Characterisation of Regional Human Meniscal Progenitor Cells

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Research Article

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Characterisation of regional human meniscal progenitor cells

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Abstract

Background

The surgical treatment of meniscus injury has represented a clinical challenge for decades. Stimulating meniscus regeneration using transplanted meniscal progenitor cells has been suggested as a promising new strategy. However, there is a lack of studies which decisively identify and characterise progenitor cell populations in human meniscus tissues.

Methods

In this study, donor-matched progenitor cells were isolated via selective fibronectin adhesion from the avascular (PAvas) and vascular (PVas) regions of the meniscus and chondroprogenitors (PChs) from articular cartilage (n=5 donors). In addition, whole mixed populations of cells (MAvas, MVas, MChs) from the same regions were obtained by standard isolation techniques for comparison. The colony formation efficacy of PAvas, PVas and PChs was monitored using Cell-IQ® live cell imaging. Proliferation rates of progenitors were compared with their mixed population counterparts. Cell surface markers indicative of mesenchymal stromal cells (MSCs) profile and progenitor markers were characterised by flow cytometry in all populations. The chondrogenic capacity was assessed via pellet culture assays and measuring chondrogenic gene expression levels, GAG/DNA content and morphology.

Results

All meniscal progenitor and chondroprogenitor populations showed colony forming capacity in monolayer culture, whereas mixed populations were distributed randomly at passage 0. PVas had significantly lower population doubling times compare to MVas and proliferated faster than PAvas and PChs based on colony forming efficacy. Progenitor populations

showed significantly higher positivity for CD49b and CD49c compared to their mixed population counterparts and PChs had a higher positivity level of CD166 compared to mixed chondrocytes. Collagen types II and X expression was significantly downregulated in pellets formed by progenitor populations. GAG/DNA analysis demonstrated that progenitor cells generally produced more GAG than mixed populations.

Conclusions

Our study demonstrates that the human meniscus contains meniscal progenitor populations in both the avascular and vascular regions. Meniscal progenitors derived from the vascular region exhibit enhanced proliferative and chondrogenic characteristics compared to those from the avascular region; this may associate with the enhanced meniscal healing potential in the vascular region. These findings build on the body of evidence which suggests that meniscal progenitors represent an attractive cell therapy strategy for meniscal regeneration.

Keywords: Progenitor; Meniscus; Cartilage; Fibronectin

Introduction

Menisci play a key role in joint congruence, dispersing load and protecting the articular cartilage surface of the femur and tibia [1]. Although treatment of meniscal tears has developed dramatically with such treatments as meniscus repair and replacement strategies, these surgical interventions provide limited protection against the progression of osteoarthritis (OA) [2,3]. Therefore, there is a demand to develop other novel treatments to improve meniscus repair and prevent or delay the onset and progression of OA.

The meniscus is composed of an outer "vascularised" zone that contains elongated fibroblast-like cells and an inner "avascularised" zone that contains rounded fibrochondrocytes. It has long been known that in the vascular region, tears of the meniscus tend to successfully repair themselves after a surgical procedure, whereas the inner avascular region has a low healing potential [4]. Mobilisation and homing of endogenous progenitor cells from the vascular zone of the meniscus may be responsible for some of the natural healing noted in the tissue following injury [5]. Indeed the enhanced regeneration of the vascular region of the meniscus might be due to the presence of CD34 and CD166 immunopositive progenitor cells visible via histological analysis in the blood vessels [6]. Further studies suggest the presence of progenitor cells in the meniscus promote repair of injured menisci in bovine, rabbit and mouse models [7–9]. Our previous work has demonstrated that there are fewer blood vessels carried by "tree-like" collagen fibres in the vascular zone of more degenerative menisci than in more healthy menisci [10]. These results suggest that there is a subpopulation of progenitor-like cells in the vascular region, but there is a lack of studies that decisively identify and characterise progenitor cell populations in different regions of the human meniscus .

Fibronectin-coated flasks have been commonly used to extract chondroprogenitors from articular cartilage and these progenitors have been investigated for their capability in terms of

cartilage regeneration [11]. Chondroprogenitors are an ideal candidate for cell-based tissue engineering cartilage repair strategies because they are known as a relatively undifferentiated population of chondrocyte precursors, which are less likely to become hypertrophic and terminally differentiate sooner than their counterpart mature chondrocytes [12]. In this study, an established chondroprogenitor isolation protocol [11] was used to obtain and characterise the progenitors and whole mixed populations from donor-matched avascular and vascular regions of the meniscus, as well as the two cellular fractions from articular cartilage from the same individual.

Methods

Patient Information

The provision of written informed consent was obtained from each patient prior to surgery. Favourable ethical approval was given by the National Research Ethics Service (NRES number 11/NW/0875) and all experiments were performed following relevant guidelines and regulations. Human meniscus (five patients) and cartilage (four patients) tissue samples were harvested from four patients undergoing total knee replacement (TKR) and one patient undergoing above-knee amputation (Table 1). The general workflow is shown in Figure 1.

Table 1. Patient Demographics.

Patient	Age	Sex	Procedure	Tissue type	Additional Clinical Information
1	37	male	TKR	Lateral meniscus; cartilage from LFC	Previous HTO, medial artificial meniscus and meniscus allograft transplant. Has received multiple knee wash outs and removal of osteophytes.
2	73	male	TKR	Lateral meniscus	Advanced OA with bone-on-bone in the medial compartment.
3	60	female	TKR	Lateral meniscus; cartilage from LFC	Indication of OA from imaging assessments.
4	65	male	TKR	Lateral meniscus; cartilage from LFC	Bone-on-bone medial compartment OA, presence of significant osteophytes.
5	59	female	AKA	Lateral meniscus; cartilage from LFC	Previous traffic accident, osteoporosis due to immobility.

TKR: total knee replacement; AKA: above knee amputation; OA: osteoarthritis; HTO: high tibial osteotomy; LFC: lateral femoral condyle.

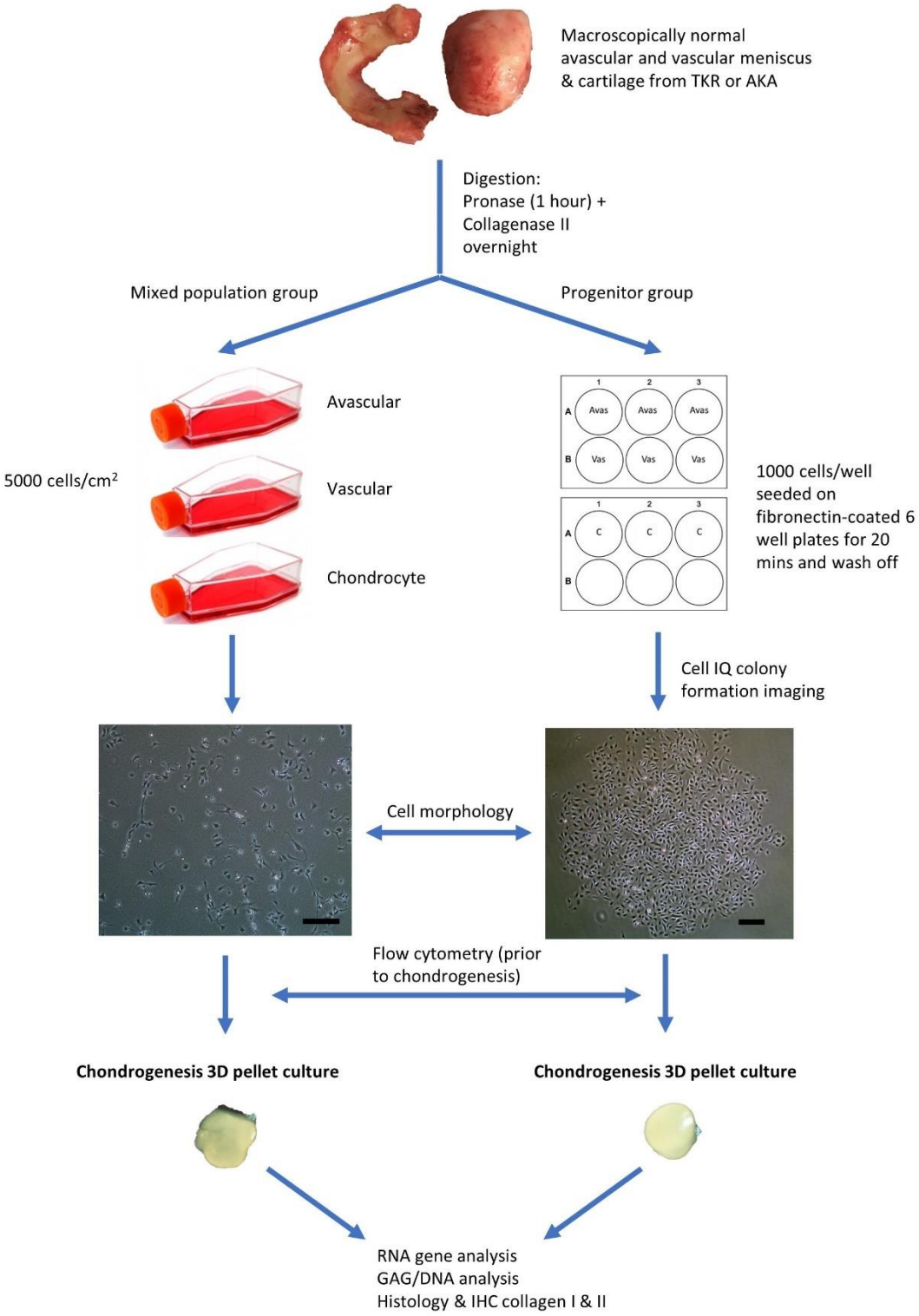

Figure 1: Flow Diagram Schematic of the Experimental Plan. Avas: Avascular meniscal cells, Vas: Vascular meniscal cells, C: Chondrocytes, TKR: total knee replacement, AKA: above-knee amputation, IHC: immunohistochemistry. Scale bars represent 250 μm.

Progenitor Cell Isolation

The meniscus was dissected longitudinally into three parts: the inner avascular zone, the middle and the outer vascular zone. The middle portion was discarded and only the extreme inner and outer avascular and vascular zones were used to derive meniscal cells in order to ensure distinct regions. Additionally, full-depth macroscopically normal human articular cartilage from femoral condyles was used to isolate chondrocytes and their progenitors. Samples were digested by sequential pronase (70U/ml, 1 hour at 37°C) and collagenase type II (245U/ml, 12 hours at 37°C; Worthington, USA) in Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1) (Gibco, USA), 1% penicillin–streptomycin (P/S) (ThermoFisher Scientific, USA) and 1% Insulin-Transferrin-Selenium-X (ThermoFisher Scientific, USA). Cells extracted from the three tissue types (avascular & vascular menisci and articular cartilage) were cultured under two conditions. The first was for the isolation and growth of a mixed population of cells (avascular meniscal mixed cells (MAvas), vascular meniscal mixed cells (MVas) and mixed chondrocytes (MChs)), which were each plated at a density of 5000 cells/cm^2. The second was for the isolation and growth of progenitor cells (avascular meniscal progenitors (PAvas), vascular meniscal progenitors (PVas) and chondroprogenitors (PChs)), which were subjected to a fibronectin selective adhesion as previously described [11]. Briefly, 6 well plates were coated with 10µg/ml fibronectin (Sigma-Aldrich, UK) in phosphate buffered saline (PBS), containing 1mM $MgCl_2$ and 1mM $CaCl_2$ overnight at 4°C. Isolated mixed populations from each tissue fraction (1000 cells/well) were seeded onto the coated wells in triplicate for 20 mins at 37°C in DMEM/F12 medium, 10% Foetal Bovine Serum (FBS) (Gibco, USA), 1% P/S and 0.1 mM ascorbic acid (Sigma-Aldrich, UK) (culture medium). After 20 minutes, medium and non-adherent cells were removed and fresh culture medium was added to the remaining adherent cells and incubated at 37°C, 5% CO_2.

Cell-IQ® Live Cell Imaging

After three days of culture in a 6 well plate, progenitor cells were imaged using a Cell-IQ® phase contrast Live Imaging Platform (CM Technologies, Tampere, Finland) to monitor colony formation. Spare wells and surrounding areas in 6 well plate were filled with distilled water to keep the plate humidified. At least one colony was monitored in each well and each colony was imaged every 30 minutes for 48 hours. The recorded images were analysed using the Cell-IQ® Analyser (CM Technologies, Tampere, Finland) software in order to measure the live cell number in each colony every 30 minutes.

Growth Kinetics

The number of monoclonal colonies (defined as a cluster of more than 32 cells which represents a population of cells derived from more than 5 population doublings of a single cell) in each well was counted under light microscopy after 5 days of culture, which was considered as the initial number of progenitors that had adhered to the plate [11]. Each type of progenitor population was then trypsinised and cultured in culture medium supplemented with 1ng/ml transforming growth factor beta 1 (TGF-β1) (PeproTech, UK), 5ng/ml fibroblast growth factor 2 (FGF-2) (PeproTech, UK). Population doubling times (PDTs) were calculated using the formula: $PDT = (t_2-t_1) \times \ln(2)/\ln(n_2/n_1)$, where t_1= the time of cell seeding, t_2 = the time of cell harvest and n = the cell population at the matching time points. PDTs at passages 0 to 2 and passage 2 to 3 were compared between mixed populations and progenitor cells, as progenitors could not be counted with sufficient accuracy at P0-1.

Flow Cytometry

Prior to chondrogenic differentiation, 120,000 avascular meniscal cells, vascular meniscal cells and chondrocytes from both mixed population and progenitor groups were resuspended in a PBS buffer consisting of 2% (w/v) bovine serum albumin (BSA; Sigma-Aldrich). Non-

specific antibody binding was blocked using a PBS buffer composed of 10% (v/v) human immunoglobulin (Grifols, Spain) at 4°C for 1 hour. Immunopositivity for 9 surface molecules which are indicative of mesenchymal stromal cell (MSC) the International Society for Cellular Therapy (ISCT) profile (CD14, CD73, CD90, CD105), progenitor markers (CD44 CD166) or cell adhesion molecules (CD29, CD49b, CD49c) were evaluated in all five donors.

Chondrogenic Differentiation Assay

The chondrogenic potential of the three donor-matched populations in both mixed populations and progenitor groups was assessed at passage 2 using a well-established 3D pellet culture protocol [13] in 5 donors (patient 2 only had matched avascular and vascular meniscal cells). Briefly, $2.5x10^5$ cells were centrifuged into a cell pellet with DMEM/F12, 1% P/S, 1% insulin transferrin selenium (Sigma-Aldrich, UK), 1 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, UK), 10 nM dexamethasone, 1mM sodium pyruvate (Sigma-Aldrich, UK) and 10 ng/ml TGF-β1 (PeproTech, UK). After 28 days in culture, three pellets from each donor and cell type were used for (i) biochemical quantitation of GAG/DNA, (ii) gene expression analysis and (iii) snap frozen in liquid nitrogen-cooled hexane and stored at -80°C until histological analysis.

Pellet Gene Expression Analysis Using Real-time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

After 28 days of chondrogenic differentiation, the mRNA of 3 pellets from each cell type was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed by a High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Warrington UK). RT-qPCR was used to assess expression levels of collagen type I (COL1A2), collagen type II (COL2A1), aggrecan (ACAN) and SOX-9, which are the markers indicative of chondrogenic potency. The

expression level of collagen type X (COL10A1) was also assessed, as a marker of hypertrophic chondrocytes. RT-qPCR was performed on the Quant Studio 3 Real-Time Quantitative PCR System (Applied Biosystems) with SYBR Green Reaction Mix. Following normalisation to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Qiagen, QuantiTect Primer Assay), the expression levels for genes of interest in progenitor cell pellets was expressed as a ratio compared with their donor matched mixed population pellets, using the comparative threshold method. A 2-fold up- or downregulated change was considered biologically significant.

GAG/DNA Analysis

After 28 days in culture, 3 pellets of each cell type were digested in papain to release GAG and DNA. The papain digestion buffer (125 µg/ml, pH 6.0) was composed of 50mM sodium phosphate (BDH), 20 mM EDTA (Sigma-Aldrich), 20 mM N-acetyl cysteine (BDH), papain (Sigma-Aldrich). Each pellet was digested in 200µl of the papain digestion buffer at 60°C for 3 hours, after which all samples were centrifuged at 1000g for 5 minutes and supernatants were stored at -20°C prior to use. The glycosaminoglycans (GAG) in pellets were assessed by 1,9-dimethylmethylene blue (DMMB) assay [14]. Briefly, 50μl of each sample and 200μl of DMMB solution were added in triplicate to a 96 well plate. The results were read immediately at A530nm and A590nm using a FluorStar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The bovine chondroitin sulphate (C9819, Sigma-Aldrich) was used to construct a standard curve, which was plotted using the following equation: (A530nm/A590nm)- (A530nm blank/A590nm bank). The total GAG content of each pellet was calculated from the standard curve. The DNA content was measured spectrofluorometrically using a PicoGreen dsDNA Assay kit (Invitrogen) according to the manufacturer's instructions. Finally, the GAG content was normalised to the corresponding DNA content per pellet.

Sectioning and Immunohistochemical Staining of Chondrogenic Pellets

Three pellets from each cell population were cryosectioned at 7μm thickness. Cryosections were immunohistochemically stained for collagen types I and II. In brief, sections were pre-treated with hyaluronidase (4800U/ml, Sigma, UK) for 2 hours and fixed in 10% formalin for 10 minutes. Sections were then washed with PBS and incubated with primary mouse collagen type I antibody (1:500, clone I-8H5, MP Biomedicals, Cambridge, UK) and collagen type II antibody (1:50, clone CIIC1, DHSB, University of Iowa, USA) in PBS for 1 hour. Negative control sections were incubated with nonspecific, isotype matched antibodies (for collagen type I: IgG2a; for collagen type II: IgG1, Dako, Denmark) instead of primary antibodies at the same concentration. Sections were then washed in PBS before incubation with the secondary biotinylated antibody at 50 µg/mL (goat anti-mouse, VECTASTAIN ABC kit, Vector Laboratories, Peterborough, UK) for 30 minutes. Hydrogen peroxide (0.3% (v/v)) in methanol (BDH) was used to eliminate endogenous peroxidase activity. Labelling with streptavidin-peroxidase was enhanced with incubation of an avidin-biotin complex (VECTASTAIN Elite ABC kit, Vector Laboratories, UK) for 30 minutes according to the manufacturer's instructions. After washing with PBS, sections were visualised with diaminobenzidine (DAB, ImmPACT, Vector Laboratories, Peterborough, UK) and then dehydrated before mounting under glass coverslips with Pertex mounting medium. The immunohistochemistry staining intensity of collagen type I and type II was semi-quantified by a previously established protocol using ImageJ Fiji Software (version 1.2; WS Rasband, National Institute of Health, Bethesda, MD) [15].

Statistical analyses

GraphPad Prism (Version 8.30, San Diego, California, USA) was used for statistical analysis. Two-way ANOVA with a multiple comparisons test was used to analyse flow cytometry,

population doubling time, pellet RNA gene analysis, pellet GAG/DNA assay and pellet collagen staining intensity. Data were presented as mean ± standard deviation (SD) in the graphs and text.

Results

Growth Kinetics and Cell Morphology in Mixed Population and Progenitor cells

Figure 2 shows the representative mixed and progenitor cell population morphologies. At passage 0 (Figure 2A), mixed population cells were distributed randomly on tissue culture plastic, whereas progenitor cells were organised into round tightly packed colonies. PAvas and PChs had a similar oval chondrocyte-like morphology whereas PVas were more fibroblast-like and spindle-shaped in appearance. At passage 2 (Figure 2B), the majority of the mixed population cells possessed extensive cytoplasmic processes of varying length [16],but these were much less common in progenitor cells.

Mixed populations demonstrated a slower growth rate in monolayer culture compared to progenitor cells at both passages 0 to 2 and 2 to 3. The mean PDT of MAvas, MVas and MChs at P0-2 were 15.46 ± 13.05 days, 35.40 ± 36.62 days and 9.47 ± 3.21 days respectively compared with 1.27 ±0.14 days, 1.25 ± 0.11 days and 1.33 ± 0.20 days for PAvas, PVas and PChs, respectively. A smaller difference in PDT was observed between mixed and progenitor cells at P2-3 with MAvas, MVas and MChs having a PDT of 15.45 ±13.16 days, 7.18 ± 3.20 days, 9.39 ± 1.16 days respectively and 2.91 ± 0.66 days, 3.25 ± 0.57 days and 3.19 ± 0.77 days for PAvas, PVas and PChs, respectively. However, the only statistically significant difference was found between MVas and PVas at P0-2 (P=0.011) (n=5 for MAvas, MVas, PAvas, PVas; n=4 for MChs, PChs) (Figure 3).

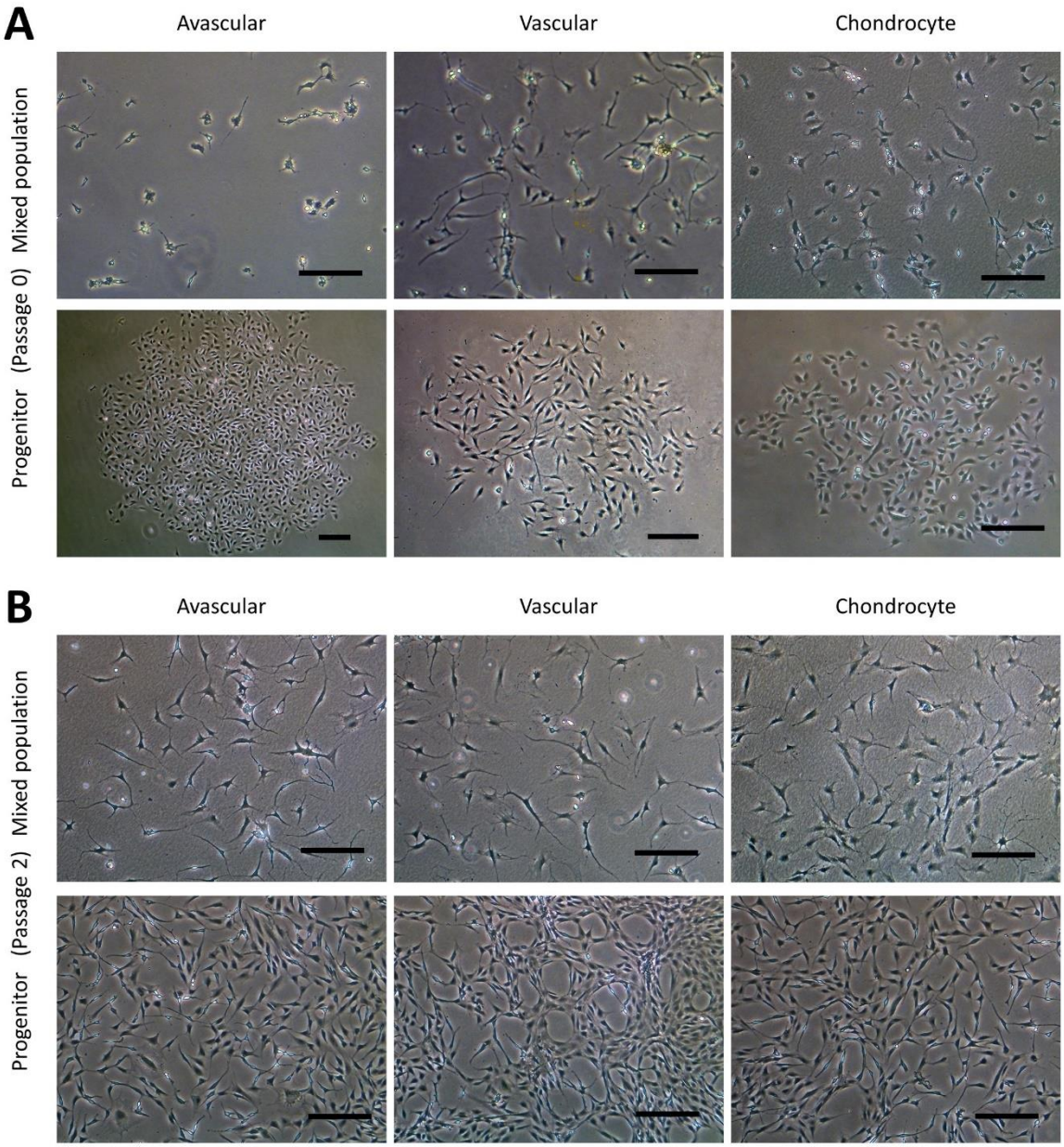

Figure 2: Comparison of progenitor cells morphologies isolated from meniscal and cartilage tissues in monolayer culture at passage 0 and 2. Representative images from a single donor for mixed population and progenitor cells at passage 0 (A) and passage 2 (B) including avascular and vascular meniscal cells and chondrocytes (left to right). Scale bars represent 250 μm.

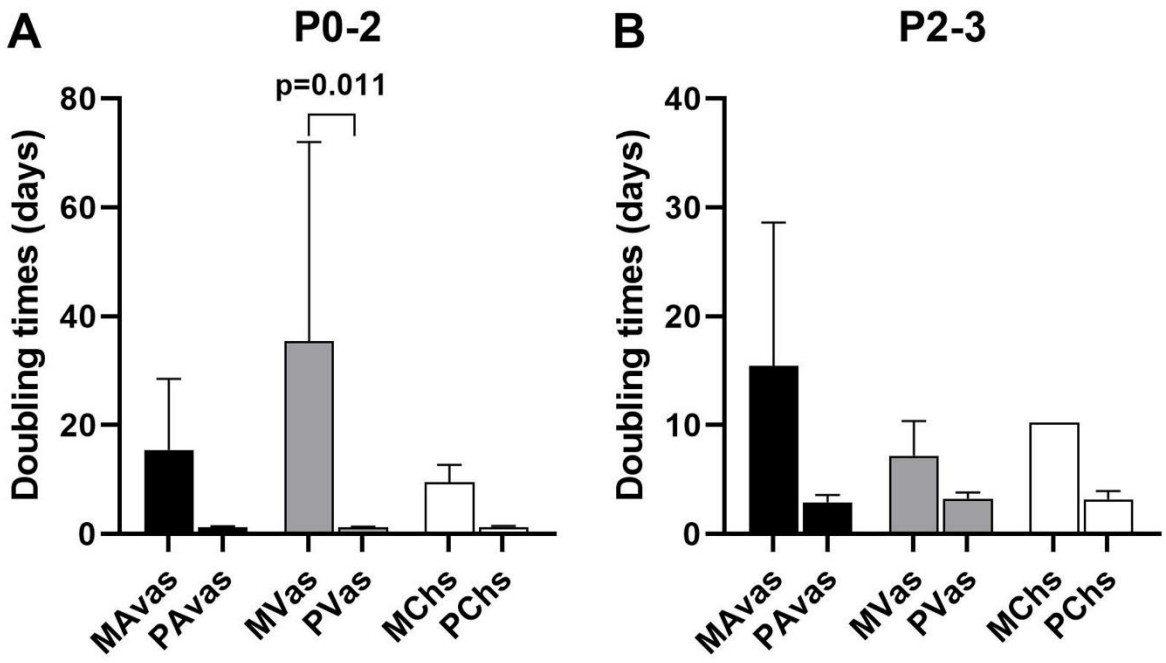

Figure 3: Population doubling time (PDT) data for progenitor and mixed cell populations**.** MAvas: mixed avascular meniscal cells, MVas: mixed vascular meniscal cells, MChs: mixed chondrocytes. PAvas: progenitor avascular meniscal cells, PVas: progenitor vascular meniscal cells, PChs: progenitor chondrocytes. Data are presented as mean ± standard deviation.

Cell-IQ® Live Cell Imaging Analysis

Four of five patients' progenitor colonies of PAvas, PVas and PChs were monitored for 48 hours in the Cell-IQ® live cell imager. For each progenitor cell type from each patient, two or three colonies were selected for colony proliferation rate analysis. Figure 4 showed the results of individual colony proliferation data. Colonies with less than 32 cells beyond 48 hours in culture were not considered to be progenitor colonies and so were excluded; these comprised three of 12 colonies for PAvas, four of 11 colonies for PVas and three of 10 colonies for PChs which were characterised as non-progenitor colonies. After excluding these non-progenitor colonies (10 of 33 colonies, 30.3%), proliferation data from progenitor colonies only was compared for PAvas, PVas, PChs fractions (Figure 5). The comparison analyses

(Table 2) showed that PVas colonies proliferated significantly faster than PAvas (P=0.0022) and PChs (P<0.0001), whilst the PChs colony proliferation rate was significantly slower than the rate of proliferation for PAvas colonies (P=0.0026).

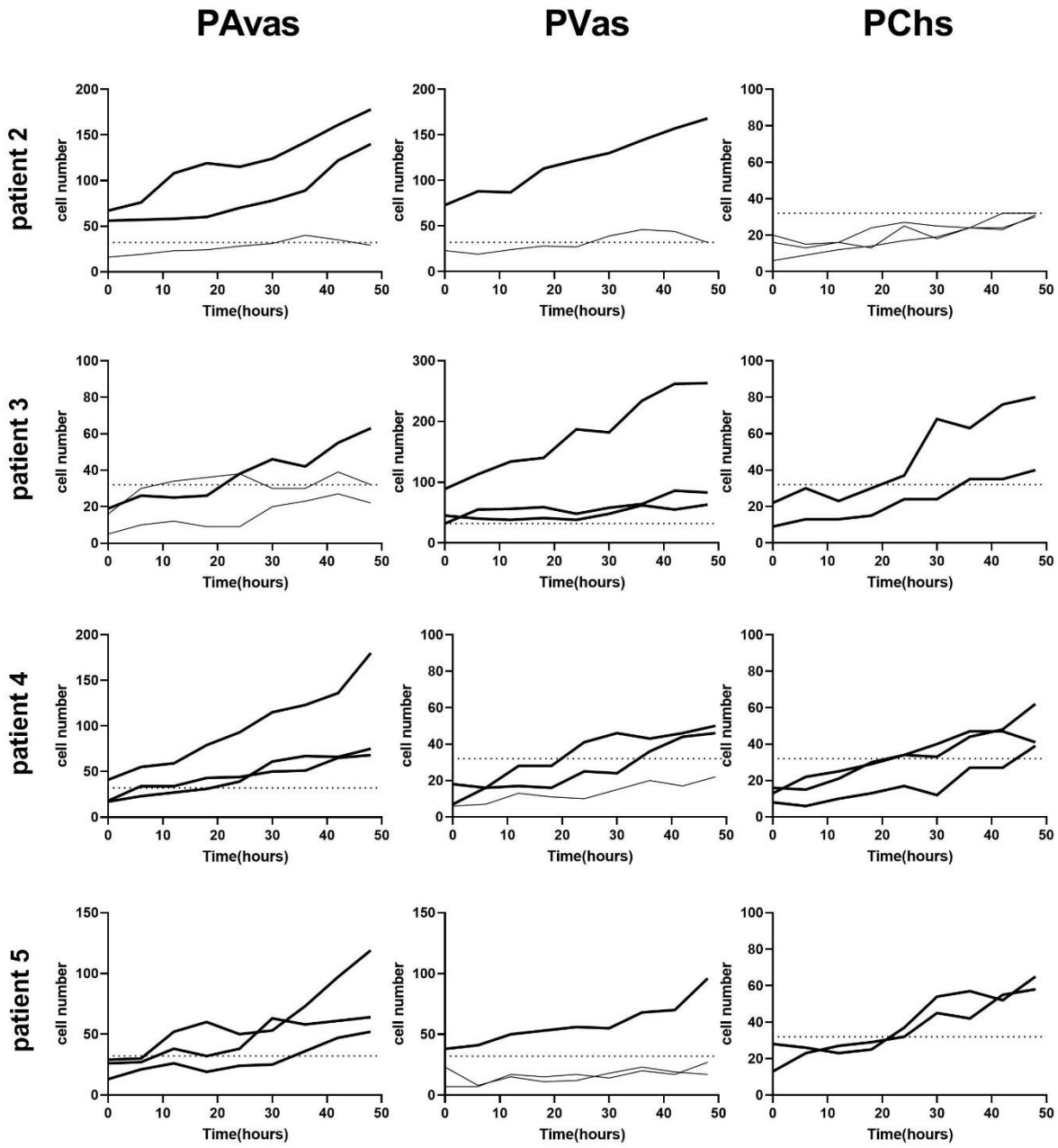

Figure 4: Diagram of individual colony proliferation rates over 48 hours in the Cell-IQ®. Progenitor colonies (cell numbers beyond 32) are indicated in bold. The dashed lines represent the threshold of the minimum cell number considered to be a progenitor colony

(n=32). PAvas: progenitor avascular meniscal cells, PVas: progenitor vascular meniscal cells, PChs: progenitor chondrocytes

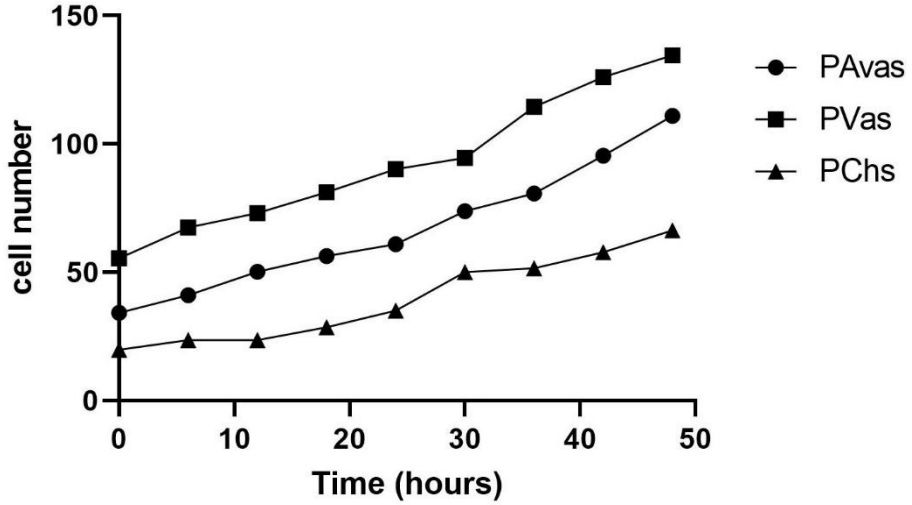

Figure 5: The comparison of colony proliferation rate (colony cell > 32 cells) for avascular (PAvas) (n=7), vascular meniscal progenitors (PVas) (n=7) and chondroprogenitors (PChs) (n=6). Date showed the mean cell number of progenitor colonies in every 6 hours.

Table 2: Multiple comparison tests for proliferation rates of progenitor colonies.

	Predict means, 95% CI	P Value
PAvas vs. PVas	(67.00, 92.98), (-43.83 to -8.127)	0.0022
PAvas vs. PChs	(67.00, 39.53), (8.298 to 46.65)	0.0026
PVas vs. PChs	(92.98, 39.53), (32.45 to 74.45)	<0.0001

PAvas: progenitor avascular meniscal cells, PVas: progenitor vascular meniscal cells, PChs: progenitor chondrocytes, CI: confidence interval.

Cell Surface Marker Immunoprofiles

Flow cytometry analyses (Figure 6) revealed that all cell populations were over 95% immunopositive for the ISCT MSC markers CD73, CD90 and CD105, as well as other matrix adhesion markers (CD29 and CD44). CD14 was present on all cell populations, ranging on

average from 5.18% to 10.75% positivity. There was no significant difference noted for any of these cell surface markers when comparing mixed populations and progenitor cells. However, differences between cell types for integrin markers CD49b, CD49c and the chondrogenic potency marker CD166 were noted. Progenitor cells showed significantly higher positivity for CD49b compared to their counterpart mixed avascular meniscal fractions (P<0.0001), vascular meniscal cells (P<0.0001) and chondrocytes (P<0.0001). Interestingly, both MAvas and PAvas had significant greater positivity for CD49b compared to MVas (P=0.0002) and PVas (P=0.0035), respectively. Progenitor cells also showed a significantly increased level of CD49c when compared to their paired mixed populations in avascular meniscal (P=0.0387), vascular meniscal (P<0.0001) and chondrocyte fractions (P=0.0035). MAvas also showed significantly higher positivity for CD49c compared to MVas, whereas no difference was noted in progenitor cell types. For CD166, MChs had a significantly lower positivity compare to MAvas (P=0.0012), MVas (P=0.0144) and PChs (P=0.0019).

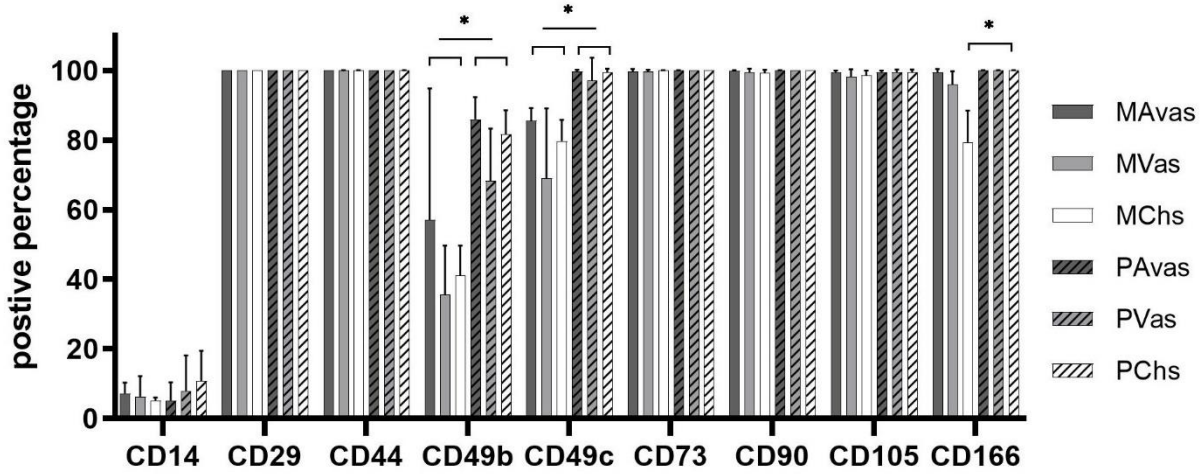

Figure 6: Meniscus and cartilage derived mixed cell and progenitor immunoprofiles

Histograms showing the percentage of cells with immunopositivity for cell surface markers on donor matched mixed or progenitor avascular and vascular meniscal cells and chondrocytes at passage 2-3. Data were presented as mean ± standard deviation (n=5).

Chondrogenic Gene Expression in Cell Pellets

Figure 7 shows relative fold change gene expression levels from pellets of progenitor cells compared to those from mixed populations after 28 days in culture. RT-qPCR analysis of *SOX9* was found to be downregulated in PAvas, PVas and PChs in all five patients compared with mixed population cells, apart from PVas in patient 5. Aggrecan was significantly downregulated in PAvas fractions from patients 1, 4, 5 and in PChs from patient 3, 4, 5. Expression of *COL1A2* was significantly upregulated in PChs from patient 1 and 5 but downregulated in PVas from patient 4 relative to the mixed population cells. *COL2A1* in PVas was significantly downregulated in patient 1 (mean fold change 50 ± 41), patient 4 (mean fold change 5288 ± 3166) and upregulated in patient 2 (mean fold change 7 ± 2) and patient 3 (mean fold change 14 ± 10) compared with MVas. Both PAvas and PChs were significantly downregulated in patient 3-5 compared with MAvas and MChs. Expression of *COL10A1* was found to be downregulated in all three progenitor populations from all five patients relative to their mixed populations, apart from PAvas and PVas which were slightly upregulated in patient 5.

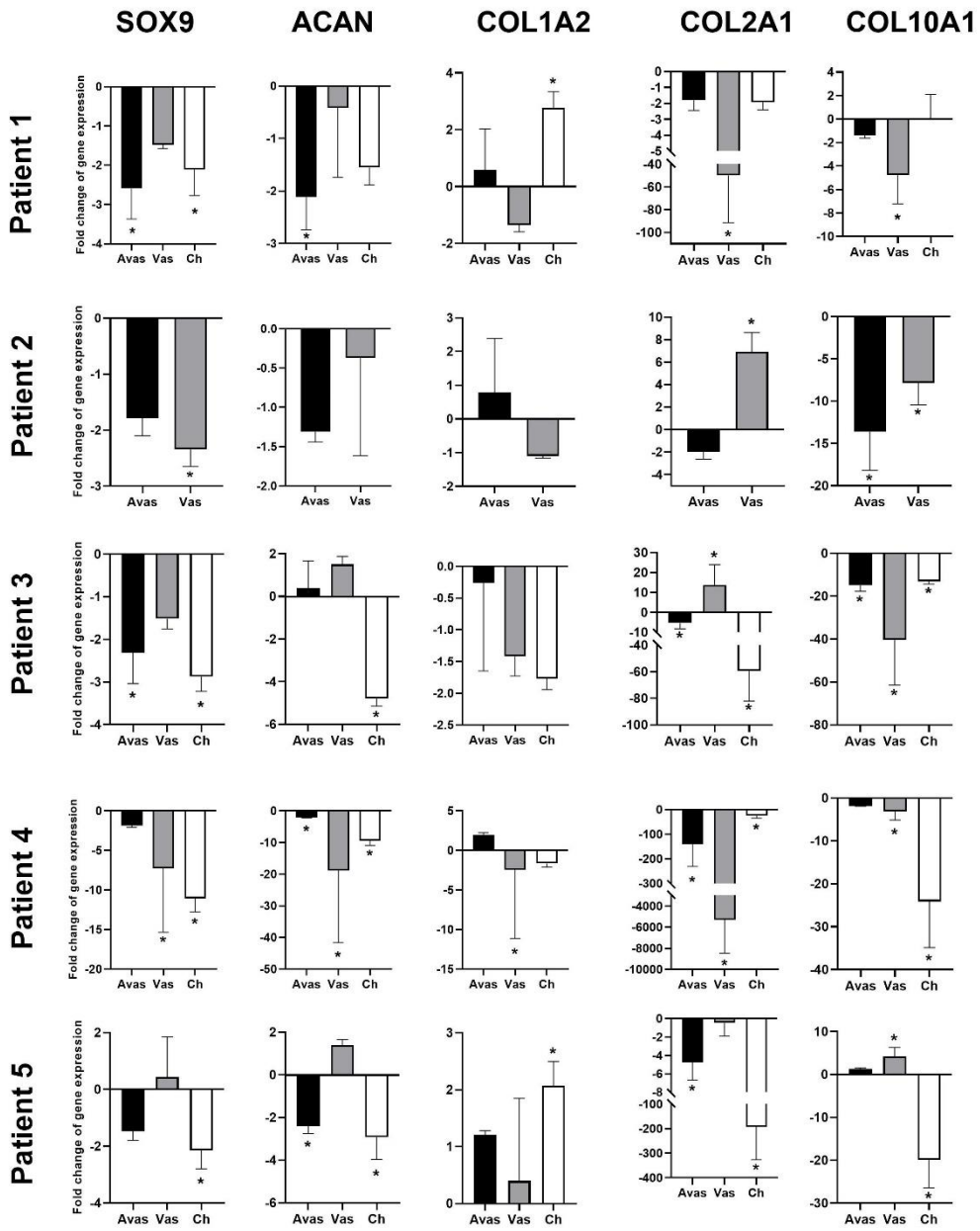

Figure 7: RT-qPCR outcomes of different cell populations (progenitor: mixed cells). Graphs showed the genes associated with chondrogenic potency and hypertrophy from 3D pellet cultures of mixed or progenitor avascular and vascular meniscal cells and chondrocytes. Gene expression for mixed and progenitor groups were normalised to the reference gene GAPDH and data for progenitor cells are expressed relative to mixed population cells. Significantly up- or downregulated genes are indicated with stars. Data were presented as mean ± standard deviation (n=3 pellets/donor).

In Vitro Chondrogenic Differentiation Analysis

GAG/DNA analysis (Figure 8) demonstrated that progenitor cells generally produced more GAGs than mixed population cells derived from all three tissues, with significant differences noted only between MVas and PVas (*P*=0.027). Both MChs and PChs had the highest production level of GAGs across all of the cell fractions analysed. Progenitor cells formed firmer and more densely populated chondrogenic pellets in terms of their matrix and cell distribution density compared with mixed population cells across all three tissue types (Figure 9). When the immunostaining was assessed semi-quantitively, there was more staining for collagen type I observed in pellets formed by all the progenitor populations compared with the mixed populations, but the difference was not significant (Figure 10A). In contrast, a trend for weaker collagen type II staining was detected in all progenitor pellets compared with mixed population pellets (Figure 10B), while only MChs pellets were found to demonstrate significantly stronger staining for collagen type II compared to PChs pellets (*P*=0.0383).

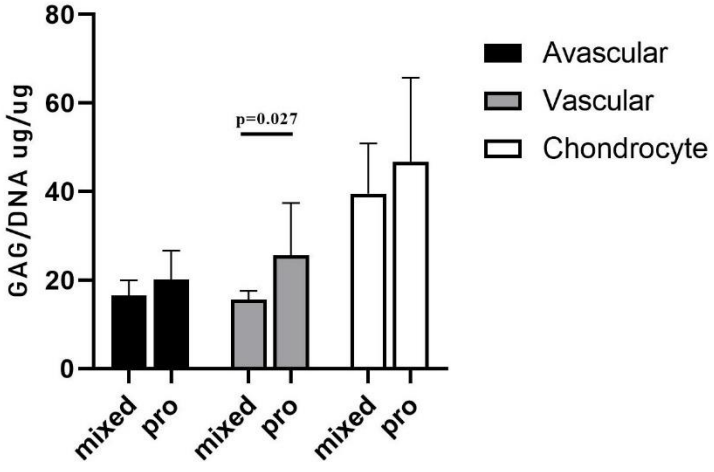

Figure 8: GAG/DNA quantitation after 28 days of 3D pellet culture. Comparison of mixed populations and progenitor (pro) avascular and vascular meniscal cells and chondrocytes from 5 donor matched samples. Data were presented as mean ± standard deviation (n=3 pellets/donor).

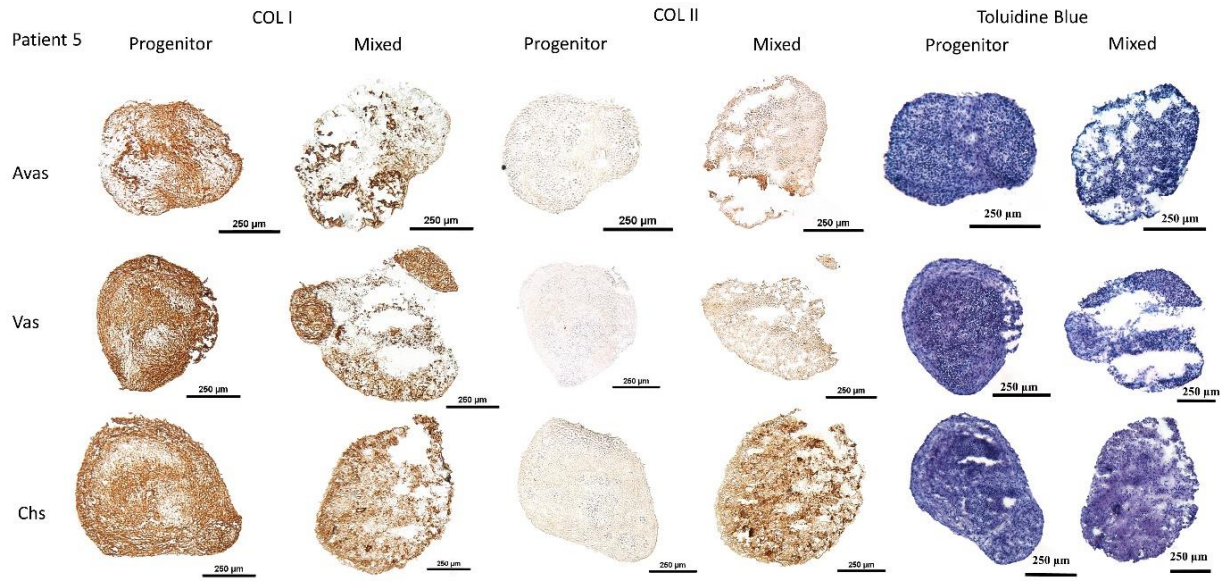

Figure 9: Chondrogenesis of both mixed population and progenitor cells from avascular and vascular meniscus and cartilage from a representative patient 5, assessed by staining sections using type I and type II collagen immunohistochemistry and toluidine blue metachromatic staining of GAGs. Calibration bars =250µm. Avas: Avascular meniscal cells; Vas: Vascular meniscal cells; Chs: Chondrocytes.

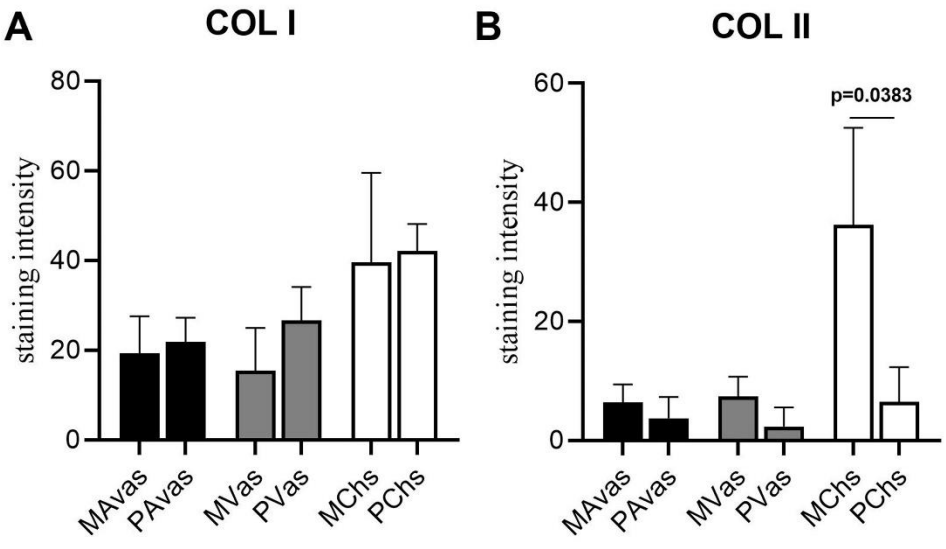

Figure 10: Semi-quantitative immunohistochemical staining measurement for collagen type I (A) and collagen type II (B) of mixed population (MAvas, MVas, MChs) and progenitor (PAvas, PVas, PChs) avascular and vascular meniscal cells and chondrocytes pellets. Data were presented as mean ± standard deviation.

Discussion

Cell-based therapies for meniscus tissue engineering are likely to represent key future meniscus regeneration strategies [17]. Recent studies have supported the hypothesis that meniscus progenitor cells are the most effective cell type for meniscus regeneration, thought to be due to their tissue specificity and histocompatibility [18]. However, the characteristics of human meniscus progenitor cells have not previously been comprehensively investigated.

Morphologically, the primary progenitors, PAvas and PChs, displayed characteristic cobblestone shaped morphologies, whereas PVas had an elongated fibroblast-like morphology. In the mixed populations, cells presented with more extensive cytoplasmic processes compared to their progenitors. Chondrocytes that display cytoplasmic process could be considered to have undergone a hypertrophic change, which is akin to changes observed in late-stage OA cartilage [19]. Samples used in this study were derived from late-stage OA TKR samples, which might explain this distinct morphological feature noted in the mixed populations. However, the progenitor cells isolated from these OA tissues retained a typical proliferative fibroblastic morphology throughout the culture period assessed.

Clonogenicity is a key feature of all types of stem cells derived from various sources including neural [20], hematopoietic [21], embryonic stem cells [22] and epidermal stem cells [23]. In our study, progenitor cells from vascular meniscus regions were shown to proliferate at a significantly higher rate compared with the mixed population cells at passages 0-2. Cell-IQ® live cell imaging analyses of colony forming capacity revealed that PAvas, PVas, PChs isolated using fibronectin substrates showed efficient colony forming potential, while the proliferation rates for PVas fractions were significantly faster compared to PAvas and PChs fractions. Seol et al. [7] created scratch and punch defects in an explant bovine meniscus, which showed the number of migrated progenitor cells in vascular regions was 8.4 times higher than

in avascular regions. This study and our results indicated that PAvas, PVas and PChs possess a capacity for self-regeneration and display stem-like properties. Our results also suggested that vascular meniscal progenitors proliferate faster than avascular meniscal progenitors and chondroprogenitors, which might relate to the higher healing potential observed in the vascular meniscus region [6]. However, based on the Cell-IQ® live imaging analysis of colony proliferation rates in PAvas, PVas and PChs, not every colony in the fibronectin coated wells showed a superior proliferation capacity. Indeed, 30.3% of colonies were not able to grow beyond 32 cells after 5 days in culture. The cell numbers in these non-progenitor colonies levelled off or even decreased during the 48 hours observed. Possible reasons for the presence of non-progenitor colonies might be that some non-progenitor cells which did not attached to fibronectin remained in the wells when media was replenished after 20 minutes. Additional rounds of PBS washing might help to reduce the number of residual non-progenitor cells, but it also poses a risk of washing away any loosely attached progenitor cells. To improve the purity of meniscal progenitor cells in the future, cloning rings could be used to isolate monoclonal progenitor cells [11], although this technique is more cumbersome and does not lend itself well to good manufacturing practices for translational cell therapy manufacture.

Gamer et al. [9] reported that progenitor cells migrate from murine meniscus explants and express stem cell markers such as CD44, CD90 and CD73. Shen et al. [24] characterised meniscus stem/progenitor cells by seeding the whole meniscus population at a very low density (300 cells/well) and carried out flow cytometry analysis which showed that these cells highly expressed CD44, CD90, CD105, CD166. Our flow cytometry data showed that both whole mixed population and progenitor cells from avascular and vascular regions had high expression levels of CD44, CD73, CD90, CD105 and CD166. Thus, CD44, CD73, CD90, CD105 might not be specific to meniscus derived progenitor populations. However,

CD166 (Activated-Leukocyte Cell Adhesion Molecule) showed 100% positive on progenitor populations, while only 90% positive for MVas and 70% positive for MChs. Brinkhof et al. [25] also identified CD166 as the candidate marker for the identification of bone marrow MSCs (BM-MSCs) in contrast to fibroblasts. Together, CD166 might be an ideal marker for discriminating between progenitors and mixed populations in meniscus and cartilage. Our data also showed that progenitor cells had a higher expression of integrin markers including CD49b, CD49c compared to the mixed populations from avascular and vascular regions, as well as chondrocytes from donor-matched cartilage. Integrins are cell membrane receptors associated with cell adhesion and recognition that are essential to cell-cell and cell-matrix interactions [26]. CD29 (integrin β1) is the most abundant integrin expressed by chondroprogenitor cells [27]. CD105+ BM-MSCs subpopulations with high expression levels of CD29 have been shown to have a superior chondrogenic capacity compared with cells that expression a lower percentage of CD29 [28]. A previous study also demonstrated that BM-MSCs have high expression levels of CD49c [29]. Together, these findings support our results which indicate that integrin marker expression levels could be used to identify progenitor cells in meniscus tissues. In terms of our flow cytometry data the differences noted between avascular and vascular meniscal cells observed were consistent with the findings in our previous study, in that avascular meniscal cells had a higher expression level of the integrin markers CD49b and CD49c compared to vascular meniscal cells from n=10 donor-matched meniscus tissues [10]. The basic principles of the progenitors isolation protocol used in this study are based on the selection of cells that highly express β1 integrins and demonstrate rapid adhesion to extracellular matrix proteins [30]. Previous studies relevant to meniscal progenitor cells have not reached a consensus on a progenitor cell isolation protocol. Table 3 summarises the meniscus progenitor cell isolation protocols used across previous studies. The table shows that the procedures used vary widely from FACS (fluorescence-activated cell

sorting) sorting, selective adhesion, low seeding density and tissue explant isolation. All of the proposed methodologies have successfully produced colonies. However, which of these protocols produced progenitor cells with a higher colony forming efficiency is unclear.

Table 3: Published meniscal progenitor cells isolation protocols

Reference	Tissue source	Progenitor cells isolation protocol
Osawa et al. [6]	Meniscus from aborted human foetuses and TKRs	FACS was used to isolate CD34 and CD146 positive meniscal cells after meniscus tissue digestion.
Seol et al.[7]	Bovine Meniscus	Progenitor cells that had migrated into injured sites *in vitro* were isolated followed by trypsin and collagenase digestion.
Shen et al. [24]	Bovine Meniscus	Meniscal cells were seeded at a very low density to form colonies (300 cells in one 6cm dish).
Gamer et al. [9]	Murine Meniscus	Menisci explant cultures in 50 µl of media for 2 hours for tissue adhesion followed by culture in 1.5 ml of media for 3 days with an additional 1.5 ml of media for a further 5-7 days. Meniscal progenitor cells migrated from explant tissues.
Huang et al. [8]	Rabbit Meniscus	Suspended digested meniscal cells in stem cell growth media and cultured for 8-10 days to form progenitor colonies.

TKR: total knee replacement; FACS: fluorescence-activated cell sorting

In the chondrogenic analyses undertaken in this study, the immunochemistry histological staining of chondrogenic pellets demonstrated that meniscal and chondrocyte progenitor cells were more chondrogenic compared with the mixed population cells. Type X collagen, a marker of chondrocyte hypertrophy, was found to be highly expressed in the OA meniscus in a previous study [31] and in our work collagen type X gene expression levels were found to be

significantly downregulated in progenitor cells compared to the mixed populations. Together this might indicate that mixed population cells undergo hypertrophic differentiation as part of the progression of OA, whereas their progenitor counterparts are less committed to differentiating and more stem cell like with their lower expression of SOX9 and type X collagen. In addition, we found that progenitor cells generally produced higher amounts of GAGs compared with mixed population cells in terms of GAG/DNA analyses (significant difference only found between MVas and PVas). These findings suggest that the progenitor population in the meniscus is a suitable cell source for use in rebuilding the proteoglycan-rich avascular zone of damaged menisci, which represents a key challenge for meniscus repair in the clinic [4]. Interestingly, we found that the collagen type II staining intensity of PChs was significantly lower than MChs, which matched the gene expression levels of collagen type II shown in PChs chondrogenic pellets (which was significantly down regulated compared with MChs chondrogenic pellets). The downregulation of collagen type II is seen as a sign of chondrocyte dedifferentiation [32]. Typically, *in vitro* cultured human articular chondrocytes become dedifferentiated and lose their ability to produce hyaline cartilage tissue as their passage number increases [33]. The dedifferentiation phenomenon of PChs may be caused by the inclusion of FGF2 growth factor in their culture media which is aimed at stimulating the proliferation of progenitor cells. Lee et al. [33] cultured costal chondrocytes *in vitro* with or without FGF2 supplementation and demonstrated that the addition of FGF2 accelerated cell expansion and dedifferentiation. However, chondrocytes cultured with FGF2 supplementation showed a better chondrogenic differentiation potential both *in vitro* and *in vivo* compared to chondrocytes cultured without FGF2. Therefore, the addition of FGF2 to culture media may have induced a rapid but reversible dedifferentiation during the *in vitro* expansion phase.

In conclusion, our study demonstrates that the human meniscus contains meniscal progenitor populations in both the avascular and vascular regions based on clonogenicity and chondrogenic differentiation capacity. Our results also suggested that meniscal progenitors derived from the vascular region of the meniscus exhibit enhanced reparative characteristics which likely associate with the better meniscal healing potential previously observed in the vascular region. The findings of this study build on the body of evidence which suggests that meniscal progenitors represent an attractive cell therapy strategy for the enhancement of meniscal repair and regeneration.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request.

Abbreviations

PAvas: Progenitor avascular meniscal cells
PVas: Progenitor vascular meniscal cells
PChs: Chondroprogenitors
MAvas: Mixed avascular meniscal cells
MVas: Mixed vascular meniscal cells
MChs: Mixed chondrocytes
MSCs: Mesenchymal stromal cells
OA: Osteoarthritis
TKR: Total knee replacement
LFC: Lateral femoral condyle
HTO: High tibial osteotomy
AKA: Above knee amputation
IHC: Immunohistochemistry

DMEM: Dulbecco's Modified Eagle Medium

PBS: Phosphate buffered saline

TGF-β1: Transforming growth factor beta 1

FGF-2: Fibroblast growth factor 2

PDT: Population doubling times

ISCT: the International Society for Cellular Therapy

RT-qPCR: Real-time Quantitative Reverse Transcriptase Polymerase Chain Reaction

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

SD: Standard deviation

CI: Confidence interval

BM-MSCs: Bone marrow mesenchymal stromal cells

FACS: Fluorescence-activated cell sorting

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JW conducted experiment. KW contributed to study design, data interpretation, manuscript writing. SR contributed to study design and manuscript writing. ZC contributed to study design and financial support. WZ contributed to supervision and manuscript writing. The authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

This study was approved by the National Research Ethics Service (NRES, 11/NW/0875). The informed consent was obtained from each patient prior to surgery.

Consent for publication

All patients signed a consent form for their data to be used for research or publication.

Competing interests

The authors declare that they have no competing interests.