

An *In Vitro* Comparison of the Incorporation, Growth, and Chondrogenic Potential of Human Bone Marrow versus Adipose Tissue Mesenchymal Stem Cells in Clinically Relevant Cell Scaffolds Used for Cartilage Repair

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Abstract

Aim. To compare the incorporation, growth, and chondrogenic potential of bone marrow (BM) and adipose tissue (AT) mesenchymal stem cells (MSCs) in scaffolds used for cartilage repair. **Methods.** Human BM and AT MSCs were isolated, culture expanded, and characterised using standard protocols, then seeded into 2 different scaffolds, Chondro-Gide or Alpha Chondro Shield. Cell adhesion, incorporation, and viable cell growth were assessed microscopically and following calcein AM/ethidium homodimer (Live/Dead) staining. Cell-seeded scaffolds were treated with chondrogenic inducers for 28 days. Extracellular matrix deposition and soluble glycosaminoglycan (GAG) release into the culture medium was measured at day 28 by histology/immunohistochemistry and dimethylmethylene blue assay, respectively. **Results.** A greater number of viable MSCs from either source adhered and incorporated into Chondro-Gide than into Alpha Chondro Shield. In both cell scaffolds, this incorporation represented less than 2% of the cells that were seeded. There was a marked proliferation of BM MSCs, but not AT MSCs, in Chondro-Gide. MSCs from both sources underwent chondrogenic differentiation following induction. However, cartilaginous extracellular matrix deposition was most marked in Chondro-Gide seeded with BM MSCs. Soluble GAG secretion increased in chondrogenic versus control conditions. There was no marked difference in GAG secretion by MSCs from either cell source. **Conclusion.** Chondro-Gide and Alpha Chondro Shield were permissive to the incorporation and chondrogenic differentiation of human BM and AT MSCs. Chondro-Gide seeded with BM MSCs demonstrated the greatest increase in MSC number and deposition of a cartilaginous tissue.

Keywords

bone marrow, adipose tissue, mesenchymal stem cells (MSCs), cell scaffolds, chondrogenesis

Introduction

The regeneration of hyaline cartilage still poses a significant clinical challenge, with current available treatments resulting in a reparative tissue with inferior mechanical properties.¹ Cell therapy using autologous chondrocyte implantation (ACI) has been used to treat cartilage defects since 1987,² but has some disadvantages, such as the production of fibrous cartilage and donor site morbidity.³ *In vitro* and preclinical animal studies suggest that multipotent mesenchymal stem or stromal cells (MSCs) can provide an alternative to autologous chondrocytes for the regeneration of cartilage, as they possess chondrogenic differentiation potential, are obtainable from a number of tissue sources

and can be culture expanded *in vitro* to provide increased cell numbers for transplant therapies.^{4,5} Bone marrow (BM) is currently the most extensively studied source of MSCs. However, harvesting an adequate number of MSCs from

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BM is problematic because of the finite volume available at any one site. Hence, adipose tissue (AT) has recently been shown as an attractive alternative,^{6,7} wherein 200 mL of lipoaspirate can readily be removed from patients, yielding 4×10^8 nucleated cells of which more than 2% constitutes the MSC population.^{8,9} The ready availability of AT MSCs is advantageous in autologous cell therapies as the time needed for costly culture expansion to generate a sufficient cell number for transplantation is considerably reduced when compared with BM. Moreover, harvesting AT through lipoaspiration makes AT MSCs an attractive cell source compared to more invasive and potentially painful iliac crest biopsies.

Whether or not AT MSCs are equivalent to BM MSCs in terms of their chondrogenic differential potential is a matter of considerable debate. Some studies have suggested that AT MSCs have inferior potential for chondrogenesis and hence use in cell therapies for cartilage repair,^{10,11} while others have reported on successful multilineage differentiation of AT MSCs, including toward chondrogenesis.^{12,13}

The aim of this *in vitro* study was to compare the incorporation, growth, and chondrogenic potential of BM versus AT MSCs in 2 commercially available cell scaffolds currently used for cartilage repair in humans, namely Chondro-Gide and Alpha Chondro Shield. *In vitro* studies have tested these scaffolds with BM MSCs and chondrocytes, but very little data are available on their use with AT MSCs in comparison.^{14,15} Chondro-Gide (Geistlich Pharma AG, Wolhusen, Switzerland) is a bilayered scaffold, composed of type I and type III collagen, with one porous side for cell attachment and a compact side to prevent cell leakage, which has been extensively used in the clinic for autologous matrix induced chondrogenesis (AMIC) procedures and ACI.^{16,17} Alpha Chondro Shield (Swiss Biomed Orthopaedics AG, Zurich, Switzerland) is intended to be used mainly as a cell-free cartilage implant to aid the migration and differentiation of mesenchymal progenitor cells from subchondral bone after a microfracture procedure. Alpha Chondro Shield is composed of fibers of polyglycolic acid (PGA) arranged in a homogeneous non-woven pattern; currently there is no clinical data available on its use with chondrocytes or MSCs, whether from BM or AT.

Methods

Before commencement of the study ethical approval was obtained from the national review body (12/EE/0136 and 06/Q2601/9) and the study was conducted with the principles of the Declaration of Helsinki (World Medical Association).

Isolation, Expansion, and Characterisation of MSCs

In total, MSCs were cultured from 3 BM donors (age range 19-80 years) and 4 AT donors (age range 27-75 years). BM was aspirated from the posterior superior iliac spine or harvested from excised femoral head during total hip replacement surgery. AT was harvested from the infrapatellar fat pad of the patients undergoing knee-reparative surgery. Mononuclear cells were isolated from BM aspirates by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, UK). AT samples were minced and treated with 0.1% collagenase type IA (Sigma; Poole, Dorset, UK) for up to 2 hours at 37°C and 5% CO₂. After this enzymatic digestion, Dulbecco's modified Eagle medium (DMEM) supplemented with 20% (v/v) fetal calf serum (FCS) (PAA, Yeovil, Somerset, UK) was added to neutralize collagenase activity and the digest was centrifuged into a cell pellet, which was then subsequently washed in DMEM/F-12, supplemented with 10% (v/v) FCS, 1% (v/v) penicillin (50 U/mL), and streptomycin (50 µg/mL) (standard medium; all from PAA), and filtered through 70-µm cell strainers to remove undigested tissue. The BM mononuclear cells and adipose stromal vascular fraction (SVF) cells¹⁸ were then plated out at a density of 2×10^7 cells per 75 cm² flask in 20 mL of standard medium and incubated at 37°C in humidified atmosphere containing 5% CO₂. After 24 to 48 hours, the nonadherent cells were washed off gently with phosphate-buffered saline (PBS; PAA) and the adherent cells were subsequently cultured until they reached approximately 70% confluence. Cells were routinely passaged at 70% confluence using 0.25% trypsin-EDTA (Life Technologies Ltd, Paisley, UK) and reseeded at 10^4 cells/cm² into fresh 75-cm² flasks to culture expand the adherent cell population. At passage II-III, culture expanded cells were characterised by their adherence to tissue culture plastic, by immunoprofiling for CD markers and by examining their differentiation potential to form osteoblasts, adipocytes, and chondrocytes. These criteria meet the MSC phenotype defined by the International Society for Cellular Therapy.¹⁹

Cell Seeding Into Scaffolds and Chondrogenesis

Bone marrow MSCs and AT MSCs were seeded at a density of 5×10^4 cells in 50 µL of standard culture medium per 9mm² piece of Chondro-Gide or Alpha Chondro Shield ($n = 4$ scaffolds per MSC donor) in non-tissue culture coated plates. After 2 hours incubation at 37°C to permit cell adhesion to the cell scaffolds, an additional 1 mL of standard culture medium was added to each well. For initial assay of cell incorporation and growth, the cell-seeded scaffolds

were maintained in standard culture media for a period of 28 days. A further analysis to examine the comparative adhesion of MSCs to Chondro-Gide and Alpha Chondro Shield was performed using scanning electron microscopy, as follows: (1) the scaffolds were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours; (2) the scaffolds were then dehydrated through a series of alcohols (20% to 100%) for 10 minutes in each solution; and (3) the scaffolds were dried overnight in hexamethyldisilazane (HMDS) and then gold sputtered and imaged using a Zeiss EVO10 scanning electron microscope (Carl Zeiss, Cambridge, UK).

In separate assays of chondrogenesis, the cell-seeded scaffolds were maintained in induction medium, consisting of DMEM (high glucose) supplemented with 2% FCS (Life Technologies Ltd), 100 nM dexamethasone (Sigma), 37.5 µg/mL ascorbate 2-phosphate (Sigma), insulin, transferrin and selenium (1% ITS-X; Sigma) and 10 ng/mL transforming growth factor-β1 (PeproTech Ltd., London, UK) (duplicate scaffolds for each MSC donor), or with control medium that contained carriers alone (duplicate scaffolds for each MSC donor) for the same period. Culture medium was replaced 2 to 3 times per week. The incorporation and viability of cells following these chondrogenesis experiments was assessed by DAPI counterstaining of sections of induced cultures harvested at day 28 and by Live/Dead staining, respectively. MSCs from 2 separate donors from both BM and AT were analyzed for the initial incorporation of cells into the cell scaffolds and MSCs from a further 2 separate donors of BM and AT were analyzed for the inductions of chondrogenesis.

Live/Dead Staining and Confocal Microscopy for Cell Viability/Growth

Cell-seeded scaffolds were assessed for cell incorporation, viability and growth using Live/Dead cell staining according to the manufacturer's guidelines (Sigma), wherein live cells fluoresce green and dead cells fluoresce red. The staining procedure was performed in the dark for 30 minutes at 37°C and 5% CO₂. Live and dead cells were visualized and scored by fluorescence imaging and confocal microscopy (Leica Microsystems DM6000B-SP57CS). This was performed by scoring the number of viable (green) and the number of dead (red) cells present in each of 4 fields of view taken through the depth of the scaffold over 2 separate regions for each MSC donor and each scaffold at each time point.

Dimethylmethylene Blue Assay for Glycosaminoglycans

The dimethylmethylene blue (DMMB) assay protocol was adapted from previously published methods²⁰ as follows: (1) the DMMB dye solution was prepared by adding 3.04 g of glycine, 2.37 g of NaCl, and 16 mg of 1,9 dimethylmethylene

blue to 1 L of deionized water; (2) the pH was adjusted to 3.0 with hydrochloric acid and the reagent was stored in a brown bottle; (3) 50-µL aliquots of culture medium harvested from the cell-seeded scaffolds at day 28 were added in triplicate to a 96-well plate; (4) 200 µL of the DMMB dye was added and absorbance was assessed at 540 nm immediately. Chondroitin sulfate from shark cartilage (Sigma) was used to provide a standard curve (0-40 µg/mL) from which the glycosaminoglycan (GAG) content in the samples of medium was calculated. The levels of absorbance for GAG content in the samples of medium were normalized to account for the background absorbance resultant from the presence of phenol red within the medium. Replicate values from 2 independent donors of BM MSC versus 2 independent donors of AT MSCs for each experimental condition were pooled and GAG content has been presented as means ± standard deviations from these pooled data.

Histology and Immunohistochemistry

Cell-seeded scaffolds were harvested at day 28 by fixation in 10% neutral buffered formalin for 24 hours, then processed and paraffin embedded. Toluidine blue staining was performed to reveal GAG content, whilst the presence of collagen type II was detected by immunolocalization as follows: (1) Antigen retrieval was performed by incubating sections in 0.1% hyaluronidase; (2) slides were incubated in a solution containing antibodies for collagen type II (6.5 µg/mL; CIIC1: Developmental Studies Hybridoma Bank, Iowa City, IA) for 60 minutes at room temperature (RT); (3) the omission of a primary antibody was used for control purposes; (4) slides were then incubated with a biotinylated secondary antibody for 30 minutes at RT; and (5) immunopositivity was revealed with a streptavidin-based tertiary step and 3,3'-diaminobenzidine (DAB) chromogen using a commercial labelling kit (Vector ABC Elite, Vector Laboratories Ltd, Peterborough, UK). Some sections of the harvested cultures were mounted in Vectamount containing DAPI (Vector Laboratories Ltd) to counterstain for cell nuclei.

Statistical Analysis

Quantitative data for CD profiling have been presented as means ± standard error of the mean from 3 independent donors for BM MSCs and 3 independent donors for AT MSCs. Quantitative data for MSC incorporation, cell viability, and GAG content have been presented as means ± standard deviations, which derived from replicate values pooled from 2 independent donors for each MSC type, that is, $n = 2$ BM MSC versus $n = 2$ AT MSC donors for each experimental condition and time point.

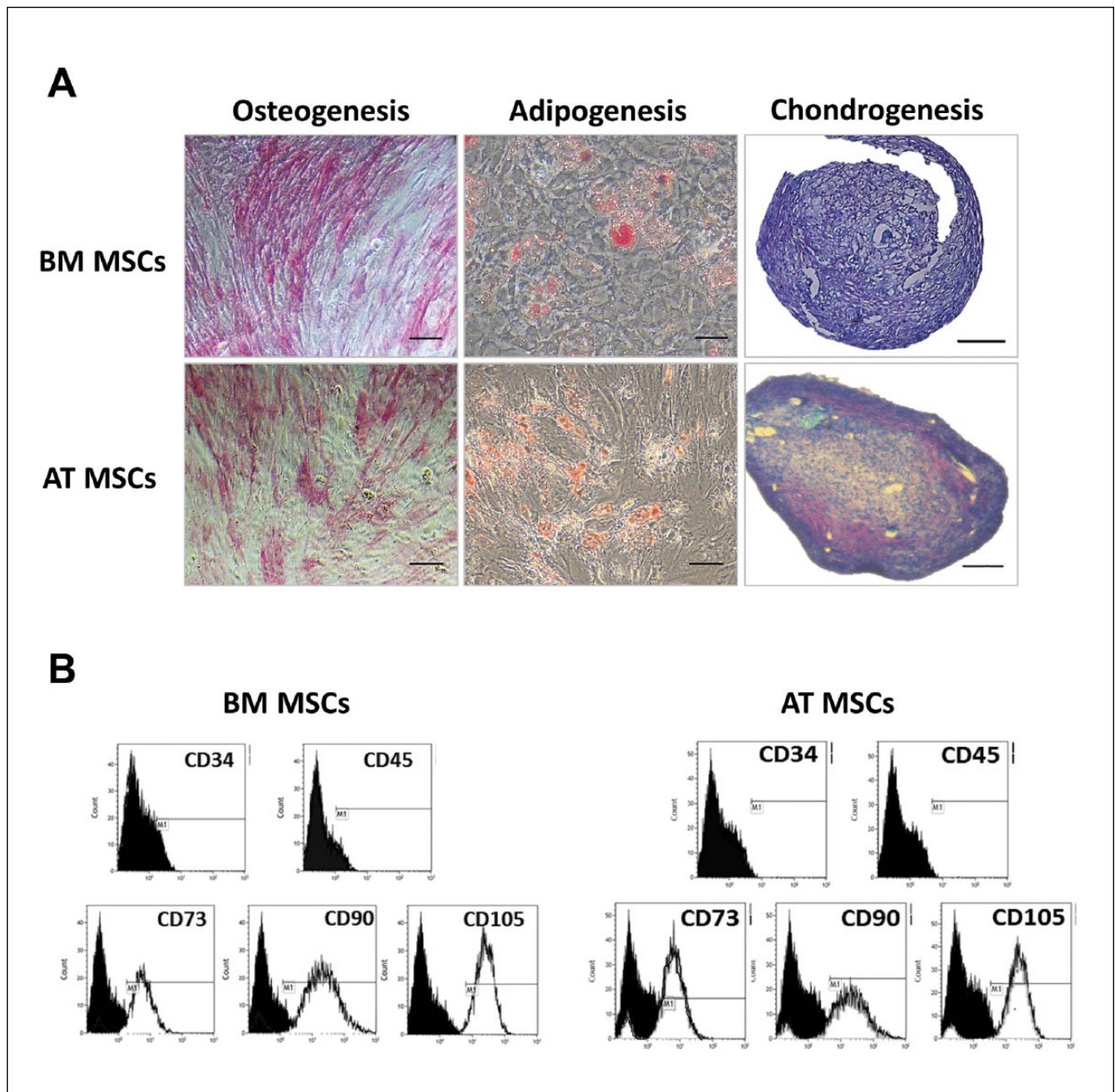


Figure 1. Characterisation of bone marrow (BM) mesenchymal stem cells (MSCs) and adipose tissue (AT) MSCs. **(A).** Culture expanded and plastic adherent cells from BM and AT differentiated along mesenchymal lineages, as indicated by the presence of alkaline phosphatase positive osteoblasts or Oil Red O positive adipocytes in monolayer cultures and metachromatic staining for glycosaminoglycans (GAGs) in pellet cultures (scale bars represent 100 μ m). **(B).** Representative histograms are shown for positivity for CD markers in BM MSCs (left panels) and AT MSCs (right panels). The white histogram shows immunopositivity for each indicated marker, which is only clearly apparent when the extent of immunofluorescence is greater than that detected following immunolabeling with an isotype-matched control antibody, indicated by the black histogram.

Results

Mesenchymal Stem Cell Characterisation

Plastic-adherent stromal cells isolated and culture expanded from BM and AT differentiated down the three mesodermal lineages, as indicated by alkaline phosphatase staining for osteogenesis, Oil Red O staining of lipid vacuoles for adipogenesis and toluidine blue metachromatic staining of paraffin sections of cell pellets for chondrogenesis (**Fig. 1A**). Using flow cytometry, these cells were immunoreactive for MSC-specific cell surface antigens, that is, CD73, CD90, and CD105 and were not immunoreactive for non-MSC markers, that is, CD34 and CD45 (**Fig. 1B**). For BM MSCs, $2.2\% \pm 0.3\%$ cells were CD34 positive, $2.7\% \pm 0.7\%$ cells were CD45 positive, $90.4\% \pm 5.1\%$ cells were CD73 positive, $84.8\% \pm 4\%$ cells were CD90 positive, and $97.8\% \pm 0.6\%$ cells were CD105 positive. For AT MSCs, $3\% \pm 1.1\%$ cells were CD34 positive, $3\% \pm 1.2\%$ cells were CD45 positive, $92.4\% \pm 4\%$ were CD73 positive, $90.4\% \pm 3\%$ cells were CD90 positive, and $93.8\% \pm 2.8\%$ cells were CD105 positive.

Bone Marrow MSCs and AT MSCs Incorporated and Remained Viable in Chondro-Gide and Alpha Chondro Shield during Long-Term Cultures

A greater number of MSCs from both tissue sources incorporated into Chondro-Gide than into Alpha Chondro Shield. Scanning electron microscopy at 30 minutes postseeding demonstrated that the cells more readily attached to the surface of the Chondro-Gide than to the surface of Alpha Chondro Shield (**Fig. 2A**). Many cells were lost during the cell seeding process. For the AT MSCs, only $1.2\% \pm 0.1\%$ of cells were retained in Chondro-Gide compared with $0.7\% \pm 0.4\%$ of cells retained in Alpha Chondro Shield at day 1. In comparison, for the BM MSCs only $0.8\% \pm 0.4\%$ of cells were retained in Chondro-Gide and $0.5\% \pm 0.4\%$ retained in Alpha Chondro Shield at day 1. Greater numbers of AT MSCs than BM MSCs appeared to incorporate in both of the scaffolds at day 1.

In longer term cultures, that is, from 7 days postseeding onward, both BM and AT MSCs appeared to become fibroblast-like in Chondro-Gide, whereas they also showed an elongated morphology and attached and spread along the length of the fibers in Alpha Chondro Shield (**Fig. 2B**). The number of viable MSCs from both cell sources increased from day 1 with further time in culture in Chondro-Gide. In contrast, the number of viable MSCs from either BM or AT did not increase in Alpha Chondro Shield (**Fig. 2C**). There were increased numbers of viable cells in Chondro-Gide compared with Alpha Chondro Shield from day 14 to day 28 for BM MSCs and from day 7 to day 28 for AT MSCs.

Fewer than 5% of cells were scored as nonviable (red) in both scaffolds and at all time points.

Mesenchymal Stem Cell Condensation under Chondrogenic Conditions in Chondro-Gide and Alpha Chondro Shield

Under chondrogenic (+CM) and nonchondrogenic (-CM; control) conditions BM and AT MSCs remained greater than 95% viable over a 28-day culture in Chondro-Gide and Alpha Chondro Shield. Under chondrogenic conditions, there appeared to be a greater increase in the number of viable BM and AT MSCs in Chondro-Gide, where the cells appeared condensed and confluent compared with the less dense network of cells seen under control conditions. Because of this growth of cells, exact cell counts were not possible. In Alpha Chondro Shield, there also appeared to be an increase in the number of viable BM and AT MSCs under chondrogenic versus control conditions, with increased cell condensations apparent (**Fig. 3A**). The distribution of cells in these experiments was examined using DAPI staining of tissue sections from harvested cultures at day 28. This demonstrated that the MSCs were evenly distributed through the porous side of Chondro-Gide only, without any cells present within the nonporous side. In addition, there was an even distribution of MSCs within Alpha Chondro Shield (**Fig. 3B**).

Cartilage-Specific Extracellular Matrix Deposition in BM and AT MSCs Seeded Scaffolds Was Seen in Long-Term Cultures Following Chondrogenic Induction

Histology and immunohistochemistry were performed to examine the deposition of cartilage-specific extracellular matrix (ECM) within MSC-seeded scaffolds. Metachromatic toluidine blue staining was seen in chondrogenic (+CM) treated scaffolds, which is indicative of the accumulation of GAGs. A greater amount of metachromatic staining in the ECM was seen in both BM and AT MSCs within Chondro-Gide compared with Alpha Chondro Shield. However, rounded cell morphologies surrounded by ECM, which is indicative of a mature chondrocytic phenotype was seen only in cultures of Chondro-Gide seeded with BM MSCs under chondrogenic conditions. None of the MSC-seeded scaffolds under non-chondrogenic conditions showed any ECM deposition. Collagen type II deposition was most markedly seen in Chondro-Gide cultures seeded with BM MSCs under chondrogenic conditions, with some collagen type II seen to a lesser extent in BM and AT MSCs in Alpha Chondro Shield, only under chondrogenic conditions (**Fig. 4A** and **4B**). Staining of human articular cartilage was

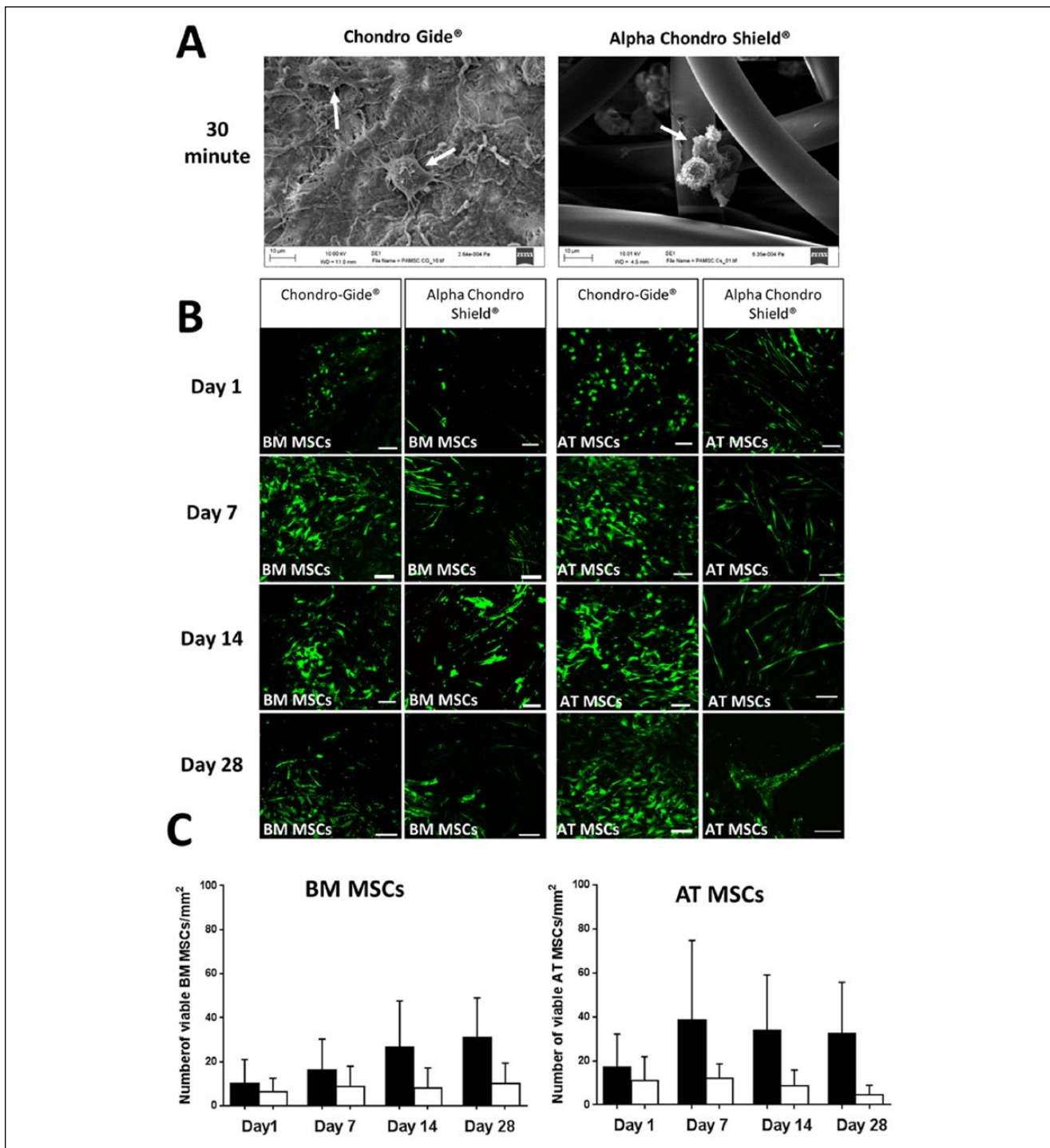


Figure 2. The incorporation and growth of bone marrow (BM) mesenchymal stem cells (MSCs) and adipose tissue (AT) MSCs in Chondro-Gide and Alpha Chondro Shield. **(A)** Representative images are shown of the appearance of AT MSCs following 30 minutes of incubation with Chondro-Gide and Alpha Chondro Shield. As shown, MSCs had already become firmly attached and spread out (arrowed) on Chondro-Gide, whereas they appeared mostly spherical in morphology and only projected 1 or 2 cell processes to attach to Alpha Chondro Shield. **(B)** Representative images are shown of BM MSCs (left panels) and AT MSCs (right panels) after Live/Dead staining. Scale bars represent 100 μm . **(C)** The number of viable BM and AT MSCs in each cell scaffold over time. BM MSCs proliferated in Chondro-Gide over time. There were more BM MSCs in Chondro-Gide than Alpha Chondro Shield at 14 and 28 days in culture, whereas for AT MSCs this difference was noticeable after 7 days in culture. Data are presented as means \pm standard deviations. Black bars = Chondro-Gide, white bars = Alpha Chondro Shield.

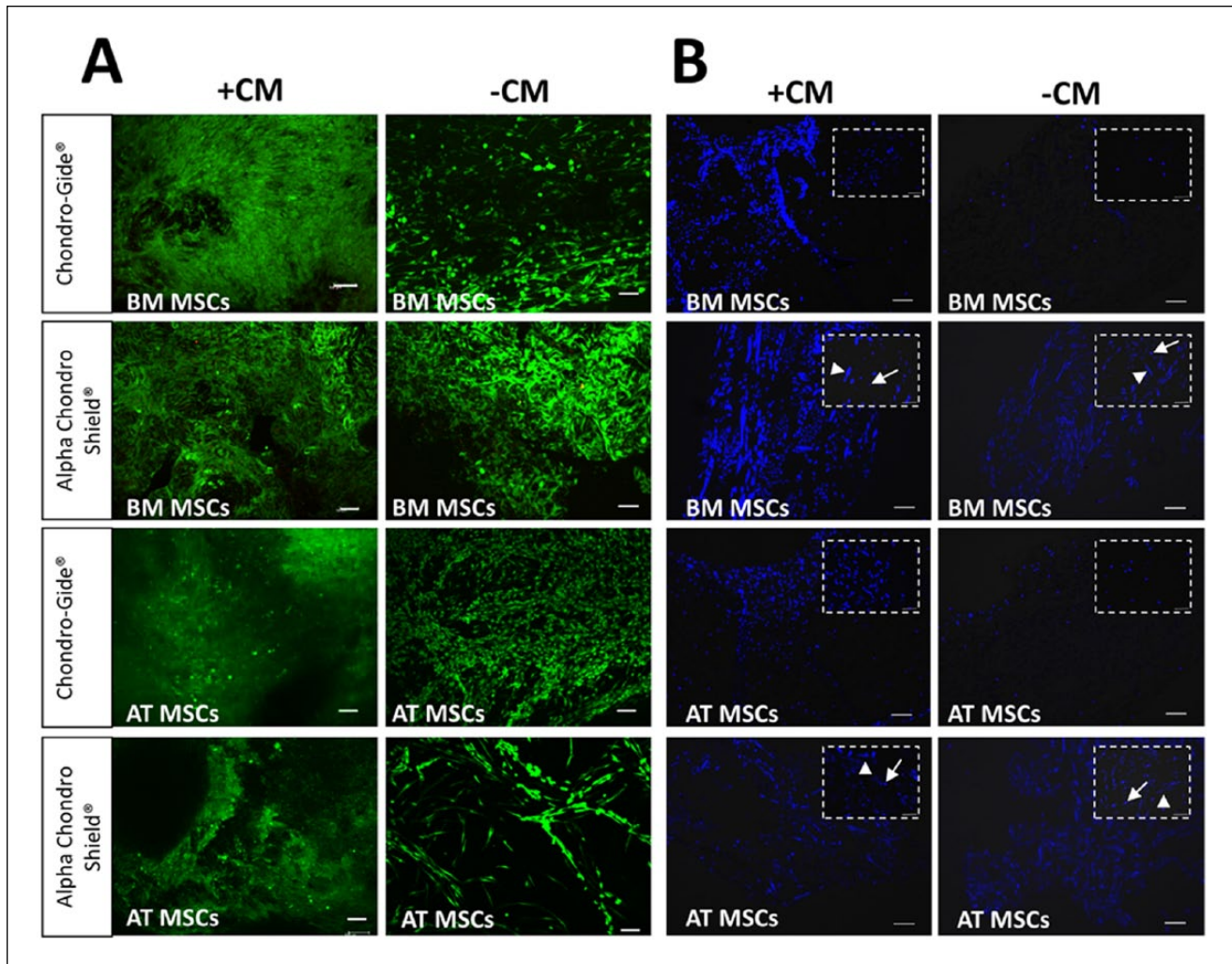


Figure 3. The viability of bone marrow (BM) mesenchymal stem cells (MSCs) and adipose tissue (AT) MSCs in Chondro-Gide and Alpha Chondro Shield under chondrogenic and nonchondrogenic conditions. **(A).** Representative images are shown of BM and AT MSCs at day 28 of culture in the presence (+CM) or absence (-CM, control) of chondrogenic inducers. More than 95% of MSCs were viable in Chondro-Gide and Alpha Chondro Shield under both conditions. Scale bar represents 100 μm, digitized images of projected z stacks following confocal microscopy. **(B).** Representative images showing the distribution of BM MSCs and AT MSCs in Chondro-Gide and Alpha Chondro Shield after 28 days in culture under chondrogenic (+CM) versus control (-CM) conditions. Scale bars = 100 μm for the main images and 50 μm for the inset images. There was an even distribution of cells throughout the porous elements of both scaffolds. MSCs have been arrowed and the autofluorescence of the scaffold fibers in the Alpha Chondro Shield indicated by arrowheads.

used to demonstrate the specificity of the histological and immunohistochemical procedures (Fig. 4C).

Higher Levels of Soluble GAGs Were Detected in Culture Medium under Chondrogenic versus Nonchondrogenic Conditions

The presence of GAGs was analyzed in cell culture supernatants harvested at day 28, that is, from medium that was harvested from the last feed only (a period of 3

days). This biochemical analysis of GAG content showed that both BM and AT MSCs secreted markedly more GAGs in Chondro-Gide than in Alpha Chondro Shield. Under chondrogenic conditions, a greater amount of GAGs was released into the medium by BM and AT MSCs in Chondro-Gide and by AT MSCs in Alpha Chondro Shield compared with control conditions. For BM MSCs in Alpha Chondro Shield, an increase in soluble GAGs was also detected under chondrogenic conditions (Fig. 4D).

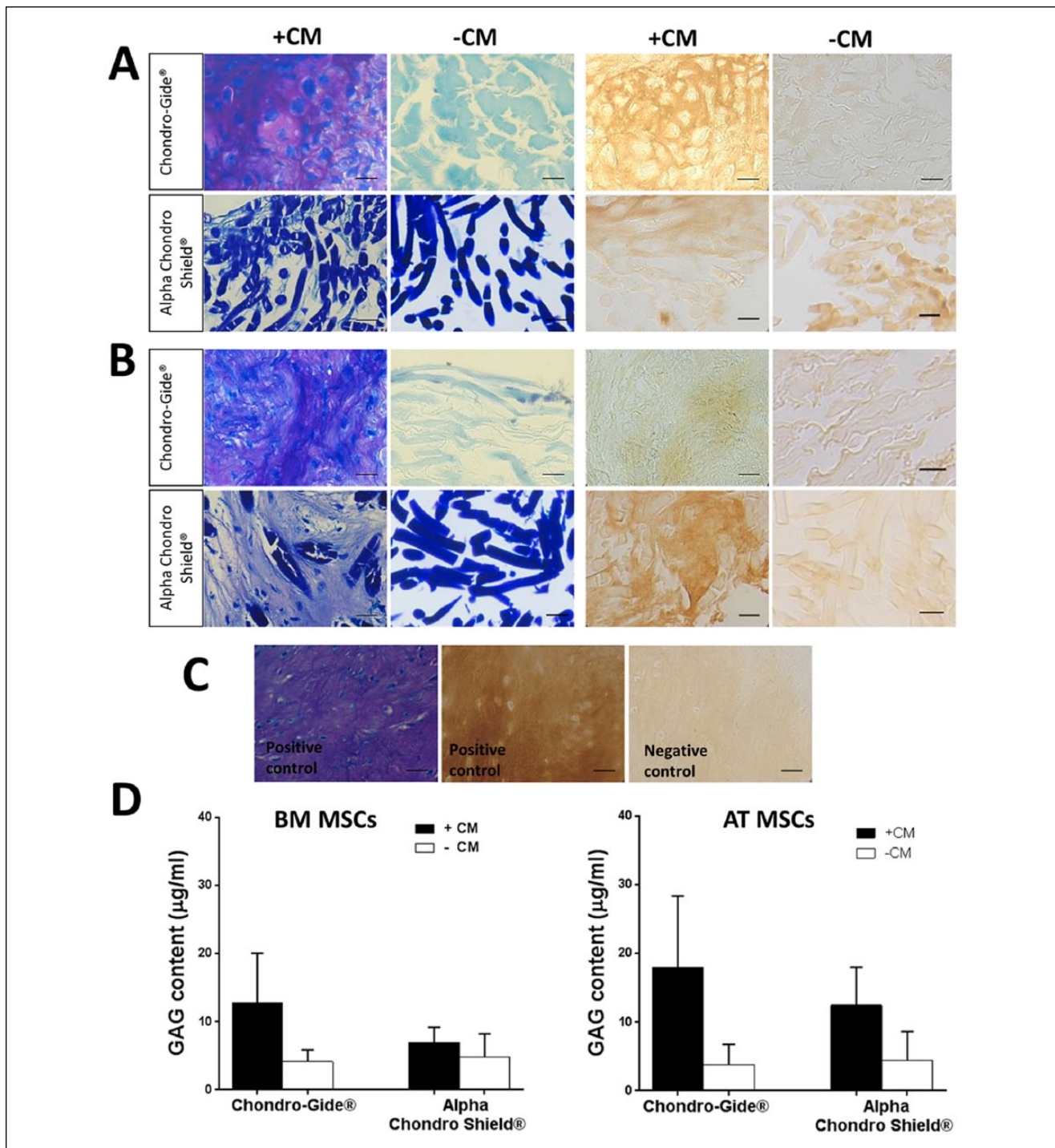


Figure 4. Histology and immunohistochemistry of mesenchymal stem cell (MSC)-seeded Chondro-Gide and Alpha Chondro Shield. (**A** and **B**). There was greater matrix deposition in Chondro-Gide cultures with bone marrow (BM) MSCs (**A**) and adipose tissue (AT) MSCs (**B**) than Alpha Chondro Shield, as shown by increased toluidine blue staining and collagen type II immunolocalization under chondrogenic conditions (+CM) compared with control conditions (-CM). Alpha Chondro Shield seeded with BM and AT MSCs only showed some localized positivity of collagen type II, which was not seen in control conditions. (**C**) A section of human knee cartilage was used as a control for both toluidine blue and collagen type II immunostaining. Scale bars represent 25 µm. (**D**) A greater level of soluble glycosaminoglycan (GAG) was detected in the culture supernatants of Chondro-Gide cultures seeded with BM MSCs and AT MSCs at day 28 under chondrogenic conditions (+CM) compared with control conditions (-CM). Data are presented as means \pm standard deviations.

Discussion

To date, there are limited clinical data available for the use of MSCs in cell-based cartilage repair therapies. One potential reason is the lack of robust *in vitro* data demonstrating their chondrogenic differentiation potential in clinically available scaffolds. This study has suggested that Chondro-Gide provides a more suitable environment than Alpha Chondro Shield for the culture and chondrogenesis of MSCs and the formation of a cartilaginous tissue. MSCs isolated and culture expanded from BM and AT underwent chondrogenic differentiation in response to chondrogenic inducers. This was evident in both scaffolds but was most marked in Chondro-Gide cultured with BM MSCs.

Bone marrow MSCs and AT MSCs were initially more readily incorporated into Chondro-Gide than Alpha Chondro Shield. One reason for this greater incorporation of cells within Chondro-Gide, which is composed of natural type I and type III porcine collagen, could be the ability of MSCs to bind to the scaffold through integrin receptors, specifically $\alpha 2\beta 1$ integrins, which is the major receptor for type I collagen and other fibril-forming collagens.²¹ In contrast, Alpha Chondro Shield is a synthetic scaffold of pure PGA that lacks specific cellular adhesion sites, which may explain how the adhesion of cells to the scaffold was minimal. The morphologies of MSCs attached to Chondro-Gide and Alpha Chondro Shield revealed by scanning electron microscopy at an early time point postseeding would support such an interpretation. The synthetic nature of Alpha Chondro Shield may facilitate its adaptation to increase MSC incorporation, for example, other researchers have used the integrin-binding peptide Arg-Gly-Asp (RGD) into polymer-based scaffolds to facilitate cell adhesion.^{22,23} Within Chondro-Gide, the increased retention and growth of cells is also possibly because of the decreased porosity of the scaffold caused by the presence of a compact surface that functions to prevent cell leakage. In contrast, Alpha Chondro Shield consists of large interconnected pores that aim to encourage cell growth and attachment *in vivo*, where blood clot formation from associated microfractures likely helps retain cells. Previous studies have shown that with time such porous scaffolds in fill with cartilaginous ECM deposition in synchrony with the degradation rate of the scaffold.^{24,25} However, in the current *in vitro* study the increased porosity of Alpha Chondro Shield may have allowed cells to have escaped the scaffold.

The differences in MSC proliferation that were observed between the 2 scaffolds may be attributed to their differing degradation rates. BM MSCs proliferated in Chondro-Gide throughout the time course. Conversely, there was no increase in BM MSC numbers in Alpha Chondro Shield with time in culture. The collagens in Chondro-Gide are slow to degrade compared with PGA fibers in Alpha Chondro Shield, which begins to lose mechanical integrity

over a 12-day period²⁶ and degrades to about 50% of its initial mass by 28 days.²⁵ In the absence of matrix production or a blood clot (generated *in vivo*), it is likely that the fast degrading Alpha Chondro Shield does not provide a suitable environment for cells to grow and proliferate and, therefore, this results in cell loss. In addition, while PGA-based scaffolds provide a good substrate for chondrocyte adhesion, cell proliferation during long-term cultures may be significantly affected by acidic products during scaffold degradation.²⁷

Overall, cell retention in both scaffolds was poor, with only approximately 0.1% to 1.5% of the MSCs attaching to the scaffolds following a 2-hour incubation period. The effectiveness of the cell-seeding process is a crucial step, which could have a significant effect on the number of cells delivered to a cartilage lesion and thus the clinical outcome of any cell therapy. For MACI procedures, chondrocytes preseeded onto Chondro-Gide have been grown for 3 days prior to implantation,²⁸ whereas ACI procedures have been adapted to preseed Chondro-Gide with chondrocytes for a recommended time of only 10 to 15 minutes prior to transplant.²⁹ Studies have previously examined the use of spinner flasks to encourage more efficient cell seeding in porous scaffolds²⁶ or of using polymerizing gels as a delivery vehicle for rapid cell seeding within collagen sponges.³⁰ However, in this study, a simple cell-seeding strategy was used to replicate the clinical setting, with the results probably representing the best case scenario given an incubation period that is in excess (2 hours) of what would be clinically acceptable. The small size of the scaffolds used in this study may have contributed to low incorporated cell numbers as they could have been of insufficient size to initially retain the total volume of medium used for cell seeding. Hence, cells may have initially leaked out of the scaffolds into the wells. Although a potential weakness of the study, this scenario commonly reflects the clinical situation. Of the two cell sources, there was better incorporation of AT MSCs into both scaffolds compared with BM MSCs. If AT MSCs are shown to incorporate into cell scaffolds more readily than BM MSCs, following analysis of increased numbers of MSC donors, there may be reasons for such differential incorporation. A recent review suggested that AT MSCs express greater levels of integrin $\alpha 4\beta 1$ (CD49d) compared with BM MSCs.³¹ The $\alpha 4\beta 1$ integrins have long been known to play a role in cell-cell and cell-matrix interactions³² and one of the ligands for CD49d is fibronectin,³³ which is present in serum-containing medium.³⁴ Hence, increased retention of AT MSCs compared with BM MSCs within Chondro-Gide may be due, in part, to the adsorption of serum proteins especially fibronectin³⁵ to the scaffolds via interaction with $\alpha 4\beta 1$ integrins. Further research is required to examine more MSCs donors from both tissue sources to ensure the reproducibility of our observations and to examine mechanisms of increasing the efficiency of

cell seeding into these scaffolds and their optimal application in cell transplantations for cartilage repair.

It is known from previous studies that scaffold structure and properties like porosity, pore size, fiber thickness, topography, and scaffold stiffness directly influence cell behavior and colonization (reviewed by Lawrence and Madhally³⁶). The cells in the Chondro-Gide scaffold attained a fibroblast-like morphology and showed progressive penetration throughout the collagen network. This has also been shown by Nuernberger *et al.*³⁷, where the dense network of collagen fibers supported a flattened cell shape of chondrocytes. In the more fibrous Alpha Chondro Shield, the cells showed a mixed morphology of both round and elongated cells. These differences in cell morphology in similar types of cell scaffolds have been observed by Schlegel *et al.*³⁸ in 2008 using chondrocytes. The chondrocytes developed a fibroblast like morphology when seeded onto a type I/type III collagen scaffold, whereas the chondrocytes showed a mix of round and elongated morphologies on a scaffold composed of hyaluronic acid.³⁸ Adoption of a spherical morphology by MSCs and chondrocytes in 3-dimensional culture can influence their synthesis of cartilage-specific ECM components,³⁹⁻⁴¹ although it is unclear if cell shape is a critical factor in influencing chondrocyte differentiation.⁴² In this study, the presence of round-shaped MSCs in Alpha Chondro Shield was not associated with increased chondrogenic differentiation compared with less rounded cell morphologies seen in Chondro-Gide, which proved to be a superior scaffold for chondrogenic differentiation.

We observed increased collagen type II and GAG deposition in the ECM of BM MSC-seeded Chondro-Gide cultures compared with AT MSC-seeded Chondro-Gide cultures. This indicates that BM may be a better source of MSCs for cartilage repair than AT. However, a potential weakness of this study was that the AT MSCs were derived from infrapatellar fat pad, which, although of use as an intraoperative cell source in the treatment of cartilage defects may be an inferior donor tissue, because cells from the damaged or diseased articular environment can possess pro-inflammatory characteristics.⁴³ Ideally, AT MSCs from peripheral fat sources and BM obtained from the same donor should have been compared to better establish the potential of these cells for chondrogenesis in autologous cell therapy for cartilage repair. In addition, TGF- β 1 was used as an inducer for chondrogenic differentiation of MSCs from both cell sources, which may not be ideal for inducing chondrogenic differentiation in AT MSCs. For example, some studies have previously reported that TGF- β 1 is not as efficient at inducing chondrogenic differentiation of AT MSCs as bone morphogenetic protein 6.^{44,45} Moreover, the faster degradation rate of Alpha Chondro Shield makes it difficult to undertake long-term cultures in order to compare chondrogenic differentiation of BM MSCs

and AT MSCs. Future *in vitro* studies to examine chondrogenesis with this scaffold should focus on improving culture conditions for longer term analysis, as well as using advanced and effective cell-seeding procedures.

In conclusion, this study has demonstrated that BM MSCs and AT MSCs undergo chondrogenic differentiation *in vitro* in cell scaffolds that have been used clinically for cartilage repair. On the basis of cell growth and ECM deposition, the use of BM MSCs with Chondro-Gide is favored. However, further study is required to test the potential of these different cell types and scaffolds for cartilage repair *in vivo*.

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Authors' Note

All the work presented was conducted at the School of Life and Health Sciences at Aston University and the Department of Musculoskeletal Pathology, Royal Orthopaedic Hospital, Birmingham, UK.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Ethical approval was obtained from the national review body (12/EE/0136 and 06/Q2601/9).

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