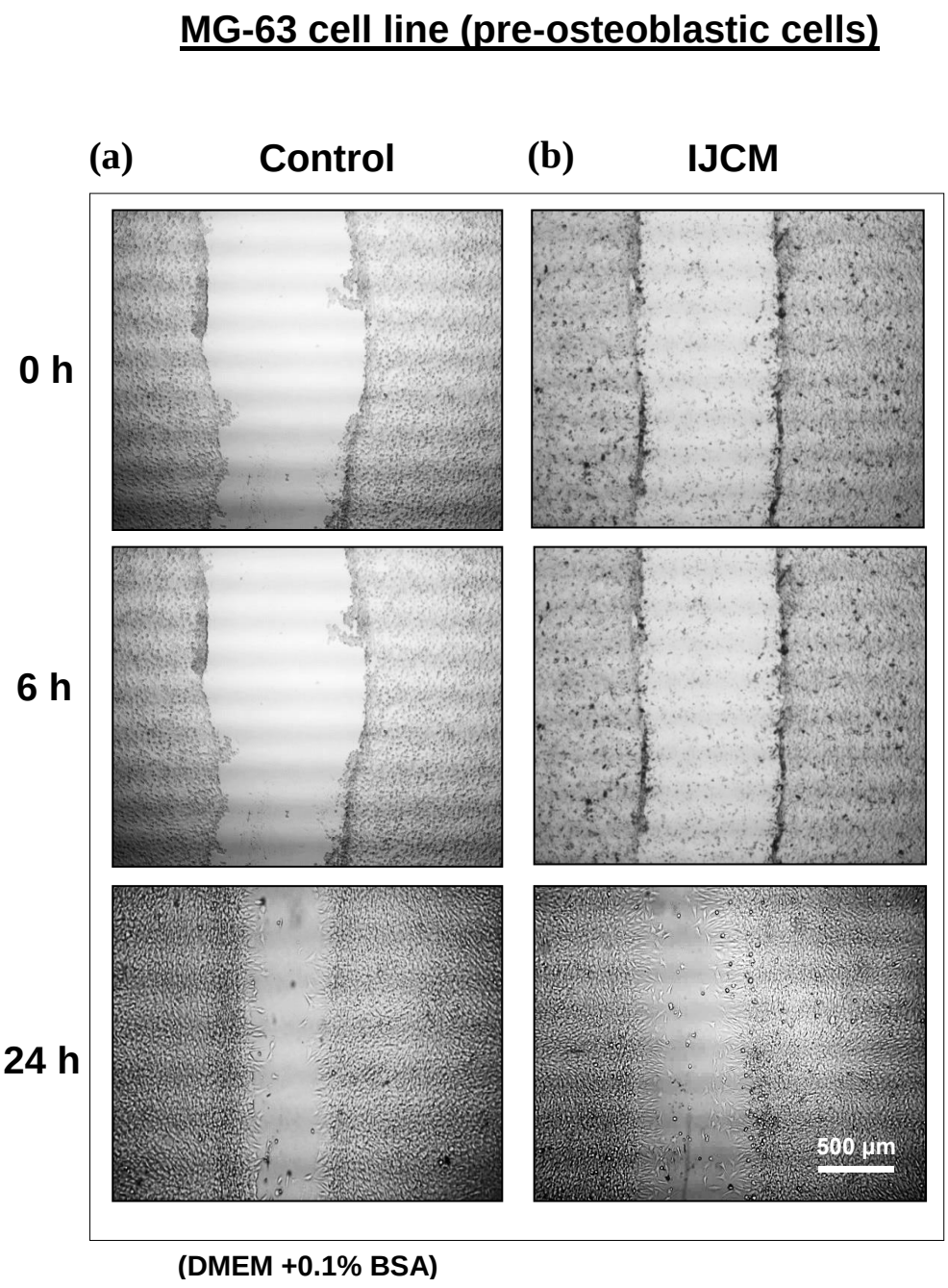


Figure 7.7: Migration of MG-63 cell lines (osteoblastic cells) in response to IJCM and serum free media (DMEM+0.1% BSA) was evaluated using the monolayer scratch assay. Migration was monitored microscopically at a regular interval and imaged at 0h (immediately following creation of the wound), 6 h and 24 h. Initially, wound closure was slow and no cell movement was observed in the first 6 h. However, rapid wound closure was observed with both serum free media and IJCM following 24 h assay.

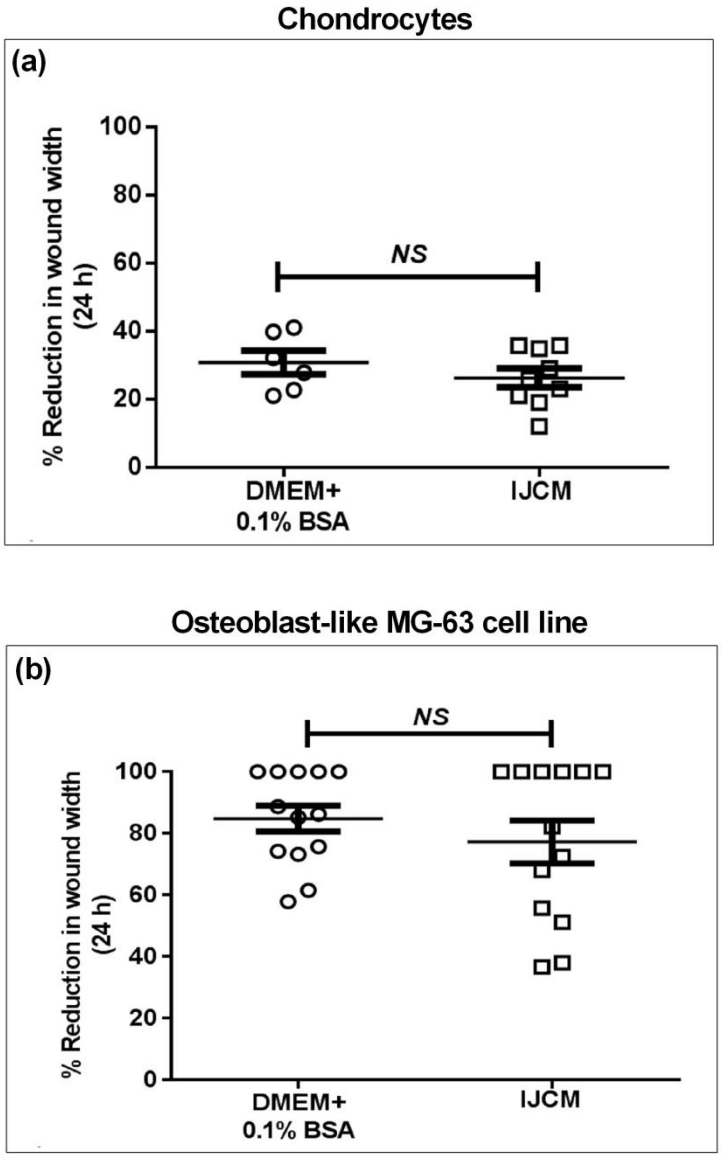


Figure 7.8: Migration of (a) bovine articular chondrocytes and (b) MG-63 cell lines (pre-osteblastic cells) in response to IJCM and serum free media (DMEM+0.1% BSA) were evaluated using the monolayer scratch assay. Graph showing percentage reduction in wound width (%) ± SEM over 24h. No significant difference in wound width reduction was observed between IJCM and serum free media (negative control) for both chondrocyte and MG-63 cell line scratch assays.

7.3.4 Histological assessment of injured cartilage to evaluate MSC migration in response to injury conditioned media (IJCM)

Cartilage discs were collected using a 6mm biopsy punch. Discs were injured using a no.11 scalpel blade attached to a no.3 scalpel holder. A single cut was made in the push-through mode by passing the scalpel blade perpendicular to the long axis of the discs in a straight line. Each disc was placed in individual wells in a 24 well plate and 1×10^6 MSCs were seeded into each well re-suspended in either serum free media (DMEM+0.1% BSA) or IJCM and incubated for 7 days at 37°C and 5% CO_2. Following this, the discs were fixed for 30 min in 4% formaldehyde in ambient temperature, washed once with PBS and mounted in optimal cutting temperature (OCT) prior to cryosectioning. Cryosections were mounted onto the microscopic slides and stained with haematoxylin and eosin (H&E). MSCs were found to be present at the cut edge of the cartilage discs for both serum free media and IJCM. However, higher numbers of MSC were present in cartilage discs incubated in IJCM compared to control (Figure 7.9).

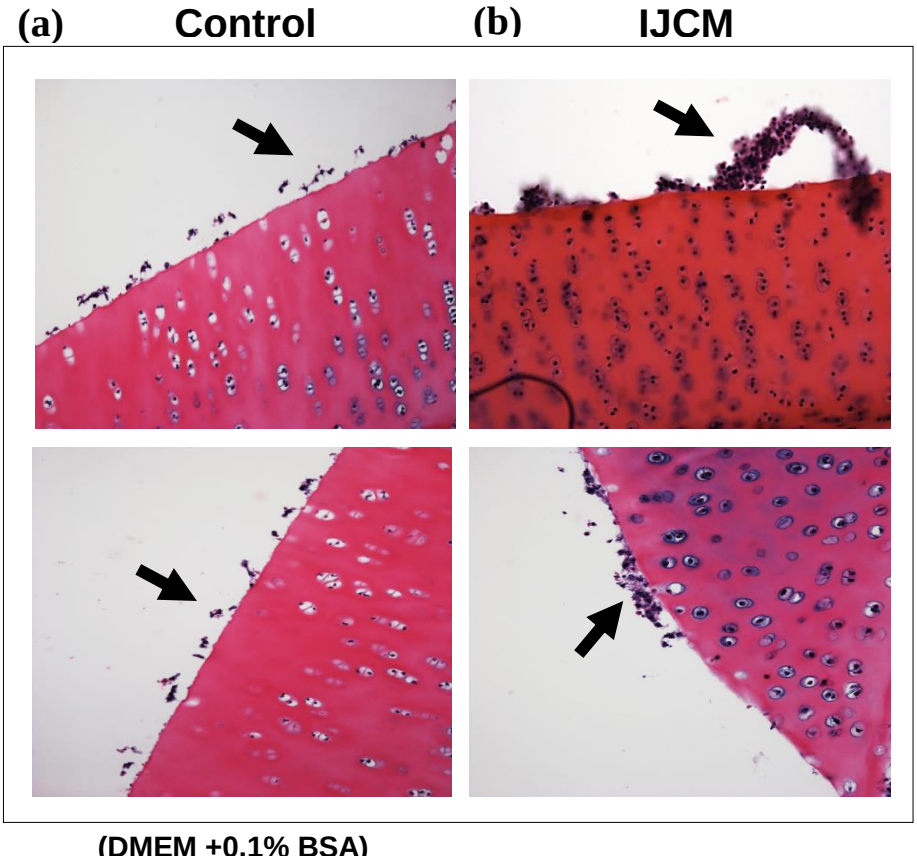


Figure 7.9: IJCM enhances SF MSC migration to the injured cartilage. Representative H&E stained sections of injured cartilage discs cultured with SF MSC for 7 days. **(a)** Control cultures were maintained in serum free media (DMEM+0.1% BSA). **(b)** Injured cartilage maintained in IJCM. Attached MSCs to the cut edge of the cartilage was seen for both control and IJCM. However, higher numbers of MSCs were seen with IJCM compared to control. Arrow indicates SF MSCs

7.3.5 Primary characterisation of injury conditioned media (IJCM) using immobilised heparin to remove heparin-binding factors from IJCM (hIJCM)

It was important to understand to what extent the migration stimulatory factors were being released from injured cartilage either by release/secretion from the chondrocytes or from an extracellular reservoir. Extracellularly, these factors are likely to be sequestered in the matrix bound to heparan sulphate containing proteoglycans. IJCM was added to microwell plates containing immobilised heparin and incubated for 24h at 4°C. The heparin-treated IJCM samples were then removed from the microplate wells, pooled and tested for their ability to stimulate MSC migration using scratch migration assays. Serum free media (DMEM+0.1% BSA) was used as control.

IJCM induced significant migration of SF MSC into the wound. Heparin-treatment of IJCM reduced the migratory stimulation of IJCM such that the extent of wound closure by SF MSCs was only approximately 49% ± (8%) compared to 75% ± (6%) by IJCM ($p<0.001$) over 24h (Figure 7.10a and 7.11a). However, migration in response to heparin treated IJCM was still significantly more compared to control (27% ± (2%)) ($p<0.001$). Similarly, heparin-treatment of IJCM also reduced the migratory stimulation of IJCM in BM MSC scratch assays (Figure 7.10b). At 17 h, when the extent of wound closure was approximately 87% ± (4%) with IJCM, it was only 57% ± (3%) with heparin-treated IJCM ($p<0.001$). But in contrast to SF MSCs, there was no significant difference in migration between heparin treated IJCM and control (48% ± (7%)) (Figure 7.11b).

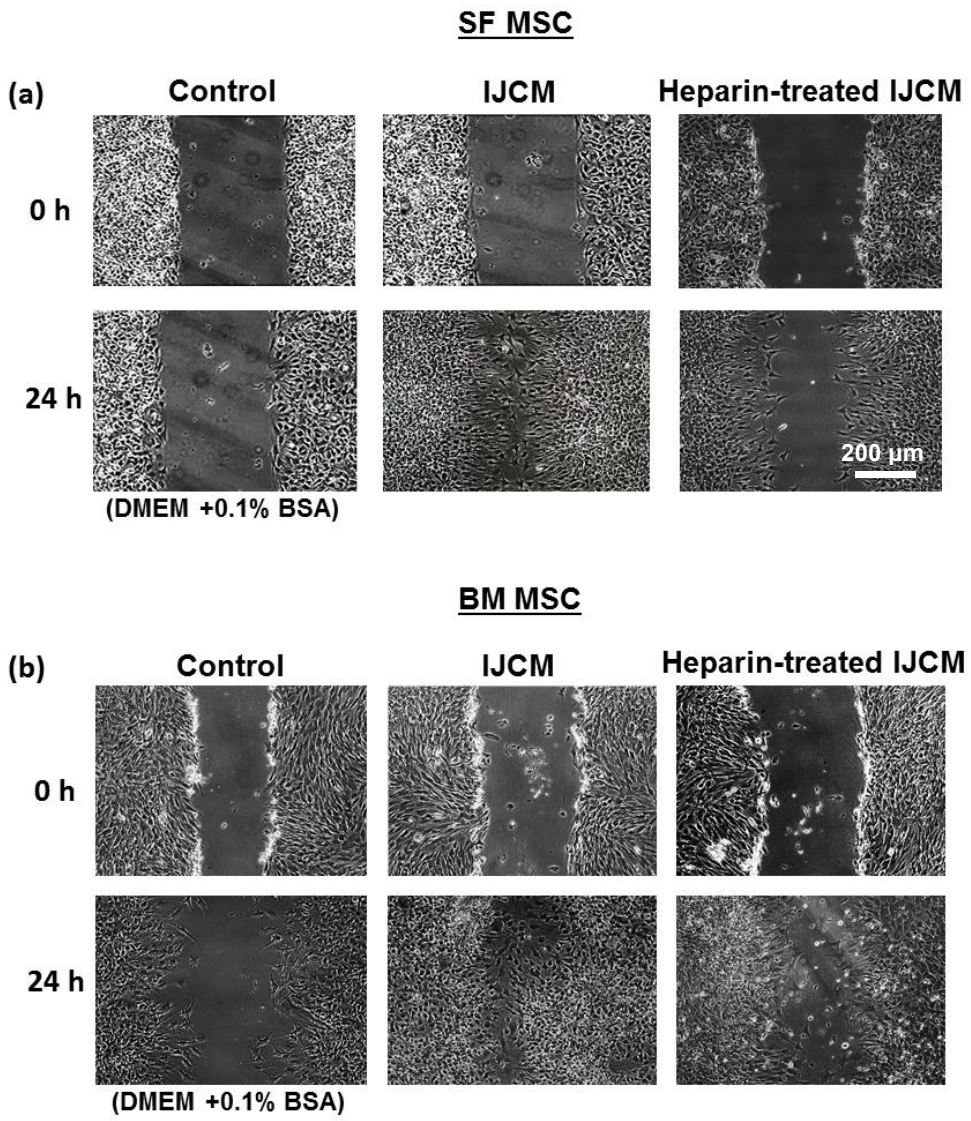


Figure 7.10: Assessment of MSC migration in response to heparin-treated injury conditioned media (IJCM) using scratch assay. Images were taken at x10 magnification. IJCM induced rapid wound closure in both **(a)** SF MSC and **(b)** BM MSC scratch assays compared to control. Pre-treatment of IJCM with heparin reduced the migration stimulatory capacity of IJCM such that reduction in wound closure was decreased compared to untreated IJCM for both (a) SF and (b) BM MSCs.

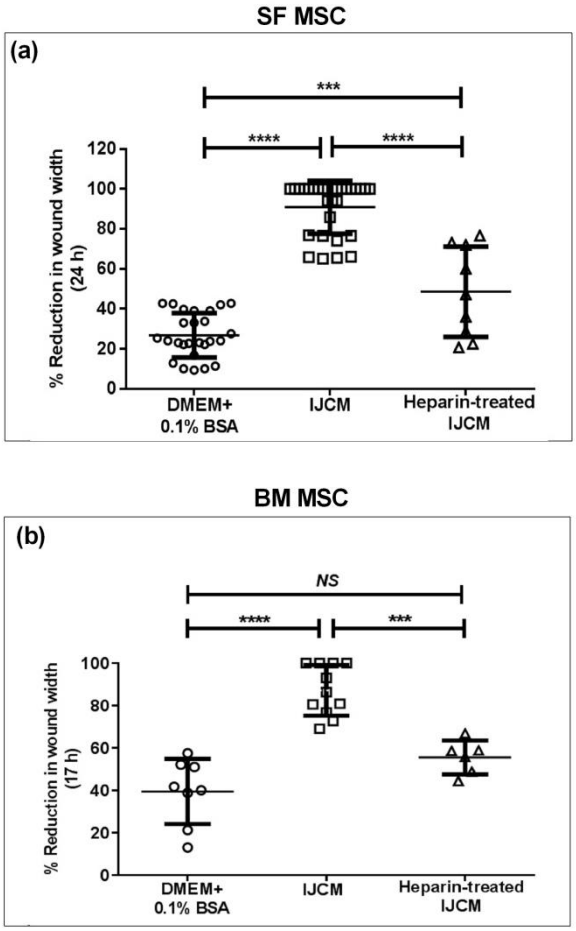


Figure 7.11: Migration of bovine (a) SF MSCs and (b) BM MSCs in response to heparin treated IJCM was evaluated using monolayer scratch assays. Graph showing percentage reduction in wound width over 24h (%) ± SEM. Significant reduction in wound width was observed with IJCM compared to control for both **(a)** SF MSC and **(b)** BM MSC. Pre-treatment of IJCM with heparin significantly reduced the migration stimulatory capacity of IJCM for both SF and BM MSCs. For example, the extent of wound closure by SF MSCs was only approximately 49% ± (8%) compared to 75% ± (6%) by untreated IJCM over 24h. And for BM MSCs it was only approximately 56% ± (3%) compared to 87% ± (4%) by untreated IJCM over 17h. However, SF MSC migration in response to treated IJCM was still significantly more compared to control (27% ± (2%)). In contrast, heparin-treatment reduced BM MSC migration to level seen with serum free negative control. *** $p < 0.001$ and **** $p<0.0001$.

7.3.6 Assessment of SF MSC migration in response to common migration stimulatory factors

We conducted scratch assays for bovine SF MSCs using seven chemokines and growth factors (CXCL12, TGFβ3, FGF2, CCL2, CCL3, CCL5 and TNFα) and compared their migration stimulatory capacity with control and IJCM. IJCM had the most potent chemotactic effect compared to both control and all 7 chemoattractants tested ($p<0.0001$) (Figure 7.12 and 7.13). Approximately, percentage reduction in wound width with IJCM was 90% ± (2%) ($p<0.0001$) compared to approximately 27% ± (2%) with control (DMEM+0.1% BSA). Among the 7 chemoattractants tested, CXCL12 (52% ± (5%); $p<0.0001$), TGFβ3 (54% ± (3%); $p<0.001$), CCL2 (48% ± (6%); $p<0.01$) and CCL5 (61% ± (4%); $p<0.0001$) showed significant reduction in wound width compared to control. No significant reduction in wound width was observed with FGF2 (47% ± (6%); NS), CCL3 (35% ± (4%); NS) and TNFα (42% ± (8%); NS) compared to control (Figure 7.14).

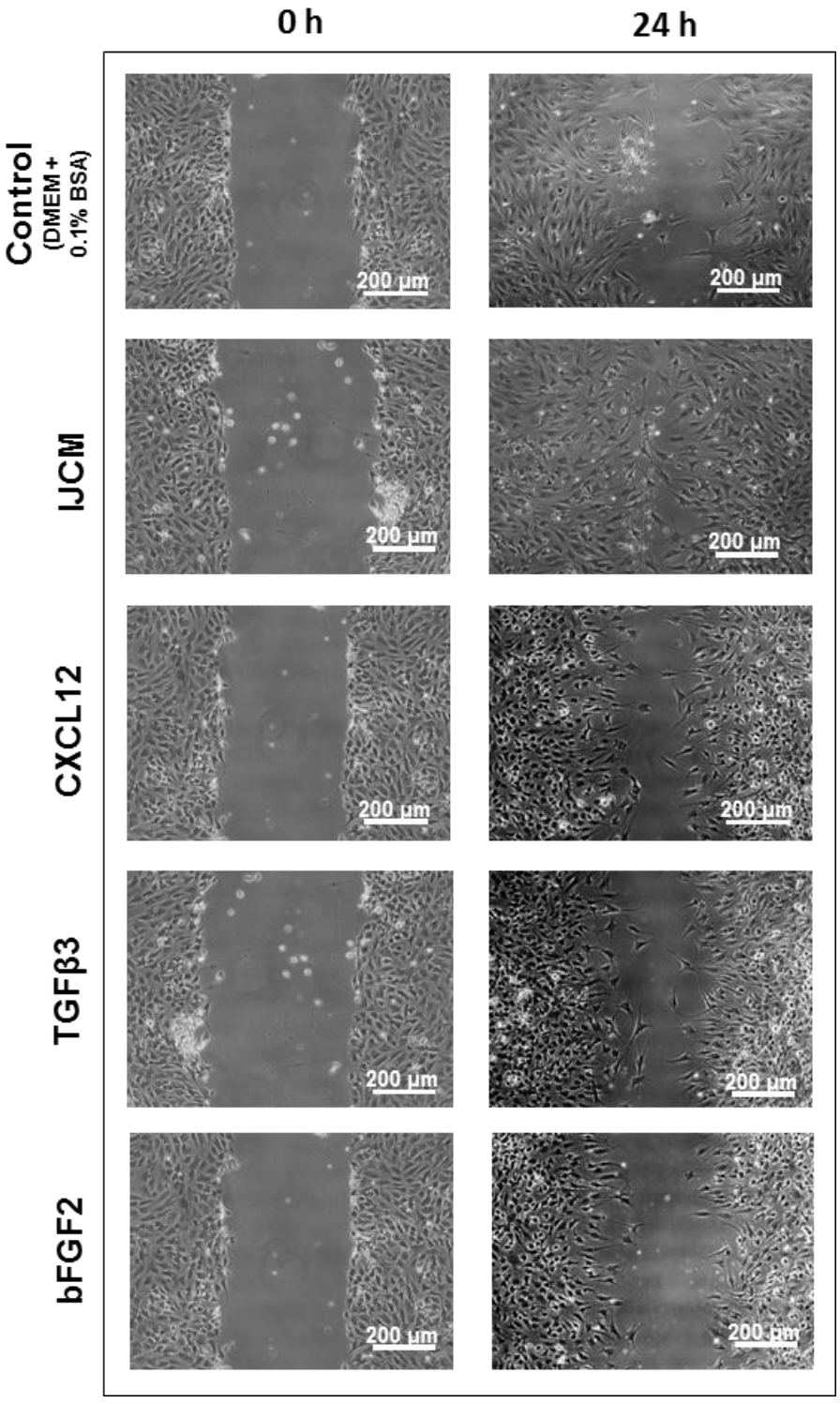


Figure 7.12: Assessment of SF MSC migration in response to CXCL12, TGFβ3 and FGF2 compared to conditioned media (IJCM) using scratch assay over 24 h. Images were taken at x10 magnification. Serum free media was used as control. All three growth factors induced MSC migration but complete wound closure was only observed with IJCM.

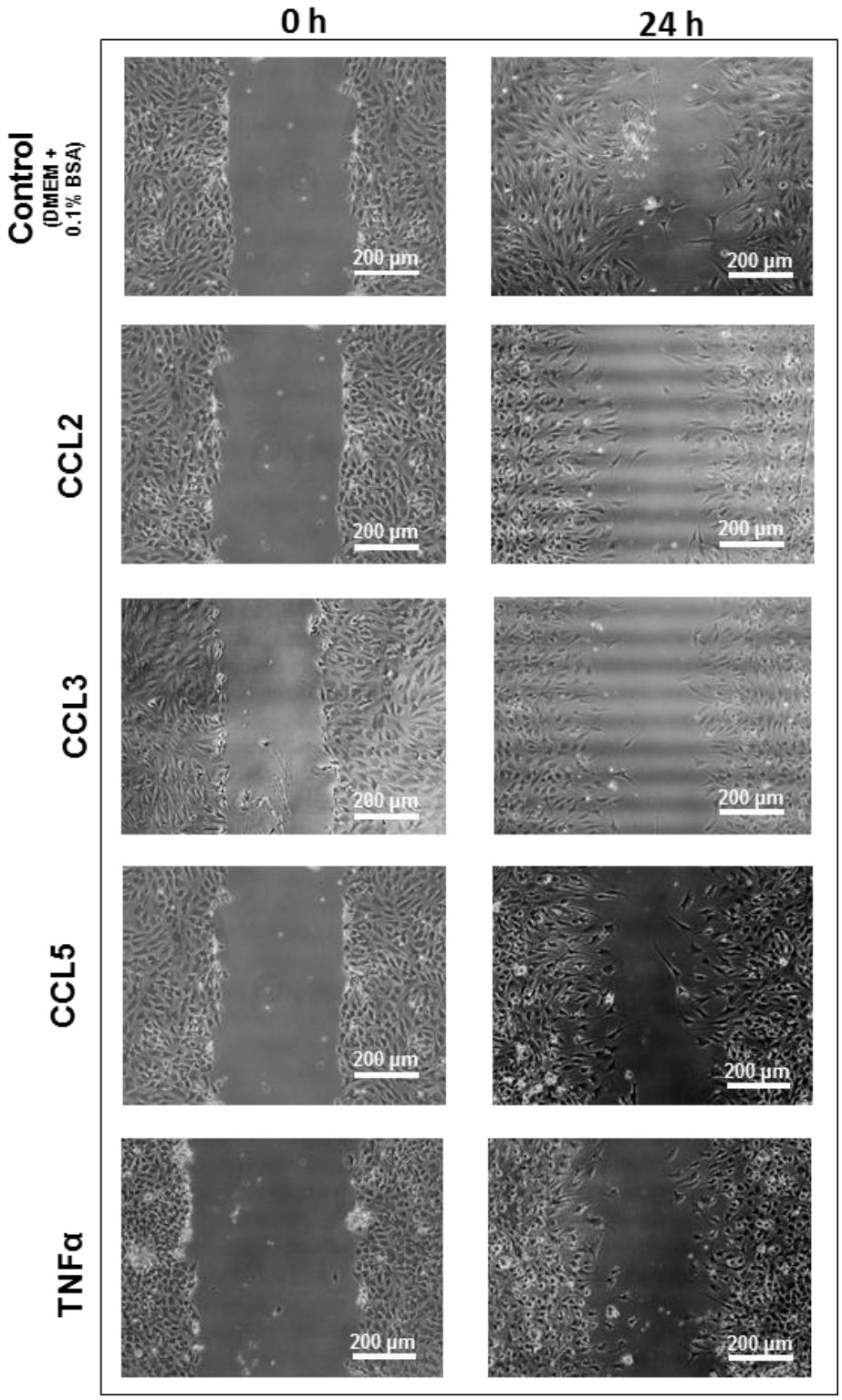


Figure 7.13: Assessment of SF MSC migration in response to CCL2, CCL3, CCL5 and TNFα compared to conditioned media (IJCM) using scratch assay over 24h. Images were taken at x10 magnification. Serum free media was used as control. All four cytokines induced MSC migration but complete wound closure was only observed with IJCM.

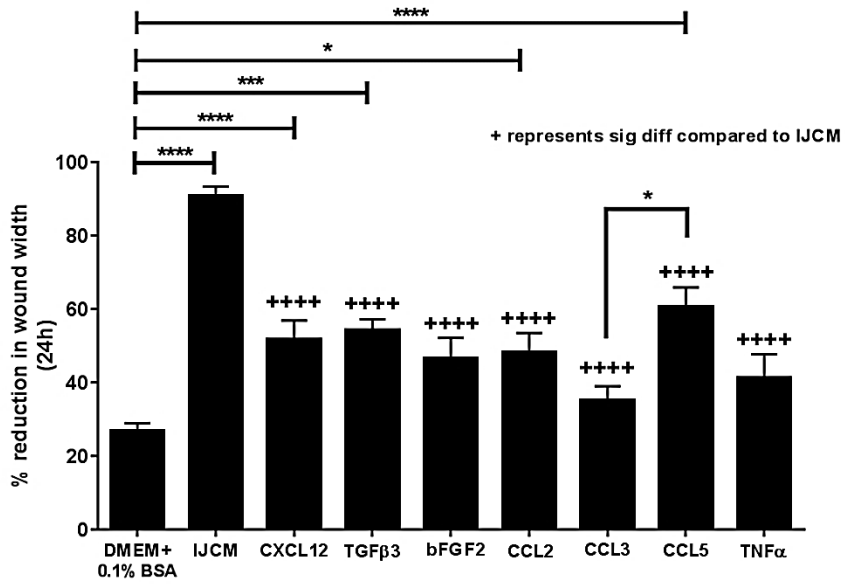


Figure 7.14: Assessment of bovine SF MSCs migration in response to seven chemokines and growth factors (CXCL12, TGFβ3, FGF2, CCL2, CCL3, CCL5 and TNFα) compared to IJCM. Graph showing percentage reduction in wound width (%) over 24h ± SEM. IJCM was the most potent inducer of SF MSC migration and induced significant MSC migration compared to both control and all chemoattractants tested. Among the 7 chemoattractants tested, CXCL12 (52% ± (5%)), TGFβ3 (54% ± (3%)), CCL2 (48% ± (6%)) and CCL5 (61% ± (4%)) showed significant reduction in wound width compared to control. No significant reduction in wound width was observed with FGF2 (47% ± (6%)), CCL3 (35% ± (4%)) and TNFα (42% ± (8%)) compared to control. * $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to control and ++++ $p<0.0001$ to IJCM.

7.3.7 Characterisation of injury conditioned media (IJCM) using antibody and receptor antagonists against common heparin interactome members

To identify migration stimulatory factors, present in the IJCM, neutralising antibodies and receptor inhibitors were used against common heparin-binding chemokines and growth factors previously reported to influence BM MSC migration. Both CXCL12 and TGFβ3 antibody and the CXCL12 receptor, (CXCR4) antagonist (AMD3100) significantly reduced the stimulatory migration effect of IJCM in SF MSC scratch assays (Figure 7.15). In contrast, a neutralising FGF2 antibody did not affect the migration stimulatory effect of IJCM in SF MSC scratch assays. Hence, when the extent of wound closure was 91% ± (3%) in the presence of untreated IJCM, it was 52% ± (4%) (P<0.0001 compared to IJCM) in the presence of CXCL12 antibody in the IJCM, 50% ± (3%) (P<0.0001 compared to IJCM) when cells were pre-treated with AMD3100, 45% ± (5%) (P<0.0001 compared to IJCM) in the presence of TGFβ3 antibody in the IJCM and 89% ± (4%) (compared to IJCM) in the presence of FGF2 antibody in the IJCM (Figure 7.17). . Controls of serum-free medium (DMEM + 0.1% BSA) and the antibodies and receptor antagonists were also tested on basal migration levels. No significant difference in wound closure was observed between the presence and absence of antibodies in the basal media.

Since, both CCL2 and CCL5 elicited significant MSC migration compared to serum free media (Figure 7.14), the effect of receptor inhibitors against CCL2 (also known as MCP-1) and CCL5 were also tested. Blocking either of the receptors did not have any significant effect on the stimulatory migratory effect of IJCM such that with CCL2 receptor antagonists approximately 92% ± (8%) and CCL5 receptor antagonists approximately almost 100% ± (1%) reductions in wound width were observed (Figure 7.16 and 7.17). Furthermore, the effect of a neutralising antibody against CX3CL1 was investigated since previous studies have reported CX3CL1 to be potent inducer of human BM MSC migration. However, no significant difference in MSC migration was observed (approximately 91% ± (5%)) was observed in the presence of the CX3CL1 neutralising antibody compared to IJCM (Figure 7.16 and 7.17).

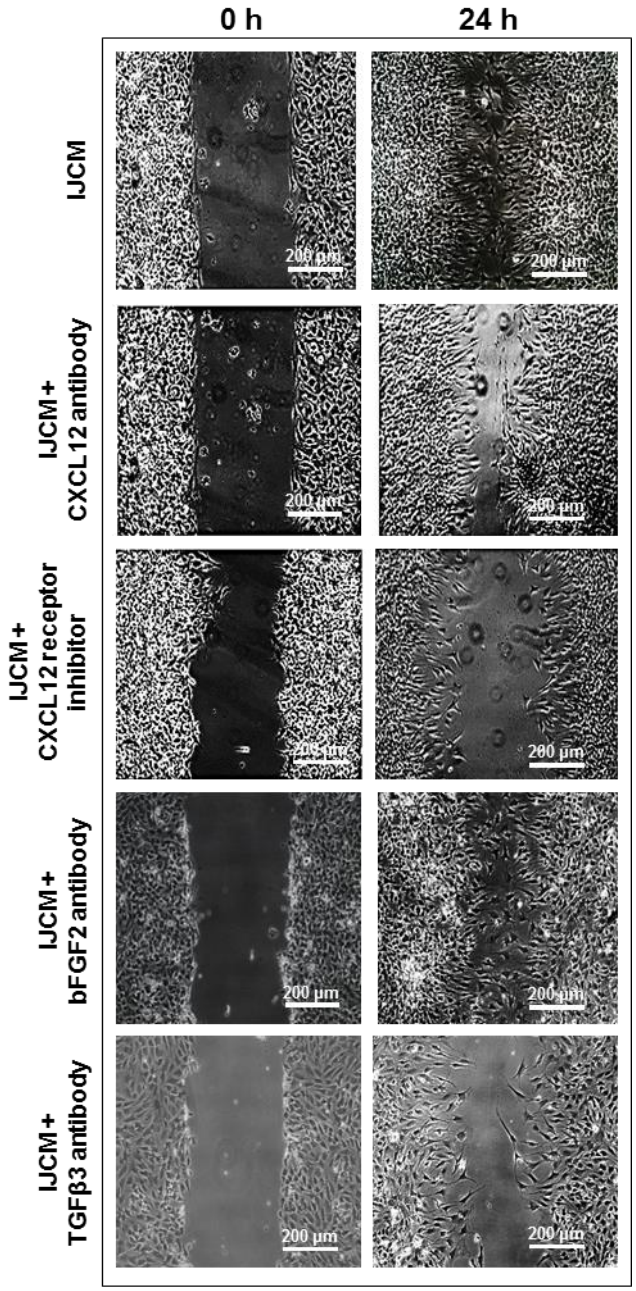


Figure 7.15: Release of migration stimulatory factors from injured cartilage following sharp injury was studied using antibody and receptor antagonists. SF MSC migration was studied using scratch assays.
Representative image of one experiment. Images were taken at x10 magnification. Serum free media (DMEM+ 0.1% BSA) was used as negative control. Almost complete wound closure was observed with IJCM. Both treatment of IJCM with CXCL12 antibody and pre-treatment of MSCs with CXCL12 receptor (CXCR4) antagonists (AMD3100) reduced the migration stimulatory capacity of IJCM. Treatment of IJCM with FGF2 antibody had no effect on IJCM's ability to induce MSC migration and almost complete closure of wound was observed.

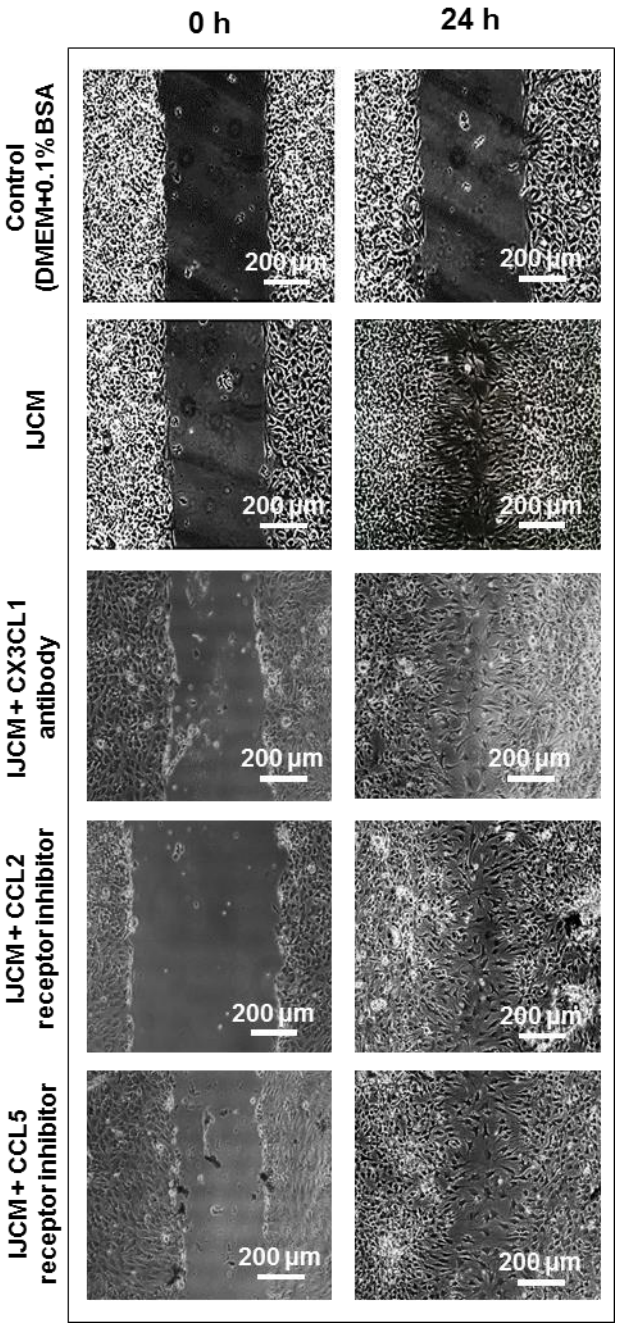


Figure 7.16: Release of migration stimulatory factors from injured cartilage following sharp injury was studied using antibody and receptor antagonists. SF MSC migration was studied using scratch assays. Representative image of one experiment. Images were taken at x10 magnification. Serum free media (DMEM+ 0.1% BSA) was used as negative control. Almost complete wound closure was observed with IJCM. Treatment of IJCM with CX3CL1 antibody, CCL2 receptor (CCR2) antagonists or CCL5 receptor (CCR5) antagonists had no effect on IJCM's ability to induce MSC migration and almost complete closure of wound was observed following 24h.

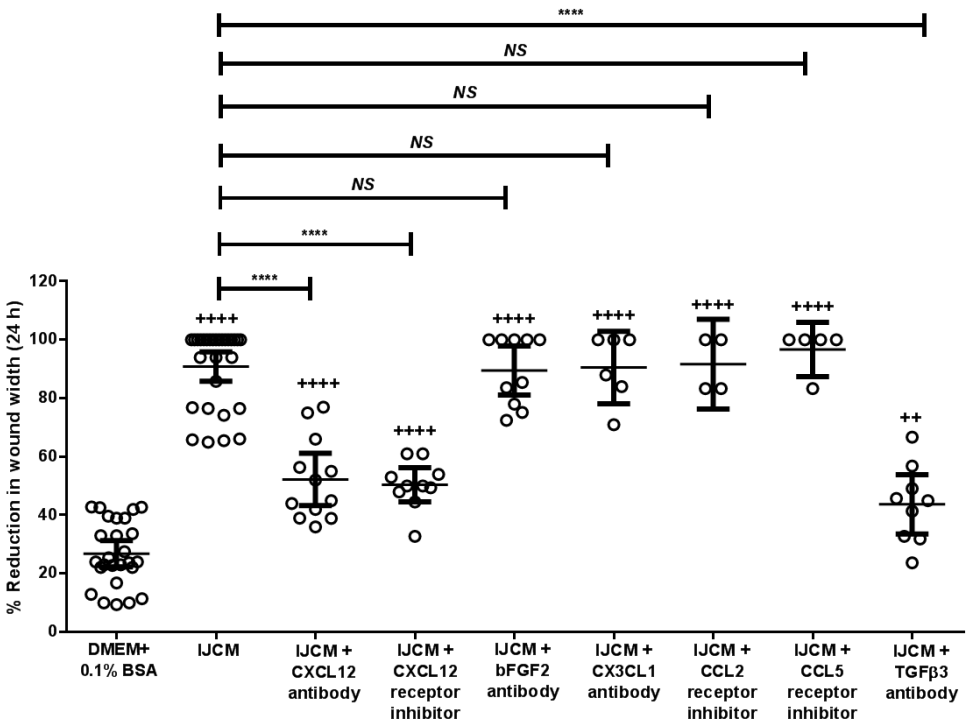


Figure 7.17: Release of migration stimulatory factors from injured cartilage following sharp injury was studied using antibody and receptor antagonists. Graph showing percentage reduction in wound width (%) over 24h ± SEM. Both pre-treatment of IJCM with CXCL12 antibody and pre-treatment of MSCs with CXCL12 receptor (CXCR4) antagonist (AMD3100) significantly reduced the migration stimulatory capacity of IJCM. The neutralising antibody significantly reduced MSC migration by approximately 39% ± (4%) and CXCR4 antagonist AMD3100 significantly reduced MSC migration by approximately 40% ± (3%). Other antibodies and receptor antagonists had no significant effect on the migration stimulatory capacity of IJCM. **** $p<0.0001$ compared to control and ++++ $p<0.0001$ to IJCM.

7.3.8 Assessment of pre-treated of SF MSC (pre-treated with AMD3100) migration in response to IJCM in transwell assay

In order to validate the results from scratch assays, SF MSCs were pre-treated with CXCL12 receptor inhibitor (AMD3100) before seeding into the upper wells of the Transwell chambers. We also tested the presence of FGF2 by blocking the growth factor activity using FGF2 antibody in the IJCM. Serum free media (DMEM+0.1% BSA) was used as negative control and 5% serum (DMEM+5% serum + 0.1% BSA) was used as positive control Similarly to the previous results from scratch assays, pre-treatment of MSCs with ADM3100 significantly reduced MSC migration in response to IJCM by approximately 0.4 fold ($p<0.01$) compared to non-treated MSCs (Figure 7.18d and 7.19). Further, no significant difference in migration was observed between ADM3100 treated-MSCs in response to IJCM and untreated MSCs in response to negative control. Moreover, no significant difference in migration was observed when IJCM was treated with FGF2 antibody compared to untreated IJCM (IJCM: 1.2 fold increase in migration compared to negative control ($p<0.0001$); FGF2 antibody treated IJCM: 1.1 fold increase in migration compared to negative control ($p<0.0001$)) (Figure 7.18e and 7.19).

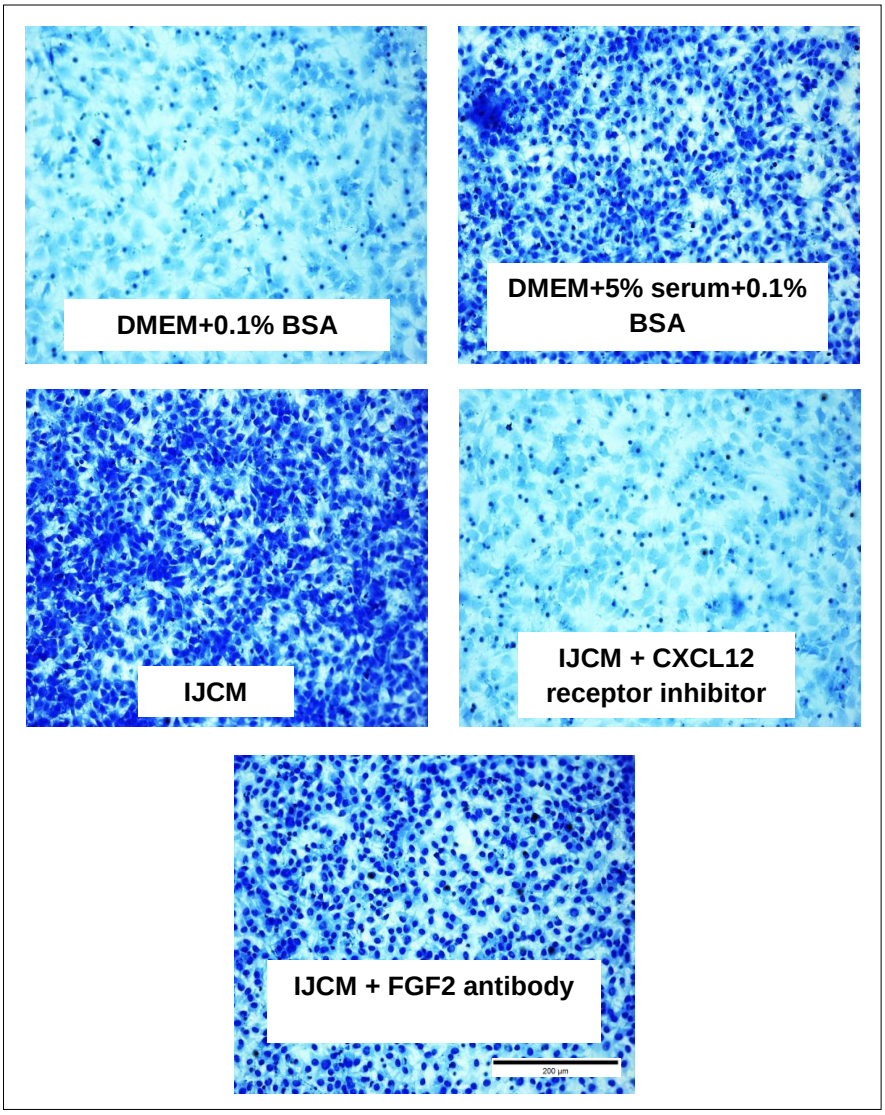


Figure 7.18: Release of migration stimulatory factors from injured cartilage following sharp injury was studied using antibody and receptor antagonists.SF MSC migration was studied using transwell assay. Membranes were stained with crystal violet. Representative image of one microscopic field at x20 magnification is shown here. Serum free media (DMEM+0.1% BSA) was used as negative control and 5% serum (DMEM+5% serum+0.1% BSA) was used as positive control. Effect of CXCL12 studied using CXCL12 receptor (CXCR4) antagonist (AMD3100). Effect of FGF2 was studied using FGF2 antibody. Pre-treatment of MSCs with AMD3100 reduced MSC migration in response to IJCM compared to untreated MSCs. Pre-treatment of IJCM with FGF2 antibody did not affect MSC migration compared to untreated IJCM.

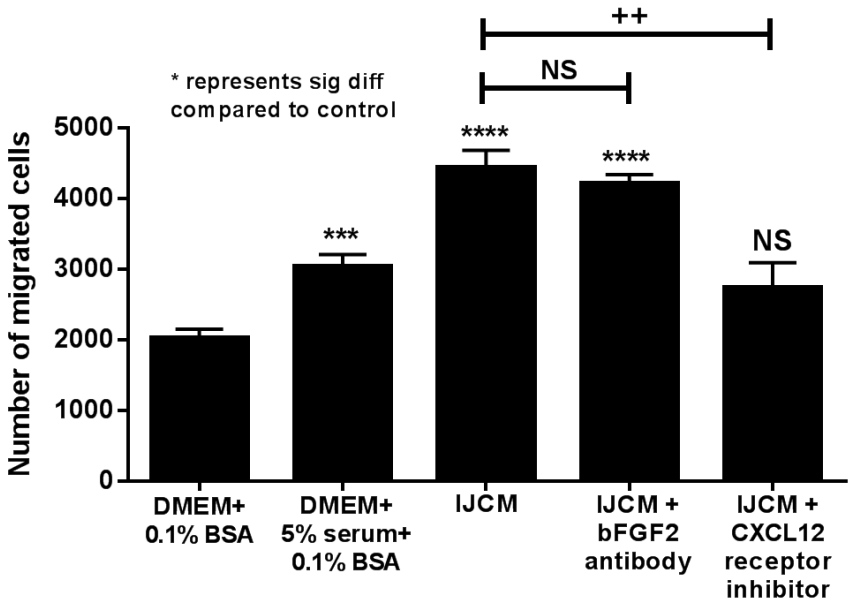
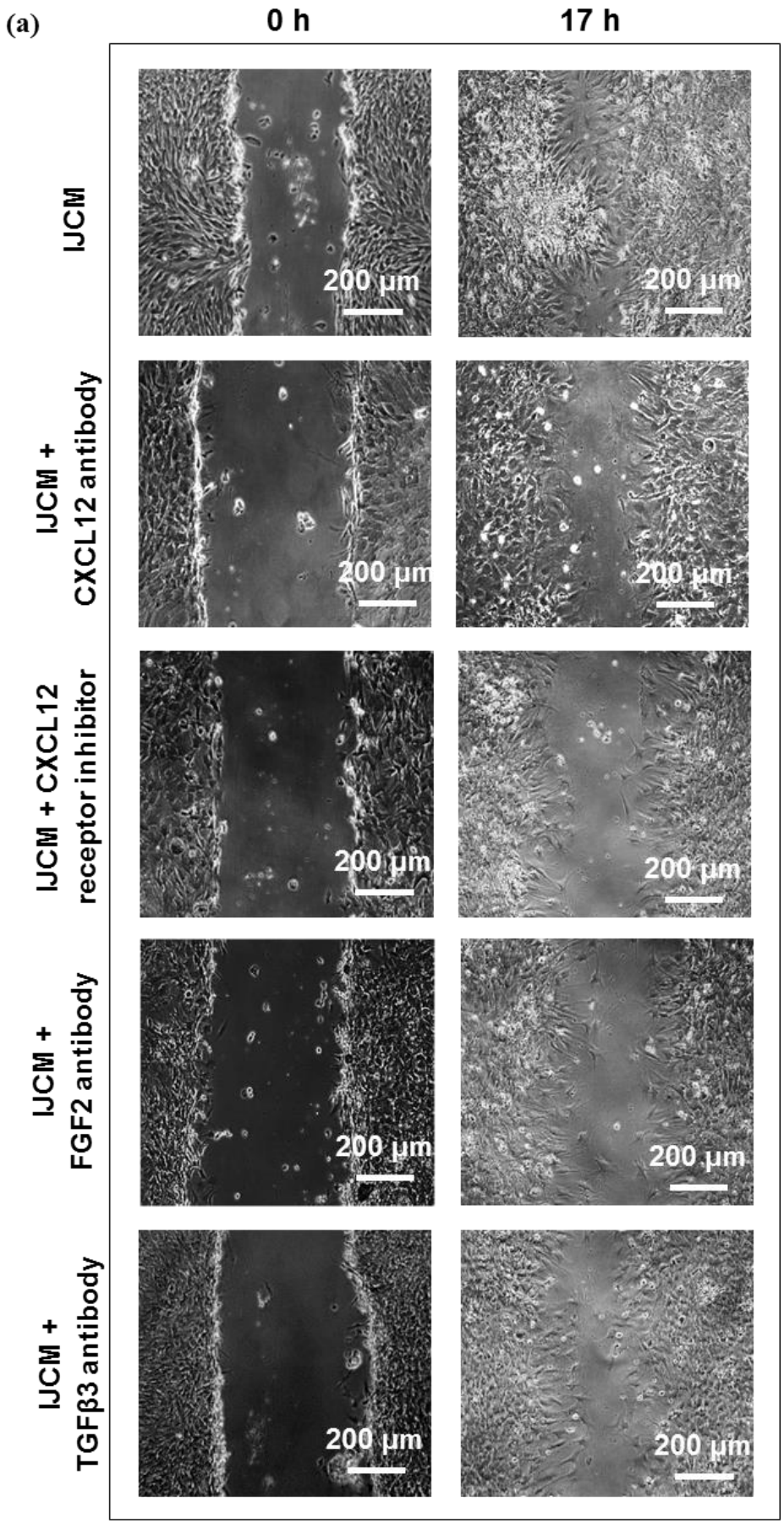


Figure 7.19: Release of migration stimulatory factors from injured cartilage following sharp injury was studied using antibody and receptor antagonists. SF MSC migration was studied using transwell assay. Graph showing mean number of migrated cells ± SEM. Serum free media (DMEM+0.1% BSA) was used as negative control and 5% serum (DMEM+5% serum+0.1% BSA) was used as positive control. ADM3100 (CXCL12 receptor inhibitor) significantly reduced MSC migration in response to IJCM by approximately 0.4 fold compared to non-treated MSCs. Further, no significant difference in migration was observed between pre-treated MSCs in response to IJCM and untreated MSCs in response to negative control. No significant difference in migration was observed when IJCM was treated with FGF2 antibody compared to untreated IJCM (IJCM: 1.2 fold increases in migration compared to negative control; FGF2 antibody treated IJCM: 1.1 fold increases in migration compared to negative control). *** $p<0.001$ and **** $p<0.0001$ compared to control.

7.3.9 Characterisation of IJCM using antibodies against CXCL12, CXCR4, TGFβ3 and FGF2

In comparison with SF MSCs, neutralising antibodies to TGFβ3 and CXCL12 and AMD3100 significantly reduced the migration stimulatory capacity of IJCM in BM MSC scratch assays. However, in contrast to SF MSCs, FGF2 antibodies also significantly affected IJCM's ability to induce BM MSC migration (Figure 7.20a). When the extent of wound closure was approximately 85% ± (4%) with untreated IJCM, it was only 55% ± (3%) (P<0.0001) in the presence of CXCL12 antibody in the IJCM, 60% ± (4%) (P<0.0001) when cells were pre-treated with AMD3100, 55% ± (4%) (P<0.0001) in the presence of TGFβ3 antibody in the IJCM and 52% ± (4%) (P<0.0001) in the presence of FGF2 antibody in the IJCM (Figure 7.20b).


(a)
0 h
17 h
IJCM
IJCM + CXCL12 antibody
IJCM + CXCL12 receptor inhibitor
IJCM + FGF2 antibody
IJCM + TGFβ3 antibody
200 µm

(b)

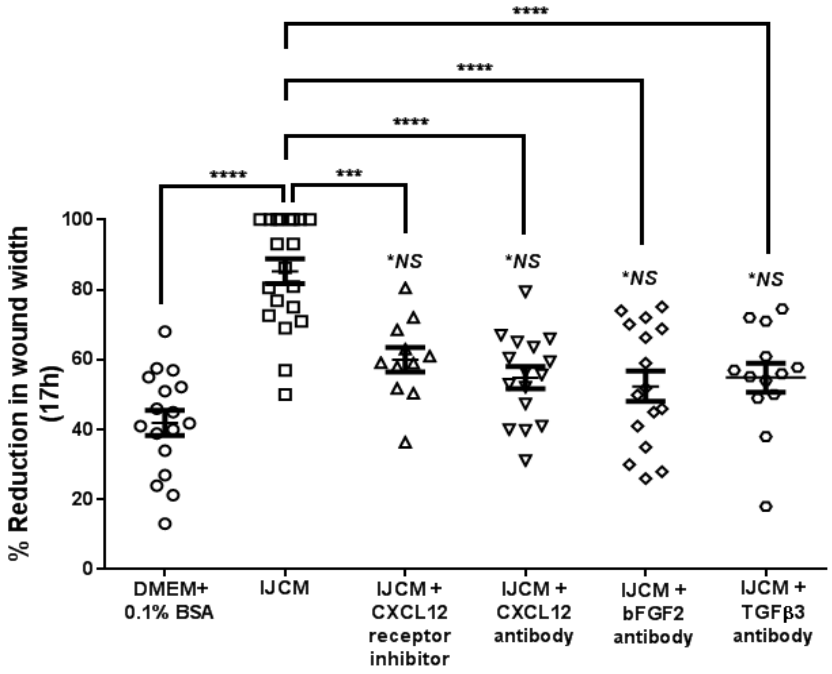


Figure 7.20: Release of migration stimulatory factors from injured cartilage following sharp injury was studied using antibody and receptor antagonists. BM MSC migration was studied using scratch assays. (a) Representative image of one experiment. Images were taken at x10 magnification. Serum free media (DMEM+ 0.1% BSA) was used as negative control. Almost complete wound closure was observed with IJCM. Treatment of IJCM with CXCL12, FGF2 and TGFβ3 antibody and pre-treatment of MSCs with CXCL12 receptor (CXCR4) antagonists (AMD3100) reduced the migration stimulatory capacity of IJCM. **(b)** Graph showing percentage reduction in wound width (%) over 17h ± SEM. Wound closure by IJCM was approximately 87% ± (4%). CXCL12 neutralising antibody significantly reduced migration stimulatory capacity of IJCM by approximately 33% ± (4%). Pre-treatment with AMD3100 also significantly reduced the effect of IJCM by approximately 28% ± (5%). The migration stimulatory capacity of IJCM was reduced by approximately 22% ± (4%) by TGFβ3 antibody and by approximately 28% ± (6%) by FGF2 neutralising antibody.

7.3.10 Investigation of mechanisms for the release of migration stimulatory factors from the cartilage following sharp injury

Two mechanisms for the release of factors following injury were investigated: synthesis of factors in response to injury and metalloproteinase (MMP) activity using the general protein synthesis inhibitor, cycloheximide and the MMP inhibitor marimastat. Cycloheximide and not marimastat significantly reduced the migration stimulatory capacity of IJCM in SF MSC scratch assays (Figure. 7.21). When the extent of wound closure was 100% ± (0%) with untreated IJCM, it was only approximately 34% ± (2%) (P<0.0001 compared to IJCM) when cartilage was injured in the presence of cycloheximide and 100% ± (0%) when cartilage was injured in the presence of marimastat.

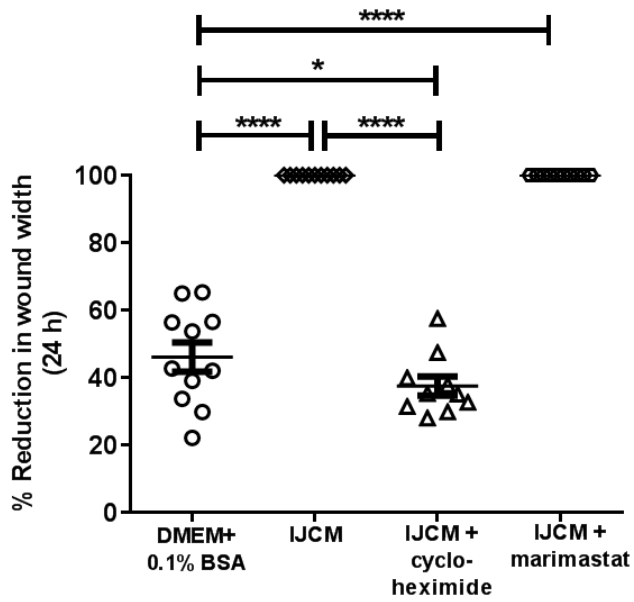


Figure 7.21: Mechanism for the release of migration stimulatory factors following injury was assessed using cycloheximide and marimastat. Graphical representation of percentage reduction in wound width over 24 h ± SEM. Statistical significances (P values) are shown in the graph. *P values represent differences compared to DMEM+0.1% BSA (control).

7.3.11 IJCM promotes MSC survival

IJCM was investigated to determine if it could promote MSC survival by plating MSCs in tissue culture flasks re-suspended in IJCM and maintained them in the IJCM only for 10 days. Both SF and BM MSCs survived in the IJCM for the 10 day culture period, however, none of the cultures reached confluency. Both MSCs formed network of cells following day 2 of culture (Figure 7.22a and d). At day 4, both MSCs appeared as elongated cells with protruding ends as seen in scratch assays during wound closure (Figure 7.22b and e). By day 10, cells aggregated to form long tubes with single cells migrating outwards (Figure 7.22c and f). These structures were similar to those seen after initial plating (passage 0) of freshly isolated cells forming colony forming units (CFU).

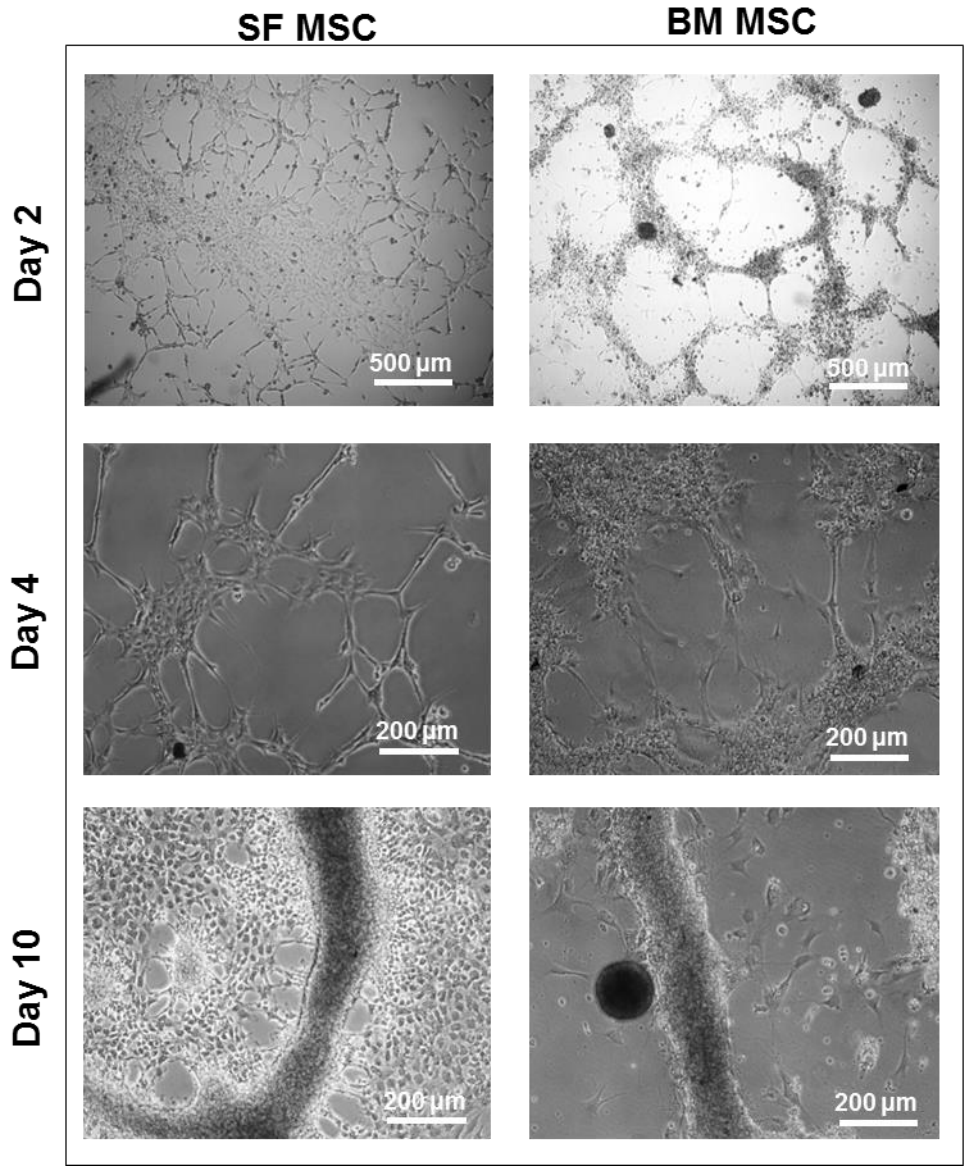


Figure 7.22: IJCM promotes MSC survival. MSCs cultured in IJCM. Representative time lapse images of SF and BM MSC cultures. Day 2 images were taken at x4 magnification to visualise the cell network. Day 4 and d 10 were imaged at x10 magnification.

7.4 Chapter discussion

In this study freshly harvested cartilage was injured in the presence of serum free media. The injured cartilage explants were then incubated in the same serum free media for 24 h and the media was then collected as injury conditioned media (IJCM). The rationale behind this technique was that if cartilage releases any migratory factors following injury, the migratory factors would be released into the serum-free media. The capacity of IJCM to stimulate MSC migration was determined using Transwell and scratch assays. IJCM stimulated significant migration of both BM and SF MSCs in both Transwell and scratch assays compared to serum free media. Furthermore, histological assessment of injured cartilage incubated with SF MSCs in the presence and absence of IJCM suggested higher MSC migration to the site of injury in the presence of IJCM compared to absence of IJCM.

IJCM was also tested for its capacity to stimulate chondrocyte and MG-63 cell migration. In these assays no significant migration was observed in response to IJCM compared to serum free media. These results suggested that following injury, articular cartilage released migratory factors which were specific for stimulating MSC migration to the site of injury. Further characterisation of IJCM suggested that the migration stimulatory factors were CXCL12, TGFβ3 and FGF2.

7.5 Chapter Summary

Previous results suggested that the mechanism through which MSCs detect and respond to injured cartilage could occur through migration along the chemotactic gradient of cytokines and growth factors released by the injury. These chemoattractants are also capable of inducing chondrogenic differentiation of both MSCs. Some of these cytokines are possibly released from the cartilage ECM, possibly bound to heparin sulphate proteoglycans. The key cytokines identified through this study were CXCL12, TGFβ3 and FGF-2. However, our results suggested that SF MSCs may have a different migration mechanism compared to BM MSCs in terms of what cytokines can trigger migration to the injury site. Our findings have the implication of designing a method that can direct a large number of endogenous MSCs towards the site of the cartilage injury, maintain these cells

within the defect and induce chondrogenic differentiation which would increase the success of endogenous cartilage repair.

Chapter 8

Priming of SF MSC using extracellular nucleotides

8.1 Hypothesis

Cell injury releases short-lived factor(s) which is required to prime MSC to home along the chemotactic gradient towards the site of the cartilage injury.

8.2 Chapter introduction

5'-nucleotide triphosphates such as ATP and UTP are ubiquitous extracellular messengers involved in various cell functions such as cell survival, cell death, proliferation, migration and differentiation. They act via purinoceptors (P2Rs) which are plasma membrane receptors further divided into P2X and P2Y subfamilies. P2XRs are selective ion channels formed by two or three identical subunits containing two plasma membrane spanning domains and a large extracellular loop, permeable to monovalent and divalent ions. P2RYs are seven transmembrane spanning G-protein coupled receptors which transduces signal via activation /inhibition of adenylate cyclase or the activation of phospholipase C [75].

Previous studies have also reported the involvement of ATP in hematopoietic stem cells recruitment to the damaged tissue. It was shown that nucleotides released from the damaged tissue were involved in paracrine signalling in conjunction with chemokines such as CXCL12 to stimulate stem cell migration to the injury site where they may participate in tissue regeneration [75]. Extracellular nucleotides have also been shown to be chondrogenic in bovine articular cartilage explants. For example, using a pellet culture of chondrocytes, it was shown that presence of ATP and UTP enhances accumulation of cartilage collagen and proteoglycans [109].

Therefore, we studied the capacity of extracellular nucleotides to affect migration of synovial fluid (SF) and bone marrow (BM) MSCs to the site of the cartilage injury. Our results suggested that while ATP and UTP released through cell injury was not required for BM MSC migration, they are required to prime SF MSCs before migration along the CXCL12 chemotactic gradient.

8.3 Results

8.3.1 Cell injury is required to prime SF MSCs but not BM MSCs

To assess the requirement for cell activation or cell injury for MSC migration, cell exclusion zones were created at the time of cell seeding by using Ibidi silicon inserts. Using these inserts also avoided problems such as unevenly thick gap created by cell scraping, and reattachment of floating cells in the wound area which then migrate towards the monolayer and have the risk of providing adulterated results. Migration into the cell exclusion zone was followed microscopically over 24 h. Serum free media (DMEM+0.1% BSA) was used as negative control. In contrast to its effect on MSC migration in scratch assays, IJCM induced little SF MSC migration into the cell free gap and was not statistically different from the negative (serum-free) control (Figure 8.2). More interestingly, heparin-treated IJCM stimulated 100% wound closure in SF MSC cultures in this assay system which was not observed with scratch assays (Figure 8.2).

In contrast, using the exclusion zone assays, 100% wound closure was observed with BM MSCs in response to IJCM. These results suggested that unlike SF MSCs, BM MSCs do not require a short-acting priming signal released through cell injury for initiation of migration (Figure 8.1).

To compare the effect of cell injury with no cell injury on SF MSC migration, a ‘wound’ was created by producing a fine scratch in the monolayer distant to the cell exclusion zone. In this case the IJCM affected rapid wound closure, inducing rapid MSC migration into the scratch but little MSC migration was observed into the cell exclusion zone. To test if the cell injury from the creation of the ‘wound area’ can induce SF MSC migration in response to IJCM into the cell exclusion zone, a fine scratch/scraping wound was created very close to the cell exclusion zone. After 24 h, SF MSC migration was induced in both the ‘wound area’ and the cell exclusion zone (Figure 8.3a). In contrast, creation of the manual wound completely inhibited SF MSC migration into the cell exclusion zone in the presence of heparin-treated IJCM (Figure 8.3b).

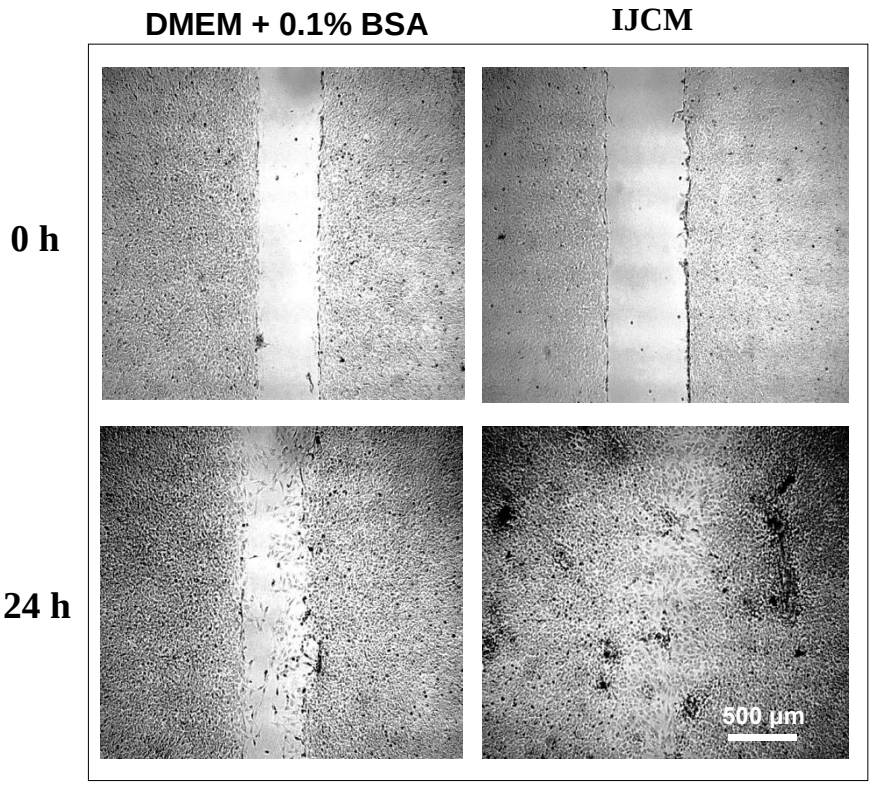


Figure 8.1: BM MSC migration in cell exclusion zone assay where creation of wound does not cause cell injury. Images were taken at x4 magnification. Cell migration was observed both in the absence of serum (control) and IJCM. However, whereas complete wound closure was observed in response to IJCM following 24 h migration, wound closure was not complete in control experiments.

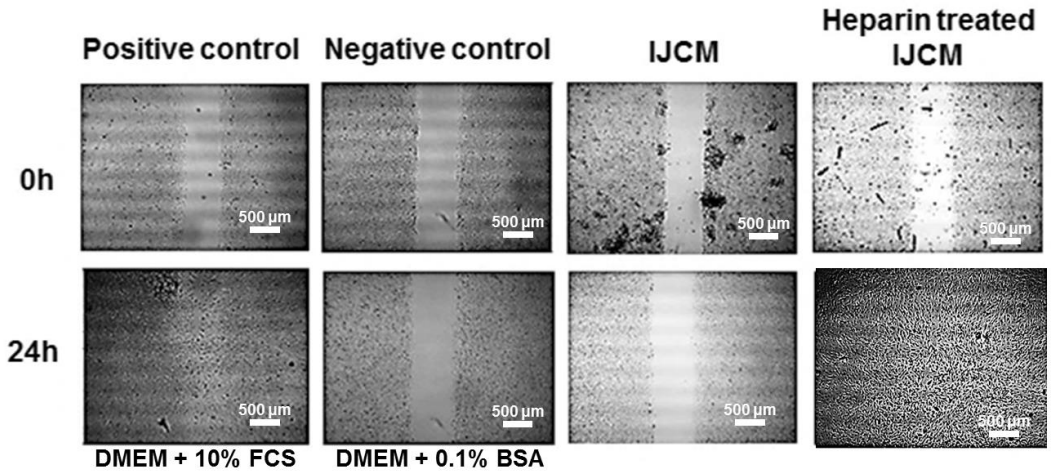


Figure 8.2: Cell exclusion zone assays were used to assess SF MSC migration in response to IJCM and heparin-treated IJCM. No SF-MSC migration was observed in response to IJCM but migration was observed in response to heparin-treated IJCM. Images were taken at x4 magnification.

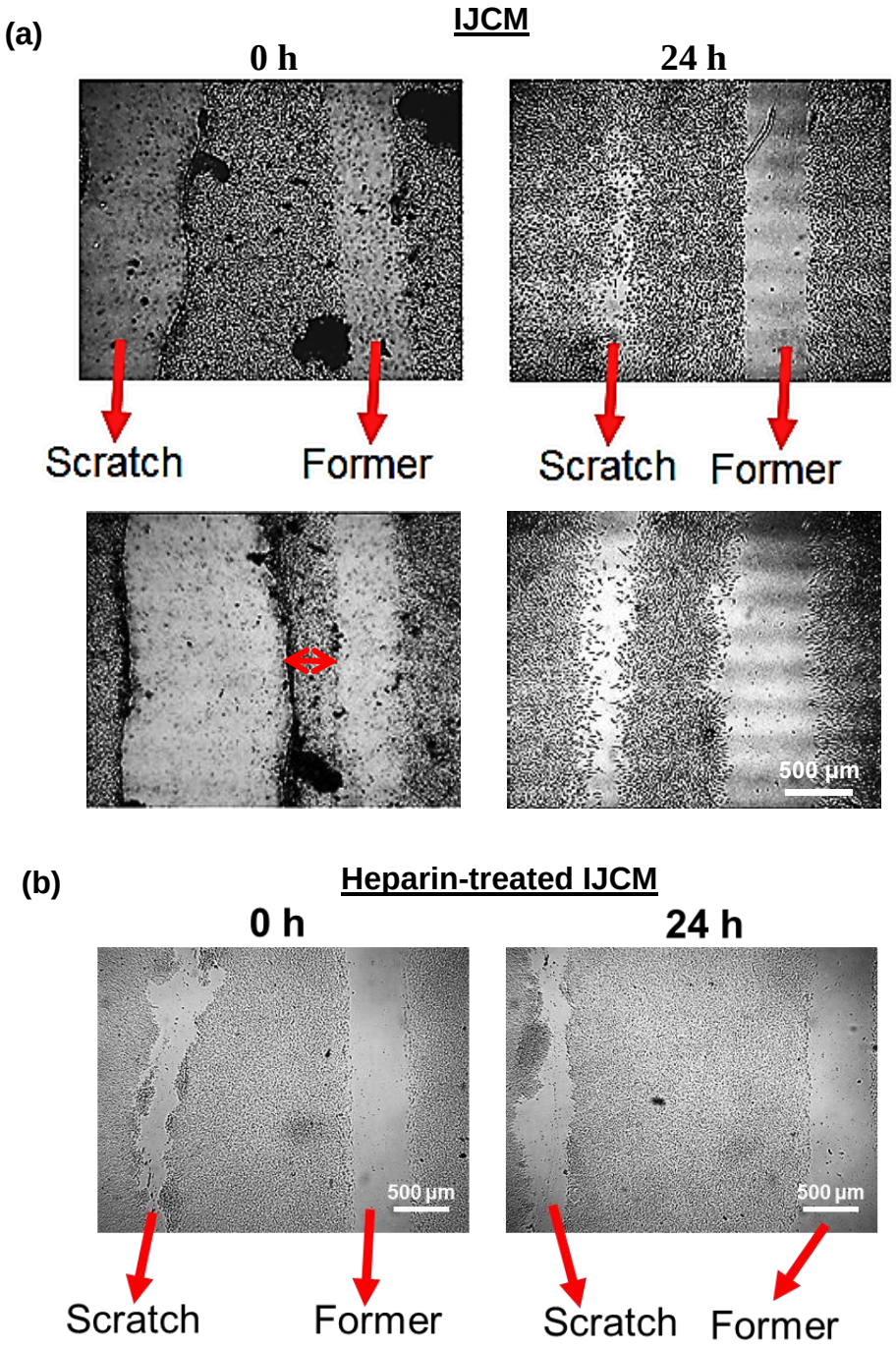
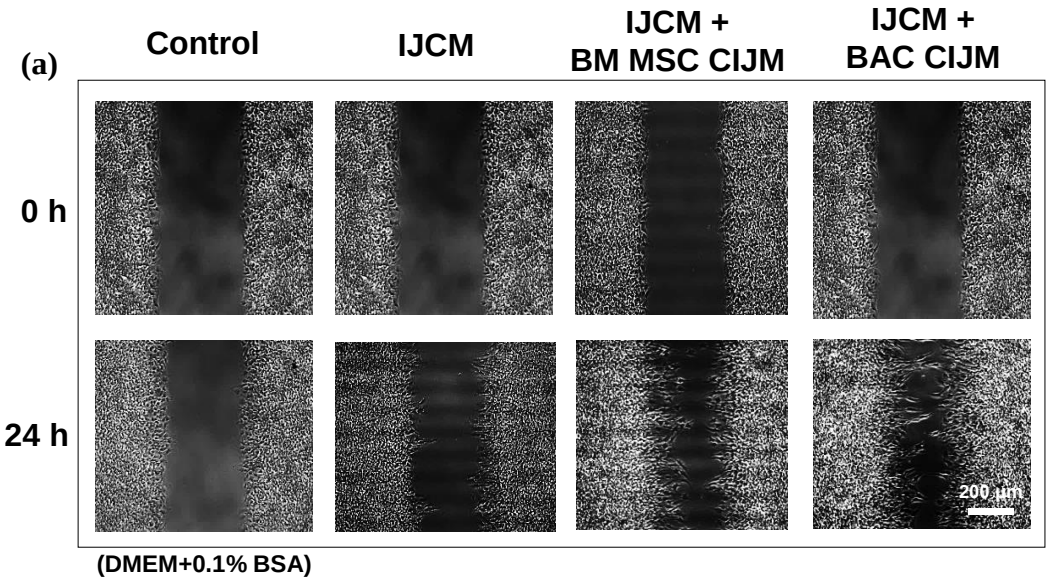


Figure 8.3: Investigation of SF MSC migration after wound was created on the same cell monolayer, side by side using both a former and manual cell scraping. Images were taken at x4 magnification. **(a)** When a scratch was created in one of the monolayers next to the cell exclusion zone, complete wound closure was observed in the manual scratch but not in the cell exclusion zone. Migration in response to IJCM was observed in this system when a cell injury was created (by scraping off cells) closer to the cell exclusion zone. **(b)** In contrast, creating a manual wound inhibited MSC migration into the cell exclusion zone in response to heparin-treated IJCM

8.3.2 Factors released through cell injury in combination with IJCM are required to stimulate migration

To test if migration in the exclusion zones were induced by release of stimulatory factors from the cell injury, chondrocytes were grown to confluency and the cell layer was then injured using 200 µl pipette tip in the presence of serum free media. The medium was collected after 30 mins for either immediate use or immediately stored at -20°C. The cell exclusion zone assay was carried out using a combination of IJCM and cell injury conditioned medium (CIJM) and the degree of migration measured after 24 h assay. Significant MSC migration was observed in response to this combined conditioned medium compared to both control and IJCM alone (Figure 8.4a). Hence, when wound closure was 77% ± (2%) with combined conditioned medium, it was only 21.1% ± (3%) with IJCM, 13% ± (3%) with CIJM alone and 16% ± (2%) with serum free control (Figure 8.3b). To test whether the cell injury factors were released specifically from the chondrocytes or simply through any cell injury, BM MSCs grown to confluence were injured similarly to chondrocytes in the presence of serum free medium and CIJM was collected after 30 mins. Cell exclusion zone assay carried out using the BM MSC CIJM combined with IJCM also induced significant MSC migration (72% ± (7%) into the cell free gap (Figure 8.4b).



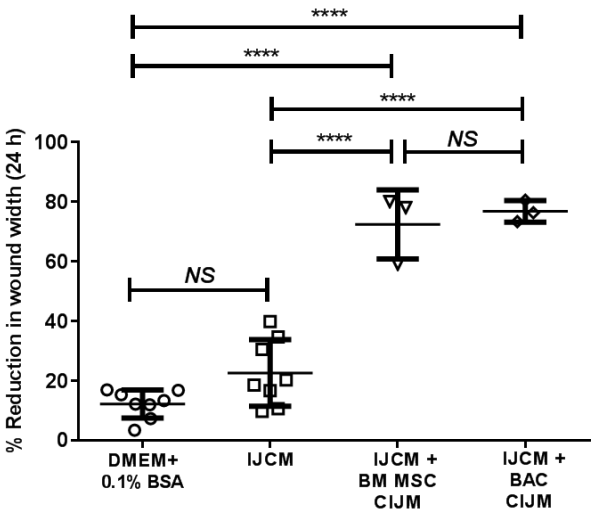


Figure 8.4: In the cell exclusion zone assay, no SF MSC migration was observed in response to IJCM. (a) Images were taken at x10 magnifications. Combination of IJCM and cell injury conditioned media (CIJM) stimulated SF MSC migration into the cell exclusion zone of non-injured MSC cultures. **(b)** Graph showing % reduction in wound width (%) ± SEM in response to IJCM and IJCM supplemented with CIJM either collected by injuring BM MSC monolayer cultures (BM MSC CIJM) or bovine articular chondrocyte monolayer cultures (BAC CIJM). Significant reduction in wound width was observed with both BM MSC CIJM and BAC CIJM supplementation with IJCM compared to control. **** $p<0.0001$ compared to negative control.

8.3.3 Effect of purines UTP and ATP on the IJCM mediated migration of SF MSC

The experiments with the cell injury conditioned medium (CIJM) strongly suggested that cell injury released active factors which were required for initiation of MSC migration in response to IJCM. Since, ATP has been reported to influence MSC migration, whether ATP/UTP accounted for all or part of the activity of the CIJM was tested. Preliminary cell exclusion zone assays were performed using IJCM supplemented with ATP (250 µM) or UTP (250 µM). These experiments were performed using two different types of silicon inserts provided by Ibidi (measures % reduction in wound width) and Platypus Technologies (Oris-FLEX, measures % reduction in wound area). Results of the assay are summarised below in table 1.

Table 8.1: Results from cell exclusion zone assays performed using two different types of silicon inserts. MSC migration was assessed in response to IJCM and IJCM supplemented with extracellular nucleotides.

	Ibidi Inserts (% reduction in wound width ± SEM); p value	**Oris-FLEX (% reduction in wound area ± SEM); p value**
Negative control	12 ± 2	17 ± 2
IJCM	23 ± 4; NS	14 ± 3; NS
IJCM +BM-MSC CIJM	72 ± 7; p<0.0001	Not tested
IJCM +BAC CIJM	77 ± 2; p<0.0001	37 ± 3; p<0.01
IJCM + ATP	43 ± 6; p<0.001	34 ± 1; p<0.001
IJCM + UTP	66 ± 3; p<0.0001	41 ± 3; p<0.0001

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A significant increase in SF MSC migration in response to IJCM supplemented with CIJM and IJCM supplemented with extracellular nucleotides was observed compared to IJCM alone with both Ibidi and Oris-FLEX inserts. However, when Ibidi inserts were used, addition of ATP could not induce similar degree of migration compared to addition of CIJM. Further, we also observed a significant increase in migration with UTP compared to ATP (Figure 8.5a and b). In contrast, no such differences were observed with Oris-FLEX inserts (Figure 8.6). However, both inserts suggested no significant increase in migration in response to IJCM compared to control. These results suggest that Ibidi inserts may be more sensitive at detecting differences in MSC migration compared to Oris-FLEX inserts.

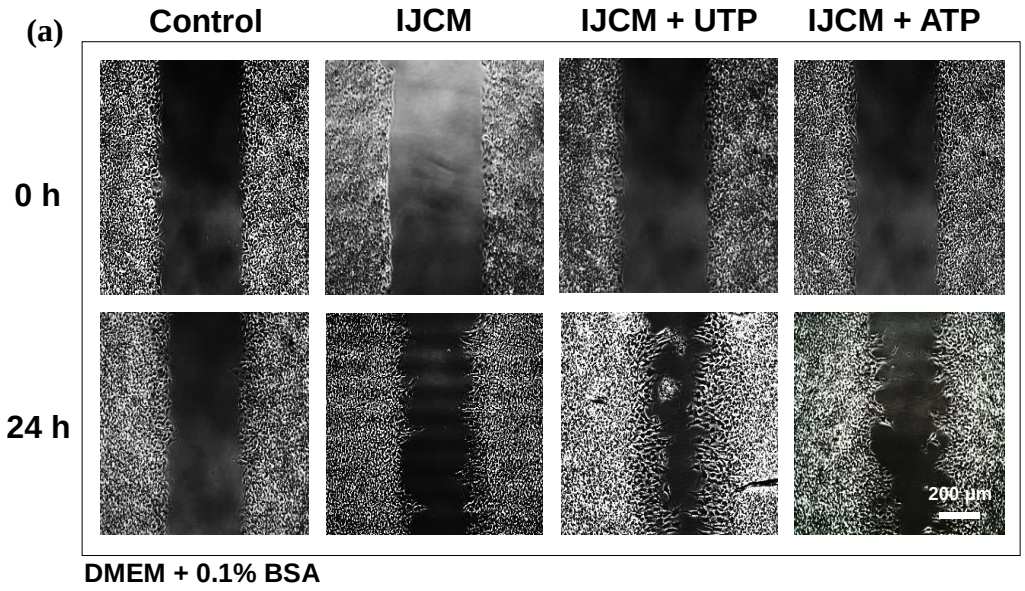
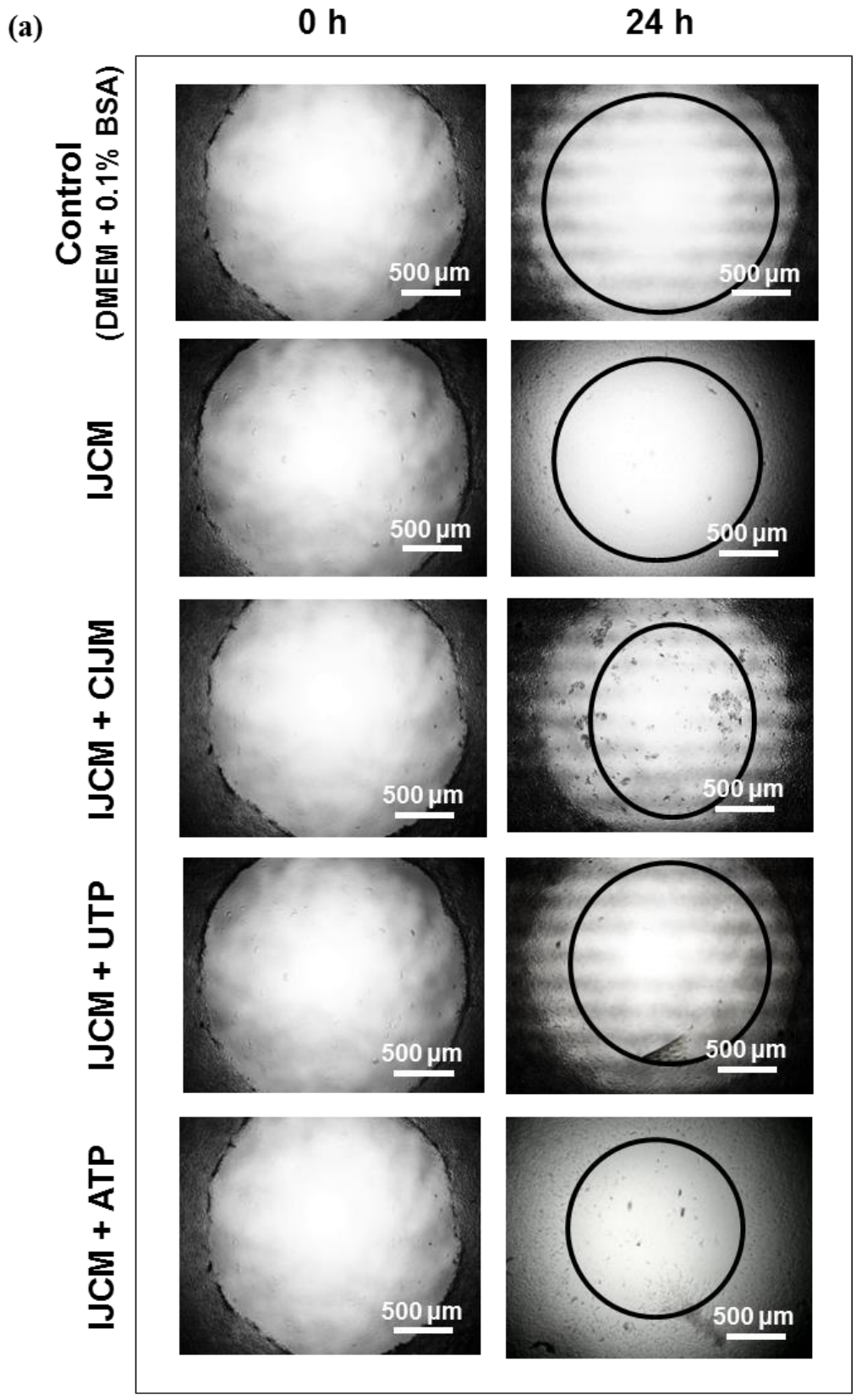


Figure 8.5: (a) Influence of extracellular nucleotide on SF MSC migration evaluated using cell exclusion zone assay. Images were taken at x10 magnification using light microscopy. Addition of both ATP and UTP to IJCM reduced wound width following 24h migration similar to BM MSC CIJM and BAC CIJM. **(b)** Graph showing percentage reduction in wound width (%) ± SEM following 24h migration in response to IJCM, IJCM supplemented with CIJM and IJCM supplemented with extracellular nucleotides. Significant reduction in wound width was observed with both IJCM supplemented with CIJM and IJCM supplemented with ATP and UTP compared to negative control. No significant reduction in wound width was observed with IJCM compared to control. Further, both CIJM and extracellular nucleotide supplementation significantly reduced wound width compared to IJCM. IJCM with BM MSC CIJM and BAC CIJM ($p<0.0001$), IJCM with ATP ($p<0.05$) and IJCM with UTP ($p<0.0001$). Moreover, significant reduction in wound width was observed with both CIJM addition (BM MSC CIJM $p<0.01$; BAC CIJM $p<0.001$) compared to addition of ATP. Similarly, UTP was more potent at inducing MSC migration ($p<0.01$) compared to ATP and no significant reduction in wound width was observed between UTP and CIJM. * $p<0.01$, **** $p<0.0001$ compared to control.


(a)
0 h
24 h
Control
(DMEM + 0.1% BSA)
IJCM
IJCM + CIJM
IJCM + UTP
IJCM + ATP
500 µm

(b)

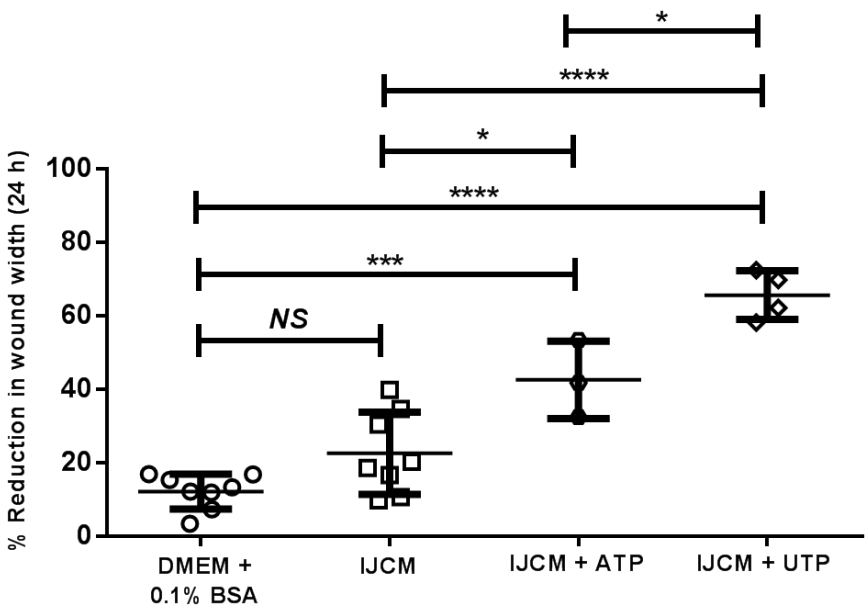


Figure 8.6: Influence of extracellular nucleotide on SF MSC migration evaluated using cell exclusion zone assay. Images were taken at x4 magnification using light microscopy. Oris-FLEX inserts bought from Platypus Technology Inc. was used. Addition of both CIJM (BAC CIJM) and extracellular nucleotides enhanced MSC migration into the exclusion zone and higher reduction in wound area was observed. (b) Graph showing percentage reduction in wound area (%) ± SEM following 24h migration in response to IJCM, IJCM supplemented with BAC CIJM and IJCM supplemented with extracellular nucleotides. Wound area was calculated by measuring diameter of the cell exclusion zone void of cells. Significant reduction in wound area was observed with both IJCM supplemented with CIJM and IJCM supplemented with ATP and UTP compared to negative control. No significant reduction in wound area was observed with IJCM compared to control. Further, both CIJM and extracellular nucleotide supplementation significantly reduced wound area compared to IJCM. IJCM with BAC CIJM ($p<0.01$), IJCM with ATP ($p<0.01$) and IJCM with UTP ($p<0.0001$). Moreover, no significant difference in reduction of wound area was observed between addition of either CIJM or extracellular nucleotides or between addition of ATP and UTP. * $p<0.01$, **** $p<0.0001$ and *NS* represents non-significant.

8.3.4 Effect of UTPγS and ATPγS on IJCM mediated migration of SF MSC

Since, ATP and UTP are rapidly degraded by extracellular endonucleases, stable nucleotide analogues were used. ATPγS and UTPγS (250 μM) (stable analogue of ATP and UTP) were added to IJCM and cell exclusion zone assay of SF MSC was performed. There were significantly enhanced wound closures for both ATPγS and UTPγS when added with IJCM (Figure 8.7). With ATPγS, the wound closure of SF MSC was 83% ± (4%) and with UTPγS it was 84% ± (3%) in 48 h assays compared to 21% ± (3%) with IJCM (Figure 8.8).

In order to characterise the nucleotide receptor identity, time dependent cell exclusion zone assay was carried out using IJCM supplemented with ATPγS and UTPγS. Higher wound closure with UTPγS suggested that the receptor responsible for MSC priming was P2Y rather than P2X which predominantly binds ATP (Figure 8.9).

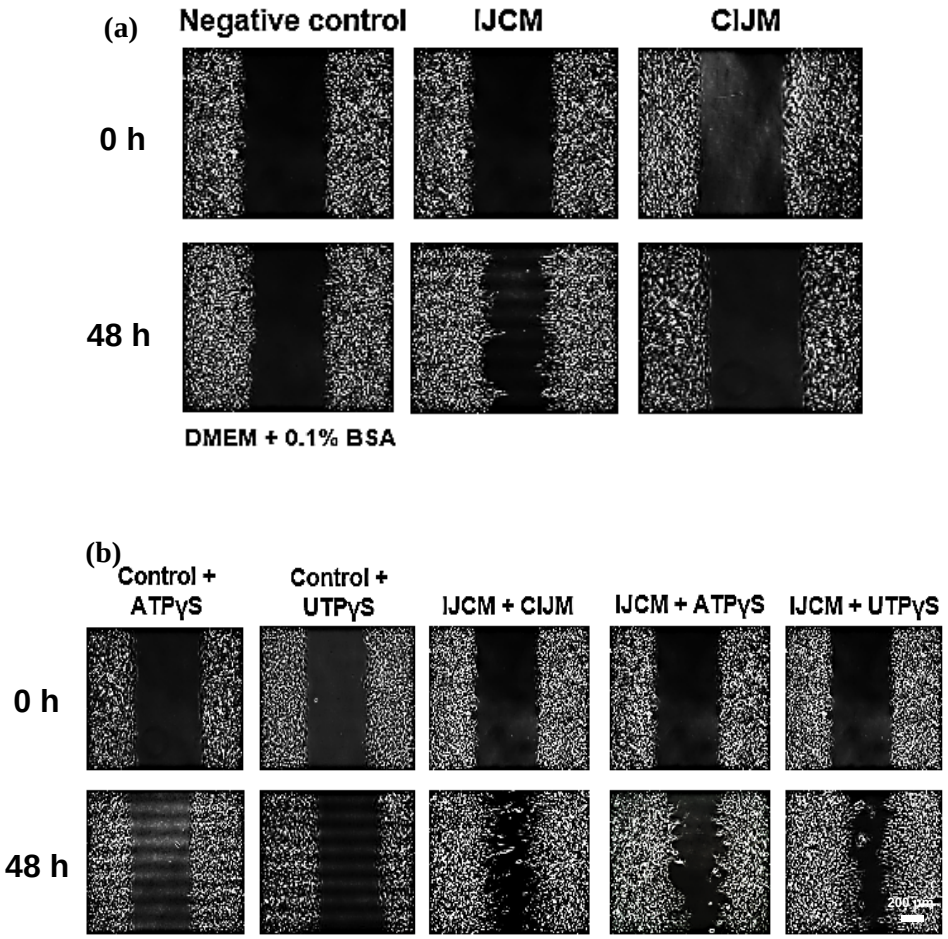


Figure 8.7: Influence of stable analogues of extracellular nucleotides on SF MSC migration evualuated using cell exclusion zone assay. Wound area was created using Ibidi inserts. Images were taken at x10 magnification. (a) IJCM and CIJM alone did not enhance MSC migration to a greater extent compared to control. (b) Similarly, the extracellular nucleotides (ATPγS and UTPγS) alone did not enhance MSC migration to a greater extent compared to control. However, addition of CIJM or extracellular nucleotides to IJCM led to enhanced MSC migration (higher reduction in wound width) compared to control.

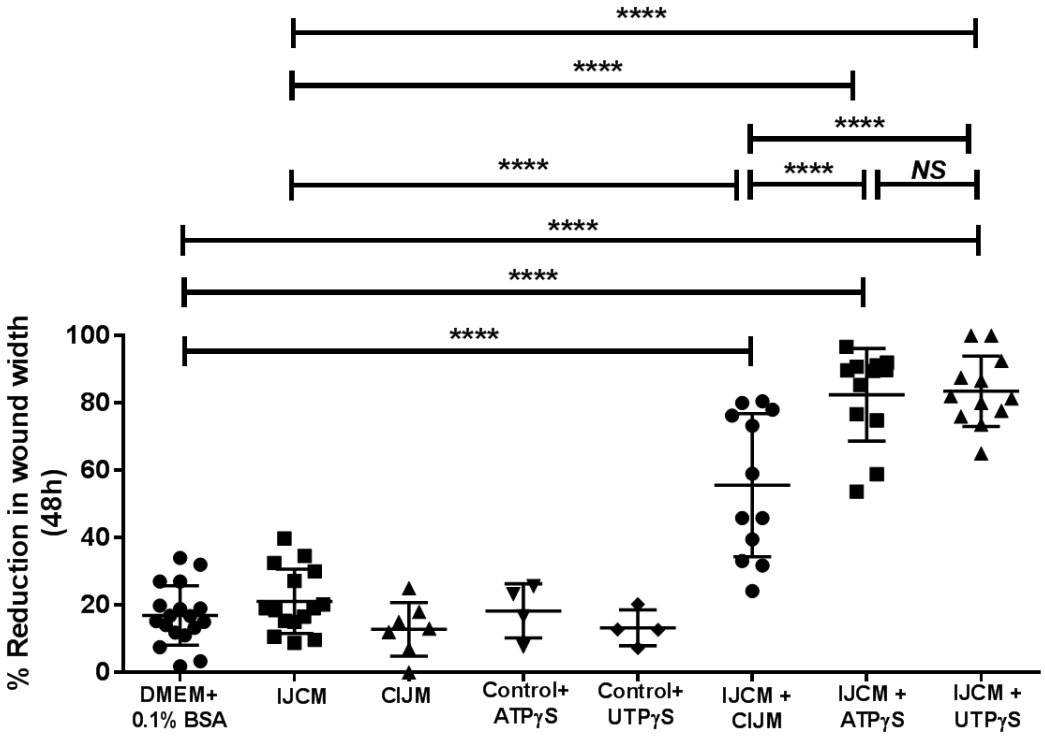


Figure 8.8: Influence of stable analogues of extracellular nucleotides on SF MSC migration evualuated using cell exclusion zone assay. Wound was created using Ibidi inserts. Graph showing percentage reduction in wound width (%) ± SEM following 48h migration in response to IJCM, IJCM supplemented with CIJM (BAC CIJM) and IJCM supplemented with stable analogues of extracellular nucleotides. No significant reduction in wound width was observed with IJCM or CIJM alone compared to control. Similarly, the extracellular nucleotides alone did not significantly reduce wound width compared to control. Addition of CIJM to IJCM significantly reduced wound width compared to both control ($p<0.0001$) and IJCM alone ($p<0.0001$). Similarly, addition of both ATPγS and UTPγS also significantly reduced wound width compared to control (ATPγS: $p<0.0001$); UTPγS: $p<0.0001$) and IJCM (ATPγS: $p<0.0001$); UTPγS: $p<0.0001$). Further, both (ATPγS and UTPγS significantly reduced wound width compared to IJCM with CIJM ($p<0.0001$). And no significant difference in wound width was observed between ATPγS and UTPγS. ****, $p<0.0001$ and *NS* represents non-significant. .

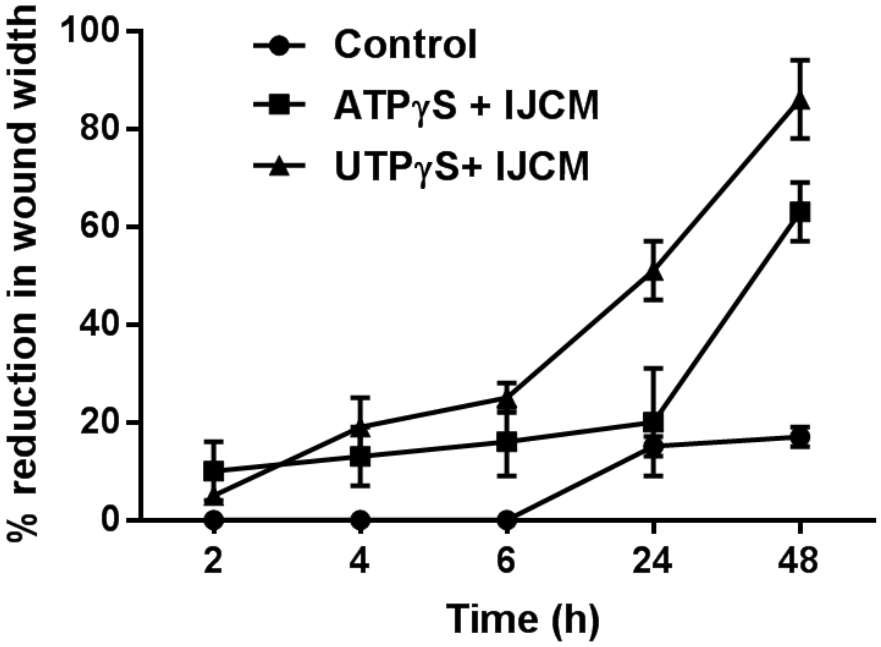


Figure 8.9: Influence of stable analogues of extracellular nucleotides on SF MSC migration evualuated using cell exclusion zone assay at various time intervals. Graph showing percentage reduction in wound width (%) ± SEM following 48h migration in response to IJCM and IJCM supplemented with stable analogues of extracellular nucleotides. Cell injury releases ATP which interacts with a purine receptor which is required to prime the MSCs for migration. Enhanced response to UTP is suggestive that the purine receptor is of P2Y classification.

8.3.5 Influence of purinergic receptor antagonists (P2R antagonists: suramin) on SF MSC migration evaluated using scratch assays.

To further test the involvement of P2R in MSC migration, MSCs were first incubated for 30 mins with a non-selective P2 purinergic antagonist (Suramin hexasodium salt) prior to determining the migratory capacity using the scratch assays. Pre-treatment with P2R antagonists significantly reduced SF MSC migration in response to IJCM by 60%. Hence, when 91% ± (3%) wound closure was observed with IJCM in untreated MSCs, only 31% ± (2%) wound closure was observed with IJCM in MSCs pre-treated with P2R antagonists. Moreover, there was no significant difference in wound closure between serum free negative control in untreated MSCs (46% (±6%)) and IJCM in pre-treated MSCs (Figure 8.10).

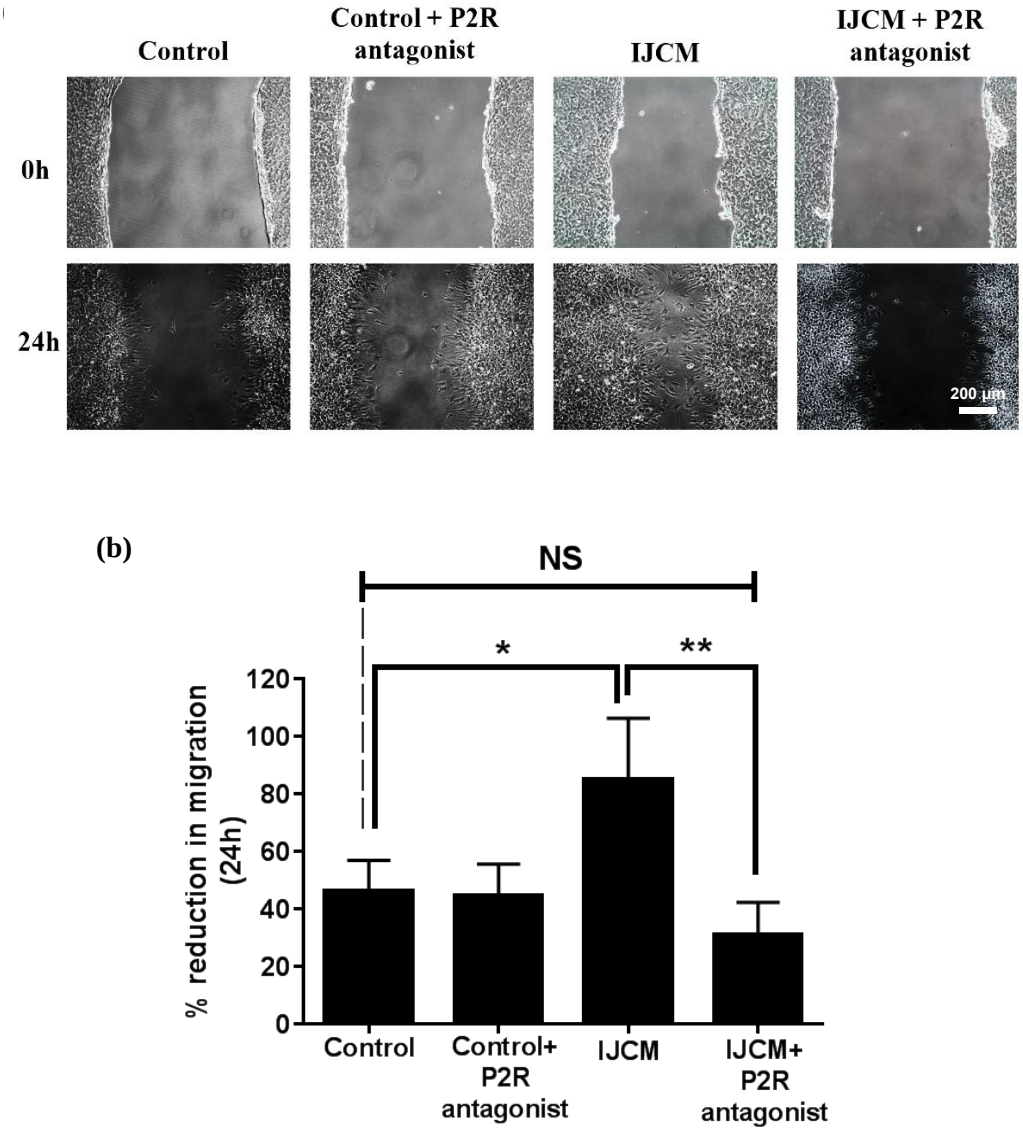


Figure 8.10: Influence of purinergic receptor antagonists (P2R antagonists: suramin) on SF MSC migration evualuated using scratch assays. Mnolayer cultures of SF MSCs were pre-treated with P2R antagonists for 1h before addition of IJCM. (a) Images were taken at x10 magnification. IJCM ehnanced SF MSC migration compared to control ($p<0.01$). This effect of IJCM on SF MSC migration was blocked using P2R antagonists. (b) Graph showing percentage reduction in wound width (%) ± SEM over 24h. IJCM significantly reduced wound width compared to control ($p<0.01$). Pre-treatment of cells with P2R antagonists prior to addition of IJCM signficantly reduced this response of IJCM ($p<0.001$) and no significant difference in migration was observed between control and IJCM in pre-treated assays. * $p<0.01$ and *NS* represents non-sgnificant. .

8.3.6 Measurement of ATP content in IJCM and CIJM

An ATP bioluminescence assay was used to validate the presence of ATP in CIJM. Both IJCM and CIJM were collected at 30 min, 1 h, 2 h and 4 h. In addition the ATP content of IJCM collected after 24 h was also determined. The results indicated the presence of ATP in the CIJM and also suggested a time-dependent depletion of ATP content in CIJM. For example, a significant depletion in ATP of CIJM was observed from 30 mins to 2 h (189.3 μM ATP to 60.8 μM; $p<0.0001$) which further depleted at 4 h (189.3 μM to 24.1 μM; $p<0.0001$). In contrast, no significant difference in ATP content of IJCM was observed from 30min (41.1 μM) to 24 h (46.8 μM) (Figure 8.11).

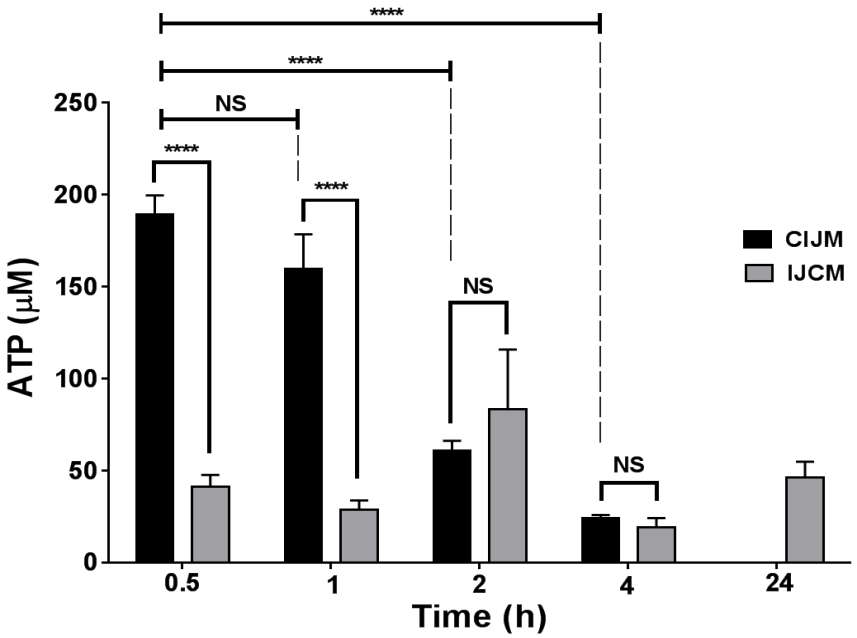


Figure 8.11: Measurement of ATP concentration (µM) in cell injury conditioned media (CIJM) and IJCM. Confluent chondrocyte monolayers were scratched extensively using a 200 µL pipette tip in the presence of serum free media and incubated for various time (h). Following incubation, media was collected as CIJM. Chondral flakes were finely cut into very small pieces using scalpel blade and incubated in serum free media for various time (h). Following incubation media was collected as IJCM. 50 µL from each sample was added into a 96-well black opaque-walled plate in triplicates and mixed with 50 µL luciferase reagent per well. The actual ATP content was calculated from the trend line equation generated from the standard curve using GraphPad Prism.

8.3.7 Potential of IJCM to stimulate MSC chondrogenesis

One question we need to answer is whether the injury factors also stimulate MSCs to mature/differentiate into cartilage tissue-forming cells (i.e. chondrocytes) which are required for the homeostatic cartilage repair process following MSC migration to the site of injury. Therefore, micromass cultures of SF and BM MSCs were stimulated with IJCM for 10 days. GAG deposition was detected using alcian blue staining. IJCM stimulated chondrogenic differentiation of both SF and BM MSCs.

8.3.8 Effect of purinergic stimulation on IJCM mediated chondrogenic differentiation of MSCs

In order to determine the effects of the extracellular nucleotide on IJCM stimulated MSC chondrogenic differentiation, micromass cultures of MSCs were treated with IJCM, ATP (250 μM) and IJCM containing ATP (250 μM). Treatment with ATP alone did not induce MSC differentiation whereas presence of ATP in the IJCM consistently produced stronger alcian blue staining compared to IJCM alone (Figure 8.12). After 10 days of culture, IJCM stimulated cultures showed focal areas of proteoglycan deposition in all micromasses. Staining was asymmetrical such that some micromasses showed stronger matrix deposition at the edges of the micromasses whereas in some micromasses stronger deposition was in the middle of the micromass. In contrast, IJCM with ATP showed more symmetrical matrix deposition and strong staining was detected throughout the micromass suggesting ATP stimulation may have enhanced IJCM stimulated differentiation. Similar alcian blue staining was observed between standard chondroinductive media and SF MSC micromass cultures maintained in IJCM supplemented with ATP whereas BM MSC micromass cultures appeared more compact under alcian blue staining.

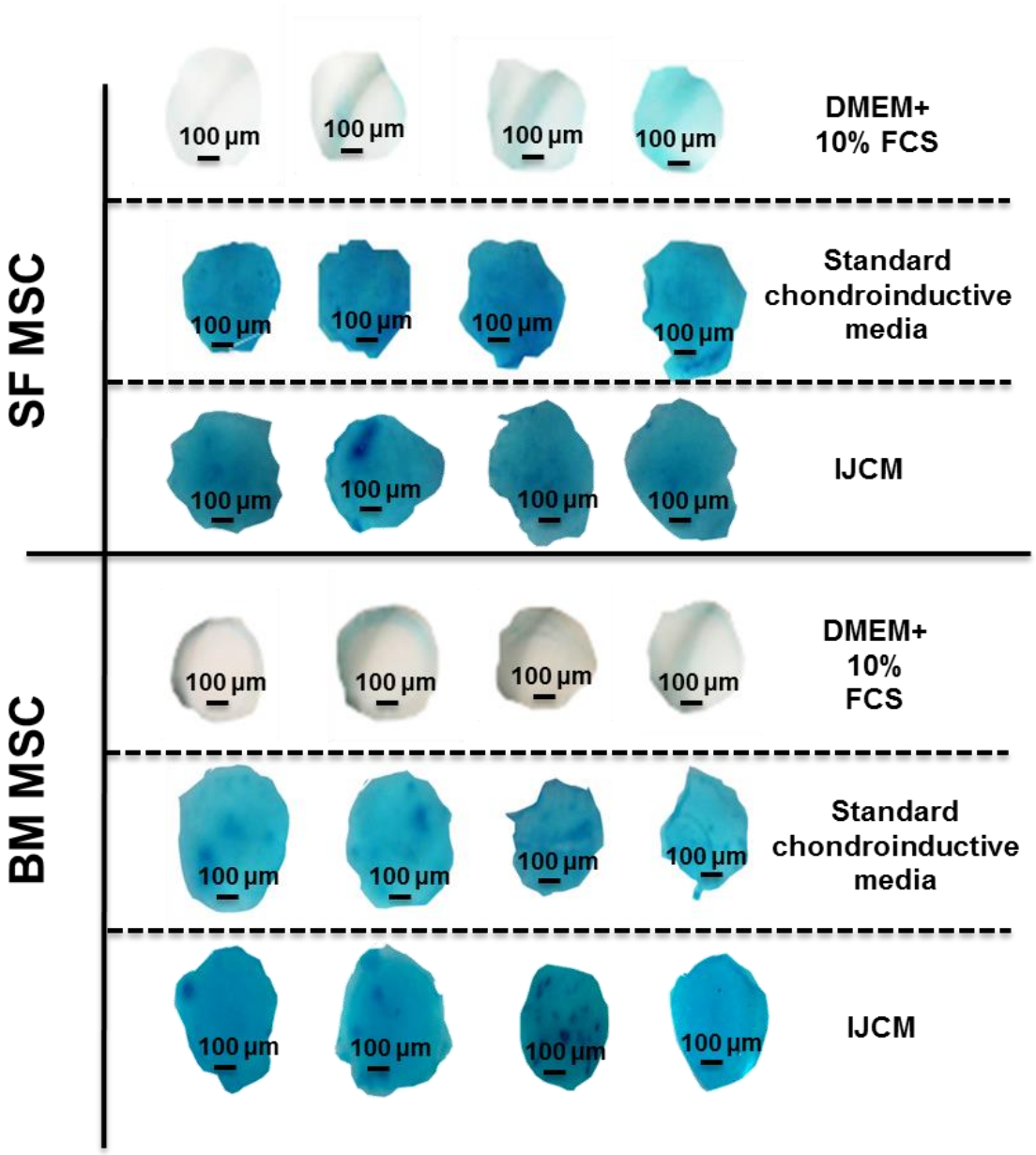


Figure 8.12: IJCM promotes MSC survival and chondrogenesis. Micromass cultures of MSCs were stimulated with IJCM for 10 days. MSC media (DMEM containing 10% serum) was used as negative control and standard chondroinductive media was used as positive control. Metachromatic staining with alcian blue, indicative of cartilage matrix was used to detect chondrogenesis. Serum containing media did not induce chondrogenesis following 10 days. Similar colour intensity suggests IJCM induced similar level of chondrogenesis as standard chondroinductive media.

We also investigated the effect of stable nucleotide analogues ATPγS and UTPγS on SF MSC chondrogenic differentiation. Micromass cultures were treated with IJCM supplemented with either ATPγS (250 μM) and UTPγS (250 μM) and presence of extracellular matrix deposition was visualised by alcian blue staining. After 10 days, both ATPγS and UTPγS stimulated chondrogenesis in all the micromasses and symmetrical matrix deposition throughout the micromass was detected (Figure 8.13). ATPγS stimulated micromasses had similar coloration as standard chondroinductive media and IJCM with ATP however, the micromasses appeared bigger compared to them. Similarly, UTPγS stimulated micromasses appeared more compact and had stronger coloration.

To test whether TGFβ3 or CXCL12 accounted for all or part of the chondrogenic ability of the IJCM we used neutralising antibody against TGFβ3 and the CXCR4 receptor blocker (AMD3100). Neither the TGFβ3 neutralising antibody nor pre-treatment with CXCR4 antagonist affected the ability of IJCM to stimulate chondrogenesis. However, less intense coloration was observed in the presence of TGβ3 antibody compared to IJCM and pre-treatment with CXCR4 antagonist (Figure 8.13).

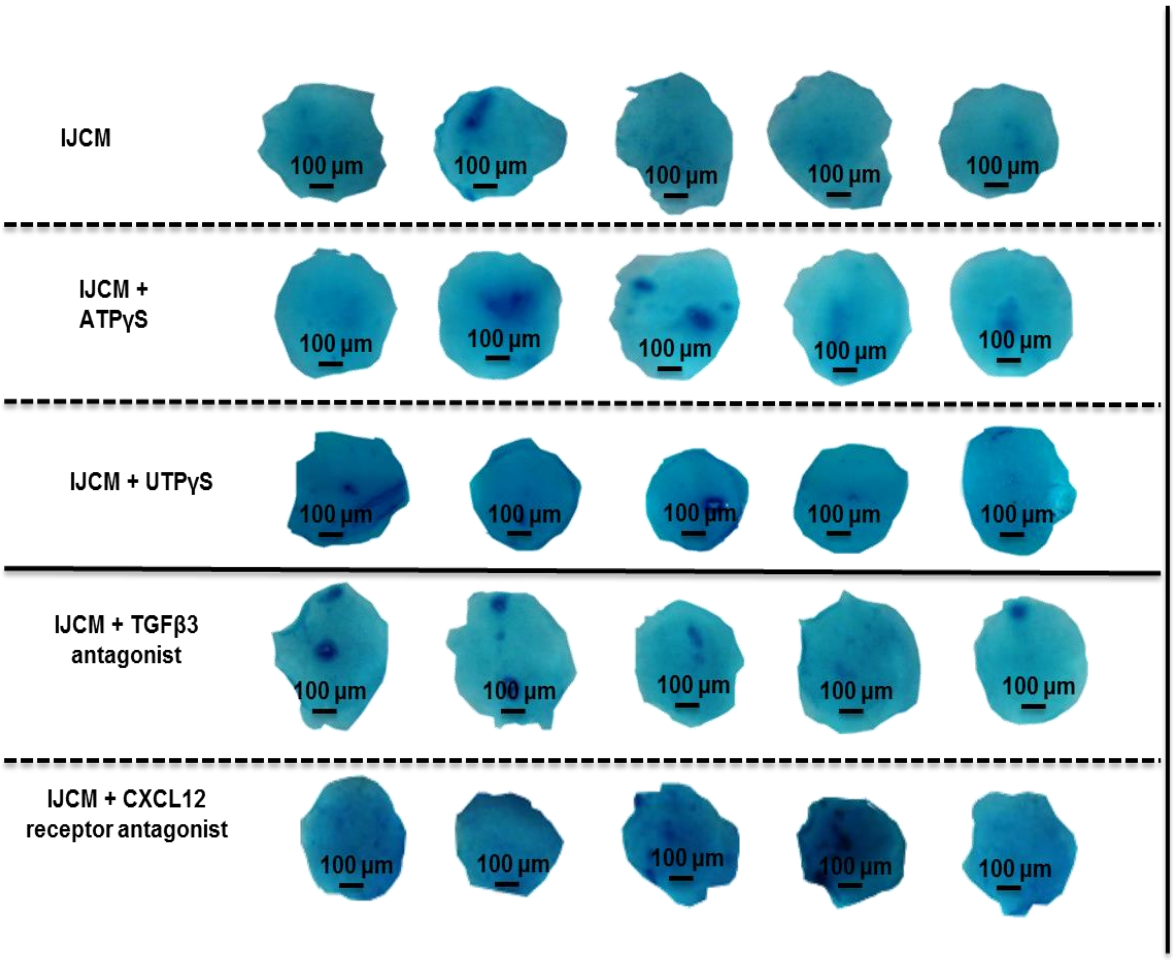


Figure 8.13: Influence of stable analogues of extracellular nucleotides, TGFβ3 antagonists and CXCL12 receptor antagonist (AMD3100) on SF MSC chondrogenesis assessed using micromass cultures. Differentiation was detected using alcian blue following 7 days stimulation. IJCM stimulated MSC differentiation. Both ATPγS and UTPγS stimulated chondrogensis in all the micromasses and symmetrical matrix deposition throughout the micromass was detected with intense coloration detected in some micromasses but not in all cultures. ATPγS stimulated micromasses had similar coloration as standard chondroinductive media and IJCM with ATP however, the micromasses appeared bigger compared to them. Similarly, UTPγS stimulated micromasses appeared more compact and had stronger coloration. Micromasses were pre-treated with AMD3100 before addition of IJCM. Even though blocking TGFβ3 did not completely block differentiation, reduced coloration was observed compared to IJCM. In contrast, blocking CXCL12 receptor did not affect chondrodrogenesis.
Note: The IJCM images are same as those shown above in figure 8.12.

8.4 Chapter Discussion

SF MSCs need to be primed by extracellular UTP and to a lesser extent ATP released through cell injury before migration along the CXCL12 chemotactic gradient. In the previous chapter we have shown that following injury cartilage releases factors which are stable for 24 h and these factors can stimulate MSC migration to the site of the injury. In this chapter exclusion zone assays were used for studying MSC migration in the absence of cell injury. Using this assay system it was demonstrated that in the absence of cell injury, SF MSCs do not respond to the chemotactic gradient of the IJCM. Furthermore, it was demonstrated that ATP and UTP enhanced MSC migration in response to the IJCM.

To separate the effect of cell injury from the IJCM, chondrocyte monolayers were injured using a 200 µl pipette tip in the presence of serum-free media and the injured cells cultured for 30 mins. It was hypothesised that factors released through cell injury would be released in this media and therefore the injury conditioned media (CIJM) was collected 30 mins after the chondrocyte monolayers had been injured. Cell exclusion zone assays of SF MSCs in the presence of IJCM and CIJM together showed rapid wound closure suggesting that CIJM contained factors which is required in conjunction with IJCM to trigger significant MSC migration. This also supports our previous results where no CIJM was required in 'classical wound healing' assays where the wound area was created by a manual scratch. It is therefore conceivable that the scrapping of the cell monolayer created cell injury, releasing the required factors for MSC stimulation.

The observation that IJCM alone cannot stimulate MSC migration in the absence of injury suggests that the factors released through cell injury are not present in the IJCM and are therefore not stable for 24 h making short-acting factors the most probable targets.

There are few studies showing the effect of purine nucleotides on MSC migration or migration. Previous studies have reported ATP to potentiate haemopoietic stem cell chemotactic response to CXCL12 and increase spontaneous migration [75]. Other studies have reported the presence of P2R subtypes such as P2X6, P2Y4 and P2Y14 playing a major role in adipose tissue-derived MSC commitment to adipogenic and osteogenic differentiation [110]. The effect of adding ATP and

UTP to IJCM in the cell exclusion zone assay was investigated and it was demonstrated that the presence of these extracellular nucleotides triggered rapid wound closure compared to IJCM alone. Although, the purinoreceptor expression, of SF MSCs was not analysed, the increased chemotactic response of SF MSCs is in line with signalling pathways initiated by P2 receptors.

Recent studies have suggested that UTP and ATP may play a role in hematopoietic stem cell motility and migration to bone marrow niches in synergy with CXCL12 via activation of Rho GTPase transduction pathway [75]. ATP and UTP functions are mediated by P2 nucleotide receptors P2Rs. P2Rs are further divided into P2X and P2Y. P2Y receptors (P2YRs) are also 7 transmembrane spanning receptors coupled to G-proteins. Signal transduction of UTP binding to P2YR is mediated by either activation of phospholipase C or activation/inhibition of adenylate cyclase. Our results of increased wound closure with UTPγS compared to ATPγS suggested that P2Y receptors (characterised by a greater binding affinity and biological activity for UTP over ATP) rather than P2X receptors (which has a greater binding affinity and biological activity for ATP over UTP) was responsible for the SF-MSC priming.

Additionally, BM MSCs did not require this priming system to migrate in response to the IJCM chemotactic gradient. This suggested that priming maybe a specific requirement for SF MSCs. Moreover, since, these extracellular nucleotides are released through cell injury; SF MSCs could be the first cell type to detect the release of the nucleotides whereas BM MSCs do not have access to the nucleotides unless the injury extends to the subchondral bone. As a result, BM MSCs have a different method of migration involving other key chemokines such as TGFβ3 and bFGF2, one of which is not required by SF MSCs for migration. This result would explain the fact that other major chemokines such as TGFβ3 (22.3%) and bFGF2 (33.6%) were also able to partiality block the activity of the IJCM in BM MSC migration assays.

Chondrocytes when either damaged or have an altered metabolic activity such as in osteoarthritis (OA) and rheumatoid arthritis (RA) may release ATP into the synovial fluid [109]. Studies analysing the MSC gene expression profile showed that in ATP-stimulated human bone marrow derived MSCs, genes involved in cell migration were strongly upregulated [75]. Therefore, it is possible that ATPs

released from the damaged chondrocytes through cartilage injury into the synovial fluid may stimulate the synovial derived MSCs and prime them through upregulation of migratory genes. This priming could then enhance MSC migration along the chemotactic gradient of CXCL12 leading them to the site of the injury. However, it is known that ATP is rapidly hydrolysed by synovial fluid from both normal and osteoarthritic joints [109] and by ectonucleotidases on the plasma membrane of chondrocytes [111]. This could prevent priming of sufficient number of MSCs and could be one of the reasons why sufficient number of MSCs does not migrate to the injury site. Therefore, inhibition of the action of ectonucleotidases in the joint may be one potential mechanism to prime sufficient MSCs for migration.

8.5 Chapter summary

The aim of this study was to investigate if cell injury released factors which were required for MSC migration to the site of the cartilage injury. We found that SF MSCs but not BM MSCs need to be primed by extracellular UTP and to a lesser extent ATP released through cell injury before migration along the CXCL12 chemotactic gradient.

Chapter 9

General Discussion

Current surgical interventions in place to treat cartilage injury such as ACI and MACI require two surgical procedures. First a biopsy of donor articular cartilage tissue is required from which the chondrocytes are isolated. After a period of *in vitro* culture to expand the numbers of isolated cells a second operative procedure is required for implantation of the cultured cells into the cartilage defect; either directly (ACI) or through implantation of a biomaterial membrane onto which the cells have been seeded (MACI). However, there are several potential concerns which can arise with these procedures such as the need for extensive cell expansion in culture, donor site morbidity, limited integration between host and repair tissue and repair through formation of fibrocartilage instead of the original hyaline cartilage [27, 49, 50, 112].. Therefore, it is important to develop new therapeutic strategies which eliminate the need for multiple surgical procedures and *ex vivo* cell manipulation prior to implantation as these strategies would minimise the, the amount of clinical time and resources required to surgically treat the damaged cartilage. One potential way to bypass the issues associated with cell-based therapies is to use the body's own resources and repairing capacities by targeting host 'repair cells' i.e. mesenchymal stem cells (MSCs) and recruiting them to the site of injury. Therefore, a model of articular cartilage damage was established in which the potential responses of bone marrow-derived (BM) and synovial fluid-derived (SF) MSCs to the injured cartilage could be investigated.

9.1 Characterisation of the optimal culture conditions for isolation and expansion of bovine BM and SF MSCs

9.1.1 Effect of serum

The data shown in this thesis indicated that culture parameters such as serum, glucose concentration, quality of the plastic surface of the culture flasks and stable glutamine greatly affected the survival and proliferation of MSCs in culture. In early preliminary experiments, MSCs were cultured using low glucose DMEM containing 10% FCS and 5 ng/ml of FGF2. However, both SF and BM MSCs failed to proliferate and appeared to senesce within 4-5 days after plating in culture flasks. The serum used for the preliminary experiments was likely to be the cause of the failure of these cell cultures The serum used is a critical step in the establishment and optimisation of MSC cultures as it contains important biological factors required for cell proliferation, binding proteins for the transport

and utilisation of other essential molecules, hormones, substrates for attachment molecules and nutrient molecules for cell growth [113]. For example, it has been suggested that replicative senescence of MSC (aging with passage) can be influenced by serum supplements and platelet lysate was found to be more potent at inducing MSC proliferation before the cells reached senescence compared to the commonly used FCS [114]. Furthermore, appropriate selection of serum has also been reported to influence MSC differentiation such that certain serums may contain inhibitors of cell differentiation [115]. Variations in the levels of biological factors among different sera means that different serum batches have varying capacities to maintain MSC characteristics *in vitro*. Hence, a commercial MSC qualified serum was tested and used throughout the project as it was quality assured by the manufacturer as supporting MSC proliferation, viability and multipotency in culture.

9.1.2 Effect of plastic surface

Quality of the culture surface is also known to affect MSC cultures [115]. For example, one study investigated the impact of plastic adherence using culture flasks from four different companies and found that compared to Greiner, Nunc and Costar, Falcon flasks allowed significantly higher MSC proliferation over 4-week culture. All four flasks tested were made from polystyrene where the culture surface was made permanently hydrophilic using corona discharge under high voltage. The usual laboratory Corning culture flasks were found to be suboptimal for SF- and BM-MSC culture in this study and changing to a class of Sarstedt flasks considerably improved MSC growth and proliferation.

9.1.3 Effect of L-glutamine

L-glutamine is a necessary component for protein and nucleic acid synthesis as well as for energy production by cells during *in vitro* culture. Hence, it is commonly supplemented into the culture media [116]. However, L-glutamine is known to be unstable in culture media and its degradation can lead to ammonia formation which can inhibit cell growth. To avoid this, alternative and stable substitutes of L-glutamine such as GlutaMax (L-alanyl-L-glutamine dipeptide) have been developed. One previous study comparing expansion efficacy of human

BM MSCs among 8 different basal media and glutamine supplementation found αMEM supplemented with GlutaMax provided the most optimal conditions for MSC expansion [117]. However, in the study reported in this thesis it was observed that L-glumatine instead of GlutaMax was more consistent in supporting bovine BM and SF MSC growth. This was probably due to a greater ease of the MSCs to utilise the glutamine over GlutaMax which requires the release of aminopeptidases by the MSCs to cleave the L-alanyl-L-glutamine dipeptide to release the L-glutamine (and L-alanine) which can then be taken up by the cells. .

Following, the optimisation of serum, culture flasks and glutamine supplementation, consistent culture of both SF and BM MSCs was achievable.

9.1.4 Passage number

During this study an apparent constant growth rate was observed in SF MSC cultures for 7 consecutive passages when maintained at subconfluency; whereas BM MSCs had a progressive reduction in the apparent rate of proliferation and appeared to enter into senescence in culture following 5 consecutive passages.

At this passage the BM MSCs started to become enlarged, appeared to have a morphology also known as ‘fried egg morphology’ and the cells appeared to have ultimately underwent growth arrest which is characteristic of senescence [118]. MSCs like any other stromal cells have limited lifespan *in vitro* and hence can only be expanded for a restricted number of times before cells start to become senescent. Moreover, many have previously reported that long-term *in vitro* expansion impairs MSC differentiation potential [119-122]. Previous studies have demonstrated that such replicative senesce was associated with gene expression alterations in MSCs similar to those observed in age-related changes between young and old donor [122, 123]. Therefore, there is a suggestion that changes observed during long-term cultures might be regulated by the same mechanism which regulates age related changes.
Hence, in this study MSCs (both SF and BM MSCs) were only expanded until passage 3when they were used for experiments.

In this study it was also observed that maintenance in culture was dependent on how long the cells were exposed to trypsin/EDTA during passaging. Hence, along

with reducing the time of the trypsinisation, the trypsin/EDTA was diluted at a 75:25 ratio with phosphate saline buffer (PBS) in order to obtain the highest number of healthy MSCs.

9.2 Difference in morphology and differentiation potential between BM and SF MSCs

9.2.1 Morphology

SF and BM aspirates were obtained from the bovine metacarpophalangeal joints. No obvious correlation between the yield of cells and the volume of SF and BM obtained with an aspirate was observed. Furthermore, visual microscopic evaluation of the cultured cells also suggested that there was no apparent correlation between the rate at which the cells proliferated and the initial seeding density of the cells. Studies investigating the optimal conditions for culturing human BM MSCs suggested the presence of three morphologically distinct cell types [124, 125]. Similarly, three morphologically distinct types of cells was also observed in our bovine BM MSC cultures: 1). small and rounded spindle shaped, 2). thin spindle shaped and 3) thin and elongated spindle shaped (as shown in Figure 4.1). However, no differences in cell morphology were observed following day 8 when the cultures reached approximately 60-70% confluency. In contrast, no heterogeneity in SF MSC morphologies was observed throughout the culture period from initial plating to 100% confluency which is in agreement with previous reports [126].

Morphologically, BM MSCs appeared to be different from the SF MSCs. Following plating into tissue culture flasks, BM MSCs appeared to be spindle-shaped whereas, SF MSCs appeared to be stellate or squat shaped.

9.2.2 Differentiation potential

Differentiation assay to validate MSC tri-potentiality suggested that BM and SF MSCs were also different in their biology. For example, BM MSCs had higher adipogenic capacity as seen from higher number of fat-laden cells following stimulation with standard adipoinductive media compared to SF MSCs (Figure 4.3). This is consistent with previous studies where SF MSCs were found to have consistently low adipogenic capacity compared to their BM counterparts [29]. In

the present study the chondrogenic differentiation capacity between BM and SF MSCs was not quantified. Both alcian blue staining, indicative of GAG deposition and immunohistochemical staining for collagen II deposition suggested that both BM and SF MSCs had strong chondrogenic capacity. However, the differentiation capacity among different individual BM and SF MSC colonies was not analysed. SF MSC clones have previously been reported to be very homogenous in their mesenchymal lineage differentiation capacity [29] compared to BM MSC clones which have been shown to be highly variable in their differentiation capacity. It is suggested that the good chondrogenic capacity of SF MSCs could be due to the expression of hyaluronan receptor CD44 which was expressed at a higher level and more consistently on the surface of SF MSCs compared to BM MSCs [126].

9.3 Models of cartilage injury

In order to study the role of the MSCs in the healing of articular cartilage defects it was first important to define the model of cartilage injury. Potential models of articular cartilage injury were investigated using standard surgical tools such as biopsy punch, post-mortem blades, no.11 scalpel blade, no. 22 scalpel blade and hammer. The range of instruments enabled study of both blunt and sharp injury on the cartilage. Damage to the cartilage was assessed using live/dead staining of chondrocyte viability following induction of injury. Injuring cartilage with a sharp cutting edge gave a more reproducible injury with less extensive chondrocyte death compared to blunt injuries (Section 6.3.2). Therefore, a sharp injury was chosen as the model of cartilage injury to investigate the MSC migration in response to the cartilage injury.

9.4 Migration assay

In this study three different types of migration assays were used to assess MSC migration in response to cartilage injury. A commercial Transwell assay system was used to study MSC migration in response to injured cartilage discs. The scratch assay system was used as a high-throughput method to screen the injury conditioned media (IJCM) produced by incubating injured cartilage with serum-free medium (section ????) and experiments characterising potential biological agents responsible for stimulating MSC migration in response to cartilage injury (Sections ???). Cell exclusion zone assays were used to assess MSC migration in response to IJCM in the absence of cell injury (Sections ???).

9.5 Migration capacity of SF- and BM MSC to cartilage injury

In the present study we investigated the response of two types of MSCs; SF-MSCs and BM-MSCs to articular cartilage injured by cutting with a scalpel. PKH67-labelled SF-MSCs specifically adhered to the freshly cut edges of cartilage but not to edges cut previously (i.e. during tissue isolation) or to the body of the cartilage. These results indicated that MSCs could 'home' to injured articular cartilage and were supported by quantitative experiments showing that both SF- and BM-MSCs significantly migrated to a greater extent to injured cartilage compared to non-injured cartilage.

9.6 Source of biological factors in the injury conditioned media (IJCM)

9.6.1 Released from extracellular matrix

A range of proteins such as proteolytic enzymes, growth factors and chemokines have binding sites for sulphated GAGs (such as heparin) and can be found sequestered in the cartilage extracellular matrix (ECM) by binding to sulphated GAG side chains of proteoglycans in the ECM. There are various sulphated GAGs in the cartilage ECM including heparan sulphate and dermatan sulphate. Heparan sulphate is a GAG that is known to bind chemokine and growth factors *in vivo* and is present in the ECM as extended O-linked chains of proteoglycans such as perlecan, versican and syndecan. The biologically active factors bound to these sulphated GAGs can be mobilised when required or can act as concentration gradients of chemokines for inducing cell migration [127]. The sequestering of biologically active factors by the sulphated GAGs is an important mechanism to prevent these factors from being chemically and proteolytically degraded. Therefore, it was feasible that the migration inducing factors in the IJCM may bind to sulphated GAGs. Hence, IJCM was treated with immobilised heparin (a mimic for heparan sulphate) *in vitro*. The rationale for treating IJCM with immobilised heparin was the following: if the factors were GAG-binding proteins (synthesised by the cells or released from the ECM) they would be removed by heparin treatment, then the treated IJCM will be less potent in inducing MSC migration compared to untreated IJCM. In fact, treatment of IJCM with heparin was found to significantly reduce the migratory capacity of IJCM for both SF and BM MSCs (Figure 7.10).

Heparin-treatment of IJCM also affected adhesion of MSCs to the plastic surface. Interestingly, when BM MSCs were cultured in 24-well plates not coated with fibronectin, some BM MSCs lost plastic adherence when exposed to heparin-treated IJCM but all BM-MSCs appeared to remain attached in the presence of untreated IJCM. All BM MSCs identified to date have been shown to need adhesion to a substrate for their isolation, survival and expansion *in vitro* [128, 129]. These results suggested that the heparin treatment removed one or more heparin-binding factors which were required for cell adhesion. Such examples could be fibronectin which is present in articular cartilage ECM.

However, it was observed that the detached BM MSCs were capable of surviving independently of adhesion as single floating cells (Section 7.3.5). This has been previously shown in one study where adult human BM MSCs were observed to survive in a stirred suspension as individual cells in a cytokine-dependent manner while preserving their capacity to differentiate into osteoblastic cells [130]. Their cytokine cocktail consisted of stem cell factor (SCF), IL-3 and PDGF which stimulated cell expansion in cell suspensions. All of these factors have been implicated as potent mitogen for MSC adherence and survival [131]. IJCM was not analysed in this project for the presence of SCF, IL-3 and PDGF. However, since all three of these factors bind to heparin [132-135], it was possible that they were removed from the IJCM by the heparin treatment and so were not present in the heparin-treated IJCM. It was observed that only 40% of cells in monolayer losing their plastic adherence in response to heparin-treatment also suggests a heterogeneous BM MSC cell population which have been extensively reported in the past [113, 115, 117, 124-126, 136-140]

9.6.2 Released from the cells

Even though treatment with heparin reduced the migration stimulatory capacity of IJCM, for both SF- and BM-MSCs, migration of the cells by heparin-treated IJCM was still significantly greater (SF-MSCS: P=0.002, BM-MSCs: P=0.0254) than that in serum-free control media (Figure 7.10). This suggested that perhaps, not all active components of IJCM were heparin-binding proteins. To assess if injury stimulated

To assess if injury stimulated synthesis of some of the factors prior to release cartilage was injured in the presence of cycloheximide (protein synthesis

inhibitor). Cycloheximide caused significant inhibition in SF-MSC migration capacity of IJCM. Therefore, production of migration factors by the injured cartilage may require *de novo* synthesis or secretion themselves or of one or more components (but not MMPs; Figure 7.21) required to release migration factors from the cartilage.

9.7 Characterisation of IJCM

9.7.1 Identification of migration factors in IJCM using neutralising antibodies and receptor antagonists

Neutralising antibodies and receptor antagonists against common heparin interactome members reported to stimulate chondrogenesis and MSC migration [89] were used to characterise IJCM-migratory factor(s).

Likely heparin-binding candidates were CXCL12 (a well-known MSC migration chemokine), CX3CL1, CCL2, CCL3, CCL5, TGFβ family and FGF family. In addition, other cytokines such as TNFα could be released during the injury process. Initial preliminary scratch migration assays showed that SF MSC migration was significantly stimulated by addition of recombinant CXCL12, CCL2, CCL5, CXCL12, TGFβ3 (Figure 7.14). However, TNFα, CCL3 and FGF2 had no significant effect (Figure 7.14) on SF MSCs although BM MSCs migrated also to FGF2. Therefore, neutralising antibodies to TGFβ3, CXCL12, CCL2, CCL5 and the CXCR4 receptor antagonist AMD 3100 were used to investigate the identity of the heparin-binding moietie(s) which caused the migratory activity of IJCM.

Experiments using the CXCR4 inhibitor, AMD3100, the CXCL12-neutralising antibody and TGFβ3 neutralising antibody strongly suggested that CXCL12 and TGFβ3 is released from the injured cartilage within 24 h of injury. When these antibodies were tested with BM MSCs, similar significant reduction in migration was observed which further validated our findings. However, in contrast to SF MSCs, BM MSC migration was also affected by the presence of FGF2 antibody in the IJCM suggesting that FGF2 was also released from the injured cartilage within 24 h of injury [Figure 6 and 7].

CXCL12 is a member of a large chemokine family and has been shown to play a major role in the migration of BM MSCs. For example, using bioluminescence

imaging Granero-Molto *et al* showed that dynamic migration of BM MSC is exclusively dependent on the CXCR4 receptor [141]. Similarly, recruitment of BM MSCs to sites of bone healing for endochondral bone repair was also shown to be critically dependent on the CXCL12/CXCR4 axis [142]. The importance of CXCL12/CXCR4 axis for MSC migration has also been implicated in non-musculoskeletal tissues. In addition, genetically modified MSCs overexpressing the CXCR4 receptor were found to home towards the coronary infarct in greater numbers as compared to untransduced MSCs [143]. This axis also mediated migration of transplanted BM MSCs to the site of the brain injury and induction of BM MSC mobilisation to the burn wounds [89].

Results presented in this thesis also suggested that CXCL12 was released from the injured cartilage within 24 h of injury. This observation is in common with others showing CXCL12 release during tissue damage. Previous studies have reported the increase in secretion of CXCL12 during tissue damage such as heart infarct [144], liver damage due to toxicity [145], excessive bleeding [144] and tissue damage following chemotherapy [146]. Studies investigating release of CXCL12 following fracture suggested that transcription factor HIF-1α may drive the increased production of CXCL12 in the damaged tissues [96]. This report [49] also suggested that in the absence of injury, PHD2 (prolyhydroxylase domain protein 2) degrades HIF-1α. However, low levels of O_2 at the damaged site reduces PHD2 activity, leading to stabilisation of HIF-1α which then translocates to the nucleus, binds to the promoter region of the CXCL12 gene and thereby increases the production of CXCL12 in the damaged tissues. Cartilage is maintained in a hypoxic environment under normal physiological condition. Therefore, it is possible that release of CXCL12 from the injured cartilage was not through the same mechanism. However, previous studies have reported that HIF-2α and not HIF-1α was critical for the hypoxia induced cartilage matrix synthesis [147]. Therefore, it is also possible that HIF-1α only increases in the cartilage following injury which then stimulates the release of CXCL12. This is also consistent with the findings presented in this thesis where a significant reduction in SF MSC migration was observed when media was conditioned by injuring cartilage in the presence of the protein inhibitor cycloheximide and the injury conditioned media was used to stimulate SF MSC migration in scratch assays

suggesting that injury may stimulate the production of migration stimulatory factors in the cartilage (Figure 7.21).

Another possibility is that CXCL12 was released from an extracellular reservoir following mechanical injury. Extracellularly, they are likely to be sequestered in the ECM bound to heparan sulphate containing proteoglycans. This concept of release of proteins from an extracellular reservoir following mechanical injury of cartilage have already been demonstrated for FGF2 where FGF2 was shown to be released immediately following cutting injury [46]. This is also consistent with our findings where significant reduction in BM MSC migration in response to IJCM was observed when neutralising antibody against FGF2 was added to the IJCM [Fig 7]. This suggested that FGF2 was released from the injured cartilage following cutting injury and was partly responsible for stimulating BM MSC migration in response to IJCM.

Application of FGF2 has been shown to promote cartilage repair in partial thickness defects in both immature [24] and mature rabbits [148]. Further, administration of FGF2 antibody into the full-thickness defects in rabbits was shown to prevent cartilage formation in the defects, even 4 weeks post-defect creation [149]. Whereas, administration of FGF2 into the full thickness defects improved cartilage repair and restored the original bone-articular cartilage junction. Mechanism of cartilage repair by FGF2 is still not clearly known. Marked increases in endogenous FGF2 production in repair tissue have been previously reported. Moreover, FGF2 has been reported to promote rapid proliferation of chondroprogenitor cells while maintaining their stemmness *in vitro* [150]. These suggest that MSCs are the target of FGF2 during the reparative process. In agreement with previous observations, in this present study it was demonstrated that FGF2 promotes cartilage repair by stimulating migration of BM MSCs to the site of the injury where MSCs then take part in the repair of the cartilage defect.

The results presented in this thesis also suggested the release of TGFβ3 from the cartilage following sharp injury. For example, significant reduction in both SF and BM MSC migration was observed in scratch assays in response to IJCM when the IJCM was pre-treated with TGFβ3 antibody. Previous studies have suggested a decrease in TGFβ3 expression in the damaged cartilage [151]. However, in that

study, they used a model of OA in contrast to this study where an immediate response following injury was investigated. Furthermore, they observed a strong increase in TGFβ3 expression in other compartments of the joint such as the synovial tissue which is consistent with TGFβ3 being released into the joint cavity following injury, similar to our results found in this study. TGFβ3 has already been demonstrated to be a potent inducer of chondrogenesis and cartilage matrix synthesis [152] suggesting that TGFβ3 plays an important role in cartilage maintenance. Therefore, our findings that TGFβ3 released from the injured cartilage stimulates both SF and BM MSC migration to the site of injury, is suggestive that TGFβ3 also may play an important role in cartilage repair.

Differences in response to injured cartilage between SF and BM MSC migration was also observed in this study. For example, with SF MSCs a significant reduction in migration in response to heparin-treated IJCM compared to untreated IJCM was observed. In contrast, in the absence of ECM coating in the plates, when BM MSCs were stimulated with heparin-treated IJCM, they lost their plastic adherence to plate and formed cell aggregates whereas cells stimulated with untreated IJCM remained attached to the uncoated plastic. This suggested that heparin-treatment might be removing factors which were required for BM MSC adherence but not for SF MSC adherence and therefore, BM and SF MSCs might use different pathways for cell attachment. Further, analysis of cell migration in scratch assays suggested that while SF MSCs migrated as single cells, BM MSCs migrated as a sheet of cells. Moreover, while BM MSCs migrated significantly more to dead cartilage compared to non-injured cartilage, this difference in migration was not observed with SF MSCs. BM MSCs and SF MSCs also responded to different growth factors in the IJCM. For example, significant reduction in BM MSC migration in response to IJCM was observed when neutralising antibody against FGF2 was added to the IJCM. However, no reduction in migration was observed with SF MSCs in response to FGF2 antibody. Therefore, it is possible that dead cartilage also released FGF2 which then promoted BM MSC migration in response to the dead cartilage.

Cartilage injury usually progress to degeneration of the cartilage since it has limited capacity to repair. Our results suggest that cartilage following sharp injury releases CXCL12, TGFβ3 and FGF2 which may act as signals to attract both SF and BM MSCs to the site of the injury. Further, in partial-thickness defects, where

BM-MSCs do not have a direct access to the injury site, SF-MSCs could be involved in the regeneration of the injured cartilage.

9.7.2 Differential response between SF – and BM MSCs in response to IJCM

Differences in response to injured cartilage between SF and BM MSC migration was also observed in this study. For example, with SF MSCs a significant reduction in migration in response to heparin-treated IJCM compared to untreated IJCM was observed. In contrast, in the absence of ECM coating in the plates, when BM MSCs were stimulated with heparin-treated IJCM, they lost their plastic adherence to plate and formed cell aggregates whereas cells stimulated with untreated IJCM remained attached to the uncoated plastic. This suggested that heparin-treatment might be removing factors which were required for BM MSC adherence but not for SF MSC adherence and therefore, BM and SF MSCs might use different pathways for cell attachment. Further, analysis of cell migration in scratch assays suggested that while SF MSCs migrated as single cells, BM MSCs migrated as a sheet of cells. Moreover, while BM MSCs migrated significantly more to dead cartilage compared to non-injured cartilage, this difference in migration was not observed with SF MSCs. BM MSCs and SF MSCs also responded to different growth factors in the IJCM. For example, significant reduction in BM MSC migration in response to IJCM was observed when neutralising antibody against FGF2 was added to the IJCM. However, no reduction in migration was observed with SF MSCs in response to FGF2 antibody. Therefore, it is possible that dead cartilage also released FGF2 which then promoted BM MSC migration in response to the dead cartilage.

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9.8 Purinergic priming factors and their importance in MSC migration

9.8.1 Comparison between Scratch and Cell exclusion zone migration assays

In this study two different types of wound healing assays were used to study SF MSC migration in response to IJCM. 'Classical' scratch assays were used to study SF MSC migration in response to IJCM in the presence of cell injury. Making the 'scratch' in the cell layers is done manually therefore to increase precision cell exclusion assays were carried out (Chapter 8).

In contrast to the results found with the scratch assay (and Transwell assays). IJCM did not promote cell migration in SF MSCs in the cell exclusion assays. Also, in contrast to scratch assays, heparin treatment significantly increased the migration stimulatory capacity of IJCM and cell migration to heparin-treated IJCM was observed in the cell exclusion assays (Figure 8.2). Hence, when no significant SF MSC migration was observed in response to untreated IJCM compared to serum free media, almost always 100% wound closure was observed with heparin-treated IJCM. These differences between the Scratch and cell exclusion assays in terms of the effects of IJCM and heparin-treated IJCM were reproducible.

However, the two assays have one fundamental difference in their mode of operation. A small injury is made in the cell layer in the scratch assay whereas no injury is made in the cell exclusion assays. To determine if this was the reason, cell migration assays were performed for 24 h in the cell exclusion assay system. After the 24 h period, a small scratch was made in the cell layer and the plates incubated for a further 24 h and the cell migration measured (Figure 8.3a). It was evident that after creating the scratch in the cell layer the cells migrated in response to the IJCM. In addition, the migration seen in the cell exclusion assays to heparin-treated IJCM was inhibited by creating a scratch (similar to one made in scratch assays) close to the cell exclusion zone (Figure 8.3b). The reason for this migration stimulatory effect of heparin-treated IJCM in cell exclusion zones is unclear. It could be deduced that IJCM contained both migration stimulatory and inhibitory factor(s). The inhibitory factor(s) were not heparin-binding proteins and hence, were not removed by heparin treatment of IJCM. However, it was clear that a small 'fresh' wound/scratch was needed to enable the migration of SF and BM MSCs in response to the IJCM. Therefore, these studies indicated that

one or more labile agents was released by the cells which was not present after 24 h (i.e. not present in the IJCM) but was present immediately after wounding i.e. scratching the cell layers. One class of biological mediators which are very labile are the extracellular nucleotides which have been indicated in various cell signalling pathways [153, 154]. Therefore, it was decided to look for ATP release by the injured cell layers and whether purine nucleotides influenced the migration of SF and BM MSCs in response to IJCM.

9.8.2 Why does injured cartilage release both migration stimulatory and inhibitory factors?

Characterisation of IJCM suggested that IJCM not only contained factors for MSC migration, it also contained factors for MSC survival and chondrogenesis. For example, SF MSCs cultured in IJCM had two main characteristics: elongated single cells with protruding ends suggestive of cell migration and condensation to form elongated tubes of cell aggregates which have previously been shown to be a pre-requisite step for chondrogenesis [155]. IJCM's ability to induce chondrogenesis was further shown by stimulating micromass cultures of MSCs with IJCM for 10 days and detecting GAG deposition using alcian blue staining. For successful repair through MSC migration and formation of repair tissue through MSC chondrogenesis, it is important that the timing between migration and tissue formation is well co-ordinated such that the necessary signals for tissue remodelling do not come before the MSCs have migrated to the site of injury. Therefore, it is possible that the migration inhibitory factors present in the IJCM plays a role in the timing between MSC migration and MSC condensation.

9.8.3 Role of extracellular nucleotides

To further understand the active components released through cell injury; monolayers of chondrocytes and BM MSCs were injured in the presence of serum free media and collected after 4 h. Addition of this cell injury conditioned media (CIJM) to IJCM in cell exclusion zone assay was able to initiate SF MSC migration but it was not sufficient to induce significant wound closure. This suggested that the factor(s) responsible for initiating migration were short lived and was possibly degraded by 4 h. Reduction of the collection time of CIJM to 30 mins strongly enhanced the effect of CIJM such that significant wound closure was observed in cell exclusion zone assays with IJCM+CIJM.

Drastic reduction in CIJM potency between 30 mins and 4 h suggested that the factor(s) responsible for initiating migration in response to IJCM+CIJM were short-lived. Previous experiments have suggested that extracellular nucleotides such as ATP or UTP are released from injured or non-apoptotic cells where it acts as a danger signal or damage-associated molecular pattern (DAMP) signal by activating the innate immunity [156]. For example, ATP has been shown to be locally released following myocardial infarction where it plays a critical role in inducing inflammation by activating NLRP3 and caspase-1 [157]. DAMPs are intracellular molecules which are hidden in order to avoid recognition by the immune system under physiological conditions. However, when cells or tissue are injured, DAMPs are either presented on the surface of the stressed/injured cells; secreted passively into the surrounding extracellular environment from the dying cells or the injured ECM or they can be actively secreted by the stressed cells [158]. Secreted DAMPs are recognised by pattern-recognition receptors (PPRs) which then activate the innate immunity.

Analysis of CIJM using bioluminescence assay confirmed the presence of micromolar levels of ATP in the cell injury media. This also suggested that immediately following cartilage damage, injured chondrocytes may release extracellular nucleotides into the synovial fluid where the nucleotides might act as damage signals. Injury-induced DAMPs play a critical role in inflammation and fibrosis-mediated wound healing [159]. Secretion of DAMPs has been shown to be correlated with the degree of severity of injury in traumatic diseases which varies from small cuts to trauma from blunt injury to bone fracture or large severe injuries. In all these cases, DAMPs were not only responsible for inducing acute inflammatory response but together with the surrounding growth factors and cytokines were responsible for wound healing [160]. This is consistent with the findings presented in this thesis where extracellular nucleotides were released immediately following injury and were responsible for priming SF MSCs to migrate along the chemotactic gradient of cytokines and growth factors (released from the ECM of the injured cartilage) to the site of injury and stimulate tissue repair.

The identity of the putative purinergic receptor causing the priming effect on the SF MSCs is unknown. However, using ectonucleotidase resistant analogues to prevent/reduce degradation of the purine nucleotides, UTP-ɣS showed a greater

potency over ATP- ɣS. Therefore, it may be possible that the priming effect of the purine nucleotides occurs through activation of a P2Y receptor. This was also supported by use of the P2 receptor antagonist suramin which inhibited SF MSC migration in scratch assays (Figure 8.10). However, this suggestion is tentative and needs to be confirmed by further research.

It was also found that SF MSCs but not BM MSCs required priming by extracellular nucleotides prior to migration in response to IJCM. One study suggested that extracellular nucleotides induced a localised and transient response to stimulate rapid cell-cell communication [78]. Furthermore, this cell-cell communication was an earlier event prior to cells becoming migratory and was actively repairing the defect caused by the injury. Therefore, it can be reasoned that by the time BM MSCs will come in contact with the extracellular nucleotides released into the synovial fluid, these MSCs have already become migratory and hence, no longer require the priming signal of the extracellular nucleotides at the site of injury.

In the study presented in this thesis, the IJCM was observed to promote chondrogenesis by SF MSCs and BM MSCs (Figure 8.12). The molecular identity of the chondrogenic factors present in the IJCM was not carried out. However, inhibition of either TGFβ3 or CXCL12 using neutralising antibodies or receptor antagonists in the IJCM which inhibited the migratory response of SF MSCs to IJCM did not inhibit SF MSC chondrogenesis by IJCM suggesting that these factors were not the only factors responsible for MSC chondrogenesis in response to IJCM (Figure 8.13). In addition, the neutralising antibody was to TGFβ3 only and chondrocytes are known to synthesis TGFβ1 which is chondrogenic. It is known that FGF2 was present in the IJCM but it was not investigated if FGF2 was responsible for the chondrogenesis. FGF2 have previously been shown to promote chondrogenic differentiation of both rat and human MSCs [161-164]. Therefore, it was possible that FGF2 present in the IJCM was responsible for the induction of chondrogenesis. Furthermore, addition of ATP-ɣS or UTP ɣS to IJCM appeared to induce deeper levels of Alcian Blue staining compare to IJCM alone suggesting a possible chondrogenic effect. It may be that purinergic stimulation promotes chondrogenic differentiation. Purinergic nucleotides have been shown to stimulate chondrogenesis in chondrocytes [155]. Since, ATP can stabilise FGF2 by binding to the growth factor through the

heparin-binding domain [165], it was also possible that this stabilisation was the reason for the enhanced chondrogenesis seen in response to ATPɣS and UTPɣS containing IJCM. However, it is likely that the IJCM contains other growth factors and biological agents in addition to CXCL12, TGFβ3 and FGF2 which were responsible for MSC chondrogenesis.

9.9 Current limitations and Future directions

While the present study has provided a novel mechanism through which injured cartilage can repair itself by stimulating MSC migration, it has limitations that must be acknowledged. These include further optimisation of the Transwell migration assay by testing a broad range of cell seeding densities. Finding an optimal cell seeding density which will reliably show the difference in migration in response to various experimental conditions will allow automation of the cell number quantification using Image J. Furthermore, even if cells were counted manually using the cell counter option on ImageJ, it would still be far easier to detect and discriminate between individual cells. In my research most of the chemokine/inhibitor concentrations were selected based on the suggested concentration on the product data sheet. This means in some cases it is possible that lack of inhibition of migration can be attributed to low concentration of inhibitors. Therefore, it is important to further investigate several concentrations of chemokines/inhibitors in order to find the optimal conditions for inhibition. Moreover, only the result of CXCL12 neutralising antibody was validated using small-molecule inhibitor AMD3100. Further experiments should also validate FGF2 and TGFβ3 signalling by blocking FGF receptor signalling using small-molecule inhibitor SB402451 and TGFβ signalling using SB431542. This study has only indirectly shown the presence of CXCL12, TGFβ3 and FGF2 in the IJCM by using cytokine specific inhibitors. Therefore, it is important to directly show the presence of these molecules in the IJCM using both ELISA and Western Blot and further validate the results using immunohistochemistry.

There are still several questions that remain to be answered with further studies. We did not investigate the contribution of chondrocytes in the production and/or release of the injury factors. To do this, further studies should investigate ‘injured’ dead cartilage along with ‘non-injured’ dead cartilage (investigated in this study)

and compare their effects on MSC migration. Furthermore, we have only identified three key molecules present in the IJCM responsible for MSC migration. It is possible there are several other molecules which are important for this pathway. Therefore, the IJCM needs to be fully characterised using mass spectrometry to identify all proteins present in the IJCM. Initially, this characterisation may be restricted to identifying only the members of the heparin interactome. Later, the identified molecules need to be further tested for their effect on the MSC migration. More importantly, all these molecules need to be tested to see how do molecules released upon cartilage injury influence MSC activity in general – by driving MSC migration, adhesion, proliferation and chondrogenic differentiation? Similarly, this study only provided preliminary data on the influence of purinergic system on MSC migration in response to cartilage injury. Further studies is needed to first show the presence of purinoceptors, identify which purinoceptors are present and then show which purinoceptors are required for the priming of the SF MSCs.

Another question yet to be answered is the translation of this data from the animal model to a human model. Although, it is relatively safe to say that human MSCs will also migrate in response to IJCM, we still need to provide experimental evidence to show that both SF- and BM MSCs from human will also migrate in response to CXCL12, TGFβ3 and FGF2. Furthermore, will we see the same differences between the SF- and BM MSC migration in a human model? Another important factor to consider is how much variability will be present among the response of the MSCs harvested from different individuals? Will we still see a homogenous response to IJCM as observed with bovine MSCs?

Lastly, it is imperative to understand is the response to injury the same in human osteoarthritic, normal aged as well as young healthy cartilage? Once, all these questions have been answered using the *in vitro* model further investigations can focus on studying this MSC migration pathway in an *in vivo* model of cartilage injury. An excellent model has already been developed by Eltawil *et al* [33]. This model can be back-crossed with knockout animals to create mutant strains s (e.g. $FGF2^{-/-}$, $CXCL12^{-/-}$, $TGF\beta3^{-/-}$) in order to see whether lack of these molecules can impair normal regeneration of the injured cartilage. An alternative strategy would

be to use small molecular inhibitors as used in this study (AMD3100) to block growth factor signalling.

MSC migration is a critical step in the repair and regeneration of the injured cartilage. However, the signalling pathway leading to the MSC migration is still lacking. Migration is a complex cellular process and results from the coordinated assembly and disassembly of focal adhesion complexes [166, 167]. Extracellular signals can stimulate specific intracellular pathways by activating mitogen-activated protein kinases (MAPK) namely extracellular signal-regulated kinases (ERK), c-Jun NH_2-terminal kinases (JNK) and p38 MAPKs [168, 169]. Once activated these MAPKs can phosphorylate other transcription factors within the cell nucleus and stimulate the activation of a number of cellular functions including survival, proliferation, differentiation and apoptosis. Previous studies have also reported the importance of MAPKs in cell migration of diverse cell types including fibroblasts, breast cancer cells, endothelial cells and Schwann cells [168, 170, 171]. Furthermore, Vincent *et al* have shown that FGF2 released from the cartilage following cutting rapidly activated ERK [46]. Therefore, it is important to investigate if CXCL12 and FGF2-induced activation of ERK1/2 is required for SF and BM MSC migration.

Data from this study suggested that when chondrocytes are injured it released ATP. One study investigating the wound healing in corneal epithelial cells have suggested that ATP released following epithelial injury acts as a signal to stimulate shedding of heparin-binding epidermal growth factor-like growth factor (HB-EGF) [172, 173]. Therefore, future studies can also test if ATP released from the injured cartilage through chondrocyte injury also induced the release of CXCL12, TGFβ3 and FGF2 which then acts as attractants for MSCs to stimulate MSC migration to the injury site.

9.10 Possible therapeutics that can be developed based on this work

Successful regeneration of damaged tissues is firstly critically dependent on identification of the range of molecules that promotes organ-specific stem cell migration and secondly on manipulation of the activity of these molecules to enhance stem cell migration.

Possible therapeutic strategies that can be developed using the knowledge from this study include developing ‘smart’ cell-free implants. These implants will be divided into four key working parts. The first part will contain inhibitors of ectonucleotidases to prevent the breakdown of ATPs released from the injury. This will allow the ATPs released from the injury to act as DAMPs and call in the necessary molecules to the injury site. Furthermore, reduced breakdown of ATP will also allow larger number of SF MSC priming which can then migrate to the injury site. The second part will be functionalised by attaching heparin-binding motifs which can then bind CXCL12, TGFβ3 and FGF2. This part will be responsible for creating a chemotactic gradient for MSC migration. The third part of the implant will contain an ATP-generating mechanism which in conjunction with the first part will stimulate priming of large number of SF MSCs. Finally, the fourth part of the implant will contain factor(s) (yet to be identified) that will stimulate chondrogenesis of the MSCs present at the injury site. These implants will be placed at the injury site through a small microfracture which will allow BM MSCs to invade the injury site.

Chapter 10

Conclusions

Conclusions

Traumatic cartilage injuries are a known risk factor for development of osteoarthritis (OA). Classically articular cartilage is seen as poorly repairing. However, cartilage injuries in very young animals can heal although the mechanism of this repair is unknown. The aim of this project was to investigate if SF and BM MSCs can respond to cartilage injury and to determine the mechanisms by which this occurred. The conclusions from this study are summarised below:

1. Articular cartilage, SF and BM MSCs were isolated from the bovine metacarpophalangeal joints and the protocol for *in vitro* culture expansion of SF and BM MSCs was optimised.
2. Multipotentiality of the MSCs using classical differentiation assays was demonstrated and cultured SF and BM MSCs could be differentiated into chondrogenic, osteogenic and adipogenic lineages. However, SF MSC showed a lower adipogenic capacity than BM MSC.
3. An *in vitro* scalpel-induced cartilage injury model was developed. Cartilage discs were collected using a 6 mm biopsy punch and a no.11 scalpel blade attached to no.3 scalpel handle. Freshly collected cartilage discs were allowed to rest in high glucose DMEM supplemented with 10% FCS and 10^{-7} M dexamethasone to obtain non-injured cartilage. Dead cartilage was obtained by irradiating cartilage discs and subsequently freezing them at -20°C. 'Rested cartilage' was injured using no.11 scalpel attached to no. 3 scalpel handle. Multiple cuts were made in the push-through mode by passing the scalpel blade perpendicular to the long axis of the discs in a straight line.
4. Significantly greater numbers of SF and BM MSCs migrated to injured cartilage compared to non-injured cartilage. However, in contrast to SF MSCs, significantly higher numbers of BM MSCs also migrated to dead cartilage compared to non-injured cartilage.
5. Use of fluorescently labelled MSCs, showed that MSCs migrated specifically to the site of the injury and adhere along the edges of the injury.
6. Incubation of freshly isolated articular cartilage explants with serum-free DMEM for 24 h yielded injury conditioned media (IJCM). The IJCM

significantly stimulated SF and BM MSCs migration compared to the serum free control media in both Transwell and Scratch migration assays which indicated that cartilage following injury released migration stimulatory factors into the media.

7. Characterisation of IJCM using neutralising antibodies and receptor inhibitors identified three key biological factors which were responsible for the migratory activity of IJCM. These were CXCL12, TGFβ3 and FGF2. Inhibition of these factors with neutralising antibodies inhibited SF and BM MSC migration. BM MSCs migrated in response to all three factors in the IJCM. In contrast, SF MSCs only migrated in response to CXCL12 and TGFβ3 present in the IJCM.
8. Cell exclusion zone assays demonstrated that in the absence of an injury in the cell layer, SF MSCs do not respond to the chemotactic gradient of the IJCM. However, migration in response to IJCM was observed in this system when a cell injury was created (by scraping off a small number of cells) close to the cell exclusion zone.
9. Creating the 'scratch' wound caused release of ATP from the injured cells which were validated using ATP-bioluminescence assay to quantify the ATP released.
10. Supplementation of ATP or UTP or stable purine analogues ATP-γS or UTP- γS with IJCM stimulated MSC migration in cell exclusion zone assays suggesting a role for a putative P2 receptor. The apparent greater response to UTP- γS compared to ATP-γS suggested that the P2 receptor may be a member of the P2Y class. Involvement of a P2Y receptor was further validated by demonstrating that IJCM-stimulated SF MSC migration could be completely inhibited in scratch assays using P2 purine receptor antagonist suramin.
11. In contrast to SF MSCs, IJCM induced significant BM MSC migration in cell exclusion zone assays suggesting a lack of requirement of extracellular nucleotides for BM MSC migration.
12. IJCM could maintain MSCs viability in *in vitro* culture in the absence of FCS. Furthermore, IJCM could also induce chondrogenesis of MSCs and this IJCM-induced chondrogenesis could be enhanced by addition of ATP or UTP to the IJCM. Chondrogenic factors in the IJCM were not identified in this study. However, inhibition of CXCL12 enhanced IJCM-induced

chondrogenesis as seen from the stronger alcian blue staining. Furthermore, inhibition of TGFβ3 reduced the IJCM-induced chondrogenesis as indicated by the reduction in the intensity of alcian blue staining.

Therefore, this study demonstrated that cartilage following injury released ATP, CXCL12, TGFβ3 and FGF2. ATP was required to prime the SF MSCs before their migration along the chemotactic gradient of CXCL12 and TGFβ3. In contrast, BM MSCs did not require priming before their migration along the chemotactic gradient of CXCL12, TGFβ3 and FGF2. Migration of MSCs in response to these chemokines enabled the MSCs to home towards the injured cartilage and specifically to the site of the injury where they may then take part in the regeneration of the injured cartilage since the injury caused release of factors which stimulated chondrogenesis. Therefore, in this study a migration mechanism through which MSCs migrated to the site of the cartilage injury and then take part in repair and regeneration of the injured cartilage was established Furthermore, migration stimulatory factors identified in this study can be incorporated into implants which could then be used to mobilise endogenous MSCs to the site of the injury. Such a technique would provide a less invasive and cost effective alternative to repair and regenerate the injured cartilage.

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Appendix

Papers

1. CXCL12, TGFβ3 and FGF2 released from injured articular cartilage stimulate mesenchymal stem cell migration to the injury site. [Submitted].

2. *Purinergic priming factor released by injured cartilage is required by SF MSCs for migration towards injured cartilage* [Title in progress].

Conference presentations and publications

Tissue Engineering and Regenerative Medicine International Society (TERMIS), Davos (June 2017)

Poster presented. Abstract published in the European Cells and Materials Journal. *'Injured articular cartilage releases both migratory/homing factors and a purinergic priming factor which are mandatory for homing of mesenchymal stem cells to cartilage lesions.'*

Tissue Engineering and Regenerative Medicine International Society (TERMIS), Sweden (June 2016)

Poster presented. Abstract published in the European Cells and Materials Journal. *'Repairing injured articular cartilage: investigation of potential repair mechanisms using mesenchymal stem cells'*

British Society of Matrix Biology (BSMB) Conference, University of Chester (April 2016)

Poster presented. Abstract to be published in the International Journal of Experimental Pathology (IJEP). *'Investigating the potential repair mechanism of injured cartilage using mesenchymal stem cells.'*

British Society of Matrix Biology (BSMB) Conference, University of Oxford (March 2015)

Poster presented. Abstract to be published in the International Journal of Experimental Pathology (IJEP). *'Repairing injured articular cartilage: investigation of potential repair mechanisms using mesenchymal stem cells'*

Local Conferences, University of Sheffield

- Poster presented at the Biomaterials and Tissue Engineering group (BiTEG) annual meeting. (2014)
- Poster presented at the 6th Annual Mellanby Centre Research Day. (2015)
- Poster presented at the Biomaterials and Tissue Engineering group (BiTEG) annual meeting. (2016)

Grants and awards received through this work

EPSRC Summer Vacation Bursary Programme (2016)

Award: £2220

Funding obtained to provide an 8 week summer project to an undergraduate student. Funded the pilot studies which established the chondrogenesis inducing capacity of IJCM.

Sheffield Undergraduate Research Experience, University of Sheffield (2015)

Award: £700

Funding obtained to provide an 8 week summer project to an undergraduate student. Funded the pilot studies which established the chondrogenesis inducing capacity of IJCM.

British Society of Matrix Biology (BSMB) Travel Grant (2016)

Award: £164

Poster Prize, British Society of Matrix Biology, Conference Presentation (2016)

Poster Prize, British Society of Matrix Biology, Conference Presentation (2015)