Characterization and application of size-sorted zonal chondrocytes for articular cartilage regeneration

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ABSTRACT

Current clinical approaches for articular cartilage repair have not been able to restore the tissue with zonal architecture, and its biomechanical and functional properties. Mimicking the zonal organization of articular cartilage in neo-tissue by implanting zonal chondrocyte subpopulations in multilayer construct could enhance the functionality of the graft, engineering of stratified tissue has not yet been realized due to lack of efficient and specific zonal chondrocyte isolation protocol. We show that by using a spiral microchannel device, the superficial, middle and deep zone chondrocytes can be separated based on cell size, and enriched from full thickness porcine cartilage in a high-throughput, label-free manner. The size-sorted cells show zone-specific characteristics in RT-PCR analysis of zonal cartilage markers. Both freshly sorted and two-passage expanded zonal chondrocytes formed cartilage tissue in 3D hydrogel, bearing respective zonal characteristics, indicated by RT-PCR, histology, extracellular matrix proteins, and mechanical compression test. In the proof-of-concept in vivo study using a rodent cartilage defect model, the size-sorted zonal chondrocytes when delivered in bi-layered hydrogel construct, facilitated better cartilage repair with mechanically enhanced cartilage tissue, in comparison to conventional chondrocytes implantation. This study provides an effective approach to obtain large numbers of zonal chondrocytes, and demonstrates the translational potential of stratified zonal chondrocyte implantation for clinical repair of critical size cartilage defects.

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1. Introduction

Articular cartilage in adult joints has poor self-regenerative capacity. Articular cartilage injuries often result in mechanical joint instability and progressive osteoarthritic development. Despite efforts by researchers over the last two decades, achieving fully functional cartilage tissue repair remains a significant challenge [1–3]. Autologous chondrocyte implantation (ACI) is currently the only FDA-approved cell-based method for treating cartilage defects [4]. Although ACI has been shown to form hyaline-like tissue in
some cases when compared to the less costly and simpler non-cell based approach of microfracture (which usually forms fibrocartilage), long term clinical studies comparing the two methods indicate similar clinical outcomes for patients with moderate baseline symptoms and relatively small lesions [4,5]. However, with larger lesions and/or more severe baseline symptoms, the ACI approach has been shown to be more effective than microfracture in short term studies [6].

Cartilage tissue engineering has been focused on achieving the bulk mechanical compressive aggregate modulus and tensile strength of native cartilage. The reproduction of surface lubrication is however essential in achieving long term cartilage integrity. A critical consideration of native cartilage tissue and its organization is pivotal for devising optimal strategies for functional tissue repair. Cartilage tissue is comprised of matrix containing abundant collagen and proteoglycans (PG), embedding a sparse population of a single cell type, chondrocyte, which is responsible for the synthesis of cartilage extracellular matrix (ECM). Despite its simple appearance, articular cartilage exhibits significant organisational complexity, comprising superficial, middle (transitional) and deep zone. The density, morphology and metabolic activity of the cells, as well as the composition and structural arrangement of the ECM components, vary greatly among these zones [7,8]. The superficial zone (SZ; constituting the top 15% of total cartilage thickness) contains flattened chondrocytes with collagen fibrils aligned parallel to the articulating surface. Chondrocytes in the superficial zone produce a specific superficial zone protein (SZP, also known as proteoglycan 4 (PRG-4) and lubricin) which acts as a boundary lubricant for efficient gliding motion during joint movement [9,10]. The middle zone (MZ; 40–50% of total cartilage thickness) contains more rounded chondrocytes, thicker collagen fibrils with random orientation. The deep zone (DZ; 30–40% of total cartilage thickness) is made up of large, spherical chondrocytes in columnar arrangement, embedded in a dense extracellular matrix rich in PGs, with thick collagen fibrils aligned perpendicularly to the articulating surface. Apart from the dominant aggrecan (Aggr) and type II collagen (Col 2), other minor collagen isoforms in the middle and deep zone cartilage such as type IX (Col 9) and XI collagen, and cartilage oligomeric matrix protein (COMP), play critical roles in the regulation of fibril size, inter fibril cross-linking, and interactions with PGs [11,12], which are critical for the compressive strength and dimension stability of the articular cartilage tissue [13,14]. These zonal variations in the collagen and proteoglycan network, and expression of zone-specific proteins, result in significant differences in compressive, shear and tensile properties according to the depth of the cartilage. The zonal biomechanical differences in turn lead to significant variations in strains and stresses experienced by the cells in different zones during joint loading.

The specific behaviours of chondrocytes derived from various depth-related zones have been well-established [15,16]. In vitro studies performed with chondrocyte subpopulations derived from different cartilage zonal tissue sections have demonstrated the ability of the zonal chondrocytes to maintain different biosynthetic activities over weeks of culture [17]. Significantly, constructs formed by a stratified framework of hydrogel incorporated with superficial and deep zone chondrocytes, mimicking the zonal organization of articular cartilage, showed better shear and compressive properties than homogeneous constructs, indicating good mechanical integration and beneficial signalling between the different chondrocytes [18,19]. In particular, restoring the superficial zone was shown to be important in establishing the appropriate signalling to the underlying tissue and regulating the proliferative and biosynthetic activities of the deep zone chondrocytes. In turn, the presence of middle zone chondrocytes upregulates the amount of PRG-4 secreted by superficial zone chondrocytes [18,20]. The accumulation of PRG-4 at the surface of cartilage is important for joint homeostasis, and is essential for cartilage integrity and joint health by reducing friction on articular cartilage surface and preventing early degeneration that stems from sliding of the joints [21,22]. These studies support the hypothesis that replicating the zonal hierarchy of native articular cartilage may facilitate the recapitulation of the cartilage mechanical properties and improve long-term function of the engineered cartilage. However, engineering of cartilage with zonal chondrocyte organization has not yet been realized, and no long-term in vivo study using zonal chondrocytes has been described [23]. Apart from the limited number of cells that can be harvested per zone, the lack of reliable and easy-to-handle zonal cell-sorting protocol severely hinders the practicality of this approach. Dissection methods, either manual [17] or using a microtome [24], have been described for the extraction of the superficial, middle and deep zone cartilage slices from the full thickness (FT) cartilage blocks. However, these dissection methods are clinically impractical because full thickness cartilage tissue with intact zonal layers might not be able to surgically obtain from patients, making identification of zonal tissue difficult. In addition, substantial amount of cartilage has to be excluded for cell harvest in order to ensure the purity of zonal chondrocytes using manual dissection method, which further limits the available cell source.

We and others have previously designed various spiral microchannel devices to separate cells of different sizes [25–30] based on Dean flow fractionation (DFF) principle [31–33]. Cells flowing in a curvilinear microchannel experience shear-induced and wall-induced lift forces, and centrifugal acceleration-induced Dean drag forces in the cross-sectional plane [31]. Interplay of these forces causes cells to migrate laterally and focus at the position where the net force equals to zero. Since the magnitudes of the lift and Dean drag forces experienced by a cell are correlated with the cell size, cells with different sizes focus at different lateral positions in the cross-sectional plane while flowing through the spiral microchannel, allowing size-based cell separation (Fig. 1D). Studies have shown diversified application of this size-based cell-sorting technique [25,27–30].

Given that chondrocytes from different zones are different in size [17], this study explored the use of spiral microchannel sorting to segregate zonal chondrocytes. Chondrocytes derived from porcine femoral condyle full thickness cartilage were subjected to serial spiral microchannel sorting to separate chondrocytes based on cell size. The size-sorted subpopulations were analysed for zonal characteristics, and the ability to form zone-specific cartilage tissue before and after culture expansion. Further, the efficacy of the size-sorted chondrocytes, implanted as bi-layered construct, to regenerate cartilage was validated in a rat osteochondral defect model. Our results demonstrate a highly efficient approach for separating zonal chondrocytes, and the translational potential of stratified zonal chondrocyte implantation for clinical repair of critical size cartilage defects.

2. Materials and methods

2.1. Chondrocyte isolation and culture

Chondrocytes were isolated from weight bearing regions of healthy pig (6–9 months old) articular cartilage with no apparent arthritic cartilage lesion. Full thickness (FT) cartilage from femoral condyle, or SZ, MZ and DZ cartilage segregated manually from FT cartilage using a dissecting microscope (Leica MZ6, Leica Microsystems, Germany) according to Kim et al. (2003) [17], were digested in TrypLE (Life Technologies, Singapore) for 30 min, then with 0.25% (w/v) type-II collagenase solution (Life Technologies) in
DMEM supplemented with 1% (v/v) penicillin-streptomycin for 12–16 h at 37 °C. FT, or size-sorted, chondrocytes, seeded in 5000 cells/cm², were expanded in low glucose DMEM (Life Technologies) with 10% FBS in the absence or presence of 1 ng/mL TGF-β, 2 ng/mL PDGF and 2 ng/mL of bFGF-1. All growth factors were from R&D Systems (USA). Expanded chondrocytes were used at passage 2 for all further experiments.

2.2. Spiral microchannel device design, fabrication and characterization

SolidWorks software (Dassault Systèmes, France) was used to design the mold for fabricating the spiral microchannel device. The spiral microchannel has 8-loops with one inlet and two outlets (Fig. 1A and D). The radius of the spiral loops decreased from 12 mm to 4 mm (from inlet to outlet). The cross-section of the microchannel was trapezoidal. The width was 600 μm, and the inner and outer heights were 80 μm and 130 μm respectively. The ratio of the inner outlet width to outer outlet width was 5:7. The mold was fabricated by micro-milling technique on an aluminium sheet for subsequent polydimethylsiloxane (PDMS) casting (Whits Technologies, Singapore). The spiral microchannel device was fabricated by casting degassed PDMS (10:1 mixture of base and curing agent, Sylgard 184, Dow Corning, USA) on the mold and allowed overnight curing in an oven at 80 °C. The PDMS device was peeled from the mold after curing, and access holes for inserting tubing at inlets and outlets were punched with the 1.5 mm Uni-Core™ Puncher (Sigma-Aldrich, Singapore). The PDMS device was then irreversibly bonded to a layer of cured PDMS base using an oxygen plasma machine (COVANCE, Femato Science, Korea). The assembled device was placed in oven at 80 °C overnight to ensure tight bonding.

The spiral microchannel device was characterized with 7.32, 9.98, and 11.81 μm polystyrene particles (Bangs laboratories, USA), representing the sizes of SZ, MZ and DZ chondrocytes, for focusing position at 0.7, 0.9, 1.2 and 1.4 mL/min sorting flow rates (Supplement Fig. S1A). Computational simulation of the flow velocity (Supplement Fig. S1B) and pressure (Supplement Fig. S1C) along the spiral micro-channel, and formation of Dean vortices in the cross-sectional plane was performed with COMSOL Multi-physics software (COMSOL, USA). The flow velocity was simulated at 40 μm distance from the bottom of the channel. The formation of Dean vortices was simulated at the last loop of the spiral micro-channel.

2.3. Chondrocyte sorting with spiral microchannel device

The spiral microchannel device was mounted on an inverted microscope (IX71, Olympus, Japan) equipped with a high-speed CCD camera (Phantom v9, Vision Research, USA) for real-time visualization of chondrocyte separation inside the channel (Fig. 1A–C). Buffers and suspending chondrocytes were loaded in syringes (Thermo Fisher Scientific, Japan), and pumped into the device through Tygon tubing using a syringe pump (PHD 2000, Harvard Apparatus, USA). The device was first primed with 1% Poloxamer 188 (Sigma-Aldrich) in Milli-Q water for 2 min at flow rate of 1 mL/min to avoid cells sticking to the channel wall during sorting, followed by PBS for another 2 min at flow rate of 1 mL/min to wash off the water in the microchannel. FT chondrocytes were
suspended in medium at concentration of 1–2 million cells/mL. Small chondrocytes (S1) were firstly collected from the outer outlet of the spiral microchannel device by pumping the cell suspension into the device at about 0.7 mL/min. Cells collected from the inner outlet were then pumped through the device again at about 1.2 mL/min, and medium-size (S2) and large (S3) chondrocytes were collected from the outer and inner outlet respectively. Real-time display of the sorting process in the device was monitored and recorded with the Phantom Camera Control software (Vision Research), and the exact flow rates for sorting the 20% smallest cells as S1, 40% medium-size cells as S2 and 40% largest cells as S3 were fine-tuned (±0.2 mL/min) accordingly to cater for inter-sample variations. The recorded sorting videos were processed using ImageJ software (NIH, USA) for images.

2.4. Cell size and zonal chondrocyte enrichment analysis

The size of chondrocytes before and after sorting was measured by Moxi Z Mini automated cell counter with type M cassette (Orflo Technologies, USA). The original CSV files generated by the cell counter were imported to MATLAB software (The MathWorks, USA) for statistics and analysis of zonal chondrocyte enrichment after sorting. To quantify the chondrogenic response, the size distribution of FT chondrocytes was first measured with the automated cell counter before sorting, and the cell size ranges of small, medium and large chondrocytes were calculated respectively based on the defined composition in this study (smallest 20% as small, next 40% medium-sized as medium and biggest 40% as large chondrocytes). After sorting, the size distribution of subpopulation S1, S2 and S3 was measured, and the composition of small, medium and large cells in each subpopulation was computed based on the cell size ranges calculated previously. Cells less than 6 μm measured by the cell counter were considered as artefacts and excluded from all analysis.

To verify whether the size-sorted subpopulations of chondrocytes were originated from different zones, manual segregated SZ, MZ and DZ chondrocytes were stained with PKH26 red fluorescence cell linker (Sigma-Aldrich), Calcium Green AM cell permeant (Thermo Fisher Scientific, Singapore), and Hoechst 33253 (Thermo Fisher Scientific) respectively, and mixed with approximate cell ratio of 2:3:5. The remixed labelled full thickness (RMFT) chondrocytes were subjected to the same sorting strategy described in Section 2.3 to obtain S1, S2 and S3 subpopulations. The subpopulations were then imaged for different fluorescent channels, and quantified for the composition of each zonal cells by manual counting the number of cells with different fluorescence.

2.5. Chondrocyte re-differentiation in fibrin hydrogel

Fibrin Sealant (TISSEEL Kit) containing human fibrinogen, thrombin, calcium chloride and aprotinin were from Baxter (Austria). Fibrinogen solution was prepared in aprotinin solution (3000 KIU/mL) to 50 mg/mL. Chondrocytes (2 × 105 cells) suspended in 10 μL of fibrinogen solution, were quickly mixed with equal volume of 10 IU thrombin solution and immediately dropped onto a tissue culture plate to form a 20 μL three-dimensional fibrin clot [34]. The resulting clot constituted cell density of 107 cells per mL in 5 IU thrombin, 20 μm/mL CaCl2, 1500 KIU/mL aprotinin and 25 mg/mL fibrinogen. The components were allowed to polymerize undisturbed at 37 °C for 30 min, before incubating in chondrogenic differentiating medium of high glucose DMEM supplemented with 107 M dexamethasone, 1% ITS + premix, 50 μg/mL ascorbic acid, 1 mM sodium pyruvate, 0.4 mM proline and 10 ng/mL of TGF-β3 (R&D Systems). The samples were cultured for 2–3 weeks, with medium change every 3 days. For hydrogel samples intended for histological analysis, monensin (0.1 μM, Sigma-Aldrich) was included in the culture media 48 h before tissue harvest to retain newly synthesised proteins in the cells.

2.6. Real-time PCR analysis

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, USA) following the manufacturer’s protocol. The concentration of RNA was determined using a NanoDrop UV–vis Spectrophotometer (NanoDrop Technologies, USA) and reverse transcription reaction was performed with 100 ng total RNA using iScript™ cDNA synthesis kit (Biorad Laboratories, USA). Real-time PCR reaction was conducted using the SYBR Green system with primer set as our previous publication [35]. Real-time RCR reaction using the ABI 7500 real-time PCR system (Life Technologies) was performed at 95 °C for 10 min and 40 cycles of amplifications, which consisted of denaturation step at 95 °C for 15 s, and extension step at 60 °C for 1 min. The gene expression level, normalized to GAPDH, was then calculated using the $2^{-ΔΔCt}$ formula with reference to the undifferentiated porcine mesenchymal stem cells (MSC). Expression of cartilaginous genes in MSC are either not detectable (eg. Col 2) or at very low levels. MSC was thus chosen as a reference to demonstrate the relative expression levels of different cartilaginous genes across the chondrocytes subpopulations.

2.7. Sulfated glycosaminoglycan (sGAG) and collagen quantification

Samples were digested with 10 mg/mL pepsin (Sigma-Aldrich) in 0.05 M acetic acid at 4 °C for 5 days, followed by further overnight elastase (1 mg/mL Sigma-Aldrich) digestion at pH 8.0. The amount of sGAG was quantified using Blyscan sGAG assay kit (Biocolor, UK) according to manufacturer’s protocol. Absorbance was measured at wavelength of 656 nm using FLUOstar Optima plate reader (BMG LABTECH, Germany). Type II and IX Collagen was quantified using a captured enzyme-linked immunosorbent assays (Chondrex, USA and ELab, China, respectively) following the manufacturer protocol. Measurement was taken on a TECAN Infinite M200 (Switzerland) plate reader at optical density of 490 nm.

2.8. Histological and immunohistochemical assessment

Tissue samples were fixed overnight in 10% neutral buffered formalin before being dehydrated and paraffin-embedded. Sections of 5 μm were cut and collected on silane-coated slides. After deparaffinized in xylene and alcohol, cartilage matrix proteoglycan was stained with 0.1% Safranin O Solution (Acrös Organics, USA), and counterstained with Accustain6 Harris hematoxylin (Sigma-Aldrich). Immunohistochemistry staining was performed for Type II collagen mouse monoclonal antibodies (Clone 6B3 at 1:500 dilution, Chemicon, USA), and PRG4 (ab-94933 at 1:100 dilution, Abcam, UK), followed by biotinylated goat anti-mouse or goat anti-rabbit secondary antibody (Lab Vision, USA). PRG4-positive cell was quantitated from 200 to 300 accounted cells per sample and expressed as % PRG4 positive cells.

2.9. Mechanical strength analysis

Compression test was carried out using Instron tester 5567 (Singapore) at 0.01 mm/s until the 10–15% strain was reached. The thickness of the samples was measured and converted to the strain of the sample $(e = L - L_0/L_0)$, where L0 and L represent the thickness before and after compression, respectively. Young’s modulus was determined using the formula $E = r/e$, where r and e represent the respective stress and strain of the sample on the stress-strain plot.
2.10. Animal experiments

All procedures were performed according to the Institutional Animal Care and Use Committee at National University of Singapore under protocol number: R15-1450. Sprague-Dawley rats (10 weeks old) were anesthetized using an intraperitoneal injection of a mixture of ketamine (10 mg/100 gm body weight) and xylazine (1 mg/100 gm body weight). An anterior midline incision was made through the skin of the knee. The knee joint was opened via a medial patellar approach and the patella was resected. An osteo-chondral defect (1.5 mm in diameter and 1.5 mm in depth) was created in the patellar groove of the distal femur. The defect was either implanted with cell-free fibrin hydrogel (Control group), FT porcine chondrocytes in fibrin hydrogel (4 μL, 5 × 10⁶ cells/mL, FT group), or with bi-layered fibrin gel construct (Bi-layered group) of MZ/DZ chondrocytes (2 μL, 5 × 10⁶ cells/mL) overlaid with SZ chondrocytes (2 μL, 5 × 10⁶ cells/mL). One minute was allowed in-between the implantation of the two hydrogel layers for the MZ/ DZ layer to solidify. SZ and mixed MZ/DZ subpopulations were obtained as S1 and S2/S3 by sorting with spiral microchannel device. To visualise the spatial distribution of cells in the bi-layered construct, SZ (S1) cells were labelled with PKH26 fluorescence (Sigma-Aldrich) and MZ/DZ (S2/S3) cells were labelled with CFDA cell tracer (Life Technologies) prior to implantation according to manufacturer protocols. For FT cells implantation, the separately labelled subpopulations were mixed before implantation. Rats were fed with cyclosporine-supplemented water (35 mg/kg; Novartis Pharma, USA) to prevent host rejection of the implanted porcine cells. Animals implanted with fluorescence tracker-labelled cells were euthanized 2 weeks post-implantation, the distal femurs were fixed, decalcified, cryo-sectioned, and tissues at the cartilage defect site were imaged using fluorescent microscope. Six weeks after surgery, the rest of the rats were euthanized and the distal femurs with defects were subjected to compressional assessment, or fixed in 10% buffered formalin, decalcified, sectioned, and underwent histological and immunohistochemical analysis. The quality of cartilage repair was assessed using the modified O’Driscoll histologic grading system (Supplement Table S1) by three blinded independent observers for parameters including cellular morphology, matrix staining, surface regularity, structural integrity, thickness, bonding, and freedom from cellular changes of degeneration [36].

2.11. Statistical analysis

The statistical significance between size-dependent subgroups within one size-sorted subpopulation in Figs. 2 and 3 was evaluated by one-way ANOVA with post hoc Scheffe’s test. The statistical significance of the composition of cell types between each two size-sorted subpopulations or unsorted (FT) chondrocytes in Figs. 2 and 3 was evaluated with two-way ANOVA with Scheffe’s test. The statistical significance of the zonal marker RNA expression, compressional modulus, and histological score between zonal chondrocyte subpopulations or unsorted (FT) chondrocytes was evaluated by one-way ANOVA with post hoc Scheffe’s test. Significance was set at p < 0.05. All statistical analysis was performed with IBM SSSP Statistics software (IBM, USA).

3. Results

3.1. Size-based separation of zonal chondrocytes with spiral microchannel device

Freshly isolated chondrocytes from full thickness (FT) cartilage of 6–9 month old pigs were subjected to size-based sorting using a spiral microchannel device with trapezoidal cross-section to separate superficial (SZ), middle (MZ) and deep zone (DZ) cells (Fig. 1A–D) [29,37]. SZ, MZ and DZ constitute the top 10–15%, the next 40–50%, and the last 30–40% of the total cartilage thickness [7]. Considering that SZ has higher cell density as compared to MZ and DZ [38], we aimed to sort the smallest 20% cells (subpopulation 1, S1) as SZ chondrocytes, the medium-size 40% (subpopulation 2, S2) as MZ chondrocytes, and the biggest 40% (subpopulation 3, S3) as DZ chondrocytes, using a two-stage serial sorting strategy (Fig. 1E–F) (See Materials and methods). All zonal chondrocytes obtained from spiral microchannel sorting showed high viability (>98%).

Results showed that size-sorted subpopulation S1 comprised 22.0%, S2 comprised 43.5% and S3 comprised 34.5% of total cells on average, which were reasonably close to the targeted 20%, 40% and 40%, respectively (Table 1). We then quantified the enrichment of small, medium and large size chondrocytes in SZ, S2 and S3 populations based on cell size range. Results showed that small cells were dominant in S1, enriched from 20% to 43% as compared to the FT chondrocytes before sorting. Medium-size cells were dominant in S2, enriched from 40% to 51%. Large size cells were dominant in S3, and enriched from 40% to 55% (Fig. 2A) (See Materials and methods). We compared the size-sorted subpopulations with zonal chondrocytes digested from manually segregated SZ, MZ and DZ cartilage tissue. Results showed that the manually segregated SZ, MZ and DZ chondrocytes were 10.71 ± 3.07, 11.76 ± 2.52 and 12.44 ± 2.96 μm, respectively, in average size; comparable to the corresponding size-sorted S1, S2 and S3 with sizes of 10.95 ± 2.93, 11.42 ± 166 and 13.33 ± 2.12 μm, respectively. The size distribution profiles were also consistent between the manually segregated and size-sorted subpopulations (Fig. 2B).

To verify whether the cell-sorting strategy actually separated the chondrocytes from different zones, manually segregated SZ, MZ and DZ chondrocytes were subjected to the same sorting strategy respectively. Results showed that at both sorting flow rates, majority of DZ cells were focused to the outer outlet of the spiral microchannel device. In contrast, majority of SZ cells were focused to the inner outlet, indicating good separation between SZ and DZ cells. Most MZ cells were focused at inner outlet at 0.7 mL/min, and separated from SZ cells. Then they were further separated from DZ cells at 1.2 mL/min, although some were still mixed with DZ cells at inner outlet (Fig. 3A). The three manually segregated zonal chondrocytes were labelled with three different fluorescence, remixed (RM-FT group) and subjected to the serial sorting to obtain S1, S2 and S3 subpopulations. The separated subpopulations were imaged, and the composition of each zonal cell types in all subpopulations were quantified based on specific fluorescence. Results showed that SZ cells were dominant in S1, enriched from the 23%–61%. MZ and DZ cells were dominant in S2, with MZ cells enriched from 29% to 43%. DZ cells were dominant in S3, enriched from 47% to 77% (Fig. 3B and C). Both S2 and S3 were depleted of SZ cells, which constituted less than 9% in both subpopulations. Together, these data indicate that the size-sorted subpopulations S1, S2 and S3 resembled enriched populations of SZ, MZ and DZ chondrocytes respectively.

3.2. Phenotype of size-sorted zonal chondrocytes

RT-PCR was performed to assess the levels of mRNAs that characterise the zonal matrix proteins. After sorting, Col 2, aggrecan and the middle deep zone proteins, Col 9, were found to be higher in S2 and S3, but generally low in S1. Superficial zone protein, PRC4, was found to be highest in S1 (Fig. 4A). The expression pattern of these cartilage markers showed good consistency with manually segregated controls (Fig. 4A) with some variations in the
expression levels for Col 2, aggrecan and Col 9 in S2 and S3, compared to MZ and DZ chondrocytes. The analysis of cartilage marker expression pattern further supported that the SZ, MZ and DZ chondrocytes were successfully enriched in S1, S2 and S3 sub-populations respectively. The size-sorted chondrocytes were subjected to 3D fibrin hydrogel culture for cartilage reformation. After three weeks, mRNA analysis for the same set of markers showed that the expression pattern of the zone-specific markers was well maintained during the tissue reformation (Fig. 4C). Quantitative ECM analysis found significantly higher sGAG and Col 2, Col 9 proteins in the S2 and S3 cartilage constructs than the S1 constructs (Fig. 4D). Relative to FT constructs, S2 and S3 constructs had higher, but not significant, protein levels of sGAG and Col 2, Col 9. The different levels of Col 2, sGAG formation across the S1, S2 and S3 were also evident in immunohistochemical analysis of the reformed cartilage tissues (Fig. 4E). Higher number of PRG4-positive cells was detected in the S1 constructs (47.9 ± 6.2%) compared to 11.8 ± 5.0%, 10.1 ± 3.1% and 7.4 ± 2.9% in FT, S2 and S3 constructs, respectively (Fig. 4E, Table 2). Mechanical testing of the tissue constructs registered significantly higher compression modulus in the S2 and S3 constructs, compared to FT constructs, while S1 constructs had significantly lower compression modulus (Fig. 4F). These results demonstrate that the three subpopulations obtained by the size-based sorting method represented the enriched populations of SZ, MZ and DZ chondrocytes, and were able to form cartilage tissues of SZ, MZ and DZ cartilage characteristics.

3.3. Cartilage regeneration with size-sorted zonal chondrocytes in rats with osteochondral defects

The ability of the size-sorted zonal chondrocytes, delivered as a bi-layered construct to repair cartilage was tested using a rat osteochondral defect model. Freshly isolated FT chondrocytes were sorted into the S1 (SZ) and mixed S2/3 (MZ/DZ) subpopulations. The two subpopulations of chondrocytes were implanted as bi-layered fibrin gel construct with sequential delivery of fibrin hydrogel of S2/3 (MZ/DZ) chondrocytes, overlaid with S1 (SZ) chondrocytes, into the osteochondral defect of rat (Bi-layered group) (Fig. 5A). Tracking of the implanted cells with fluorescence cell trackers at two weeks post implantation showed preservation of the two distinct layers at the cartilage defect site (Fig. 5B). The in vivo efficacy in cartilage repair of the bi-layered construct was compared to implantation with the unsorted FT chondrocytes (FT group) and cell-free fibrin hydrogel (Control group), with the operated joints harvested six weeks post-surgery and subjected to immunohistochemical analysis and mechanical compression test. Alcian blue and Col 2 immunostaining showed that the Bi-layered group had more cases of cartilage tissue formation throughout the depth at the defect sites (4 out of 6 cases), compared to, at best, partial tissue formation in the FT group (2 out of 6 cases) (Fig. 5C, Supplement Fig. S2). FT chondrocytes implantation resulted in tissue abundant in type I collagen (Col 1). The cases in the bi-layered implantation resulted in predominantly Col 2-hyaline cartilage tissue with significantly lower Col 1. Histologic scoring showed that the rat knees implanted with bi-layered constructs had significantly better cartilage regeneration scoring than the FT and Control.
group (Fig. 5D). Implantation of the bi-layered zonal cells also yielded tissues with better mechanical compression strength compared with the Control and FT group (Fig. 5E). These results show that implantation of size-sorted zonal chondrocytes as bi-layered constructs improved the quality of repair of cartilage defects in comparison to delivery of unsorted FT chondrocytes.  

3.4. In vitro expansion of size-sorted chondrocytes  

The size-sorted chondrocytes were subjected to two passages of expansion in media supplemented with growth factor cocktail [39,40]. The cells went through 15–20 population doublings in a period of three weeks, and as expected, underwent de-differentiation as indicated by drastic reduction of all the cartilaginous markers’ expression (data not shown). After expansion, the chondrocytes were induced to re-differentiate in 3D fibrin hydrogel with standard chondrogenic differentiation media. Analysis of the mRNA expression of cartilage markers indicated that expanded chondrocyte subpopulations after subjecting to 3D re-differentiation, had in general regained the zonal phenotypes as those of the non-expanded cells (Fig. 6A). Quantitative ECM analysis indicated similar levels of sGAG, Col 2 and Col 9 formation (Fig. 6B). Histological (Fig. 6C) and mechanical (Fig. 6D) analysis of the reformed cartilage tissue indicated that the relative levels of the

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**Fig. 3.** Verification of spiral sorting performance with manual segregated zonal chondrocytes. (A) Respective focusing positions of the manual segregated SZ, MZ and DZ chondrocytes at the two-stage serial sorting. (B) Bright field (BF) and fluorescent (Overlaid FL) images of the remixed labelled manual segregated SZ (red), MZ (green) and DZ (blue) chondrocytes before sorting, and S1, S2 and S3 subpopulations obtained after spiral sorting. (C) Quantification of the enrichment of SZ, MZ and DZ chondrocytes in S1, S2 and S3 subpopulations obtained after sorting the remixed full thickness (RM-FT) chondrocytes. One-way and two-way ANOVA showed significant difference in the composition of SZ, MZ and DZ chondrocytes within each size-sorted subpopulations, and among the three size-sorted subpopulations, respectively. * denotes significantly difference from the corresponding zonal type in the RM-FT group, and + denotes significantly difference from the corresponding small chondrocytes in each size-sorted subpopulation with post hoc Scheffe’s test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**  
Comparison of the actual percentages of cells in S1, S2 and S3 after sorting with the targeted percentages. Cell numbers collected in S1, S2 and S3 after sorting were counted using haemocytometer. Data are from 15 sorting experiments with chondrocytes from 9 pigs. Data are presented as mean ± standard deviation.

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<th>Targeted %</th>
<th>Actual % post-sorting</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 (SZ)</td>
<td>20%</td>
<td>22.0 ± 2.0%</td>
</tr>
<tr>
<td>S2 (MZ)</td>
<td>40%</td>
<td>43.5 ± 10.9%</td>
</tr>
<tr>
<td>S3 (DZ)</td>
<td>40%</td>
<td>34.5 ± 2.1%</td>
</tr>
</tbody>
</table>
ECM macromolecules and the compressional modulus of the tissue constructs were similar to that of non-expanded sorted cells. PRG4 immunostaining however found stronger expression of PRG4 and increased PRG4-positive cells in all constructs, comparing to the cell constructs of the respective non-expanded groups (Fig. 6C, Table 2). Almost all cells in S1 constructs were PRG4-positive (91.3 ± 1.4%), while FT, S2 and S3 construct has their PRG4-positive cells increased to 58 ± 9.6%, 74.1 ± 3.8% and 56.3 ± 5.9%, respectively.

### 4. Discussion

Recreation of the zonal hierarchy of native articular cartilage has
long been recognised as essential to recapitulate the bulk mechanical properties and surface lubrication of native articular cartilage for achieving long term tissue integrity of the repaired cartilage. Attempts in using zonal chondrocytes to engineer cartilage tissue with zonal hierarchy has been plagued by the difficulty in obtaining segregated zonal cells [23]. In this study, we developed a novel protocol for effective sorting of zonal chondrocytes from FT tissue-derived heterogeneous chondrocytes using a spiral microchannel device based on cell size (Fig. 1A–D). Chondrocytes obtained from FT cartilage were serially sorted into three subpopulations, S1, S2 and S3 (Fig. 1E and F), with similar average cell sizes and size distribution profiles to the respective SZ, MZ and DZ chondrocytes obtained by manual segregation (Fig. 2B). The actual zonal origin of S1, S2 and S3 was confirmed by analysing the focusing behaviour of each manually segregated zonal chondrocyte subpopulation in the spiral microchannel (Fig. 3A), and quantifying the respective zonal chondrocyte compositions after sorting the mixture of differentially-labelled zonal cells (Fig. 3B and C). The sorting protocol resulted in S1 enriched with higher proportion of SZ chondrocytes; S2 was dominant with MZ and DZ cells, while S3 was dominant with DZ cells. Significantly, the sorting resulted in the depletion of SZ cells from both S2 and S3. Using well defined ECM markers and secreted molecules associated with different cartilage zones, we further characterized the phenotype and

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**Fig. 5.** Zonal chondrocytes implantation and cartilage regeneration at rat knee osteochondral defect. (A) Schematic illustration of FT and Bi-layered chondrocyte implantation procedures. (B) Fluorescent images of the spatial distribution of cells in the FT and bi-layered constructs at the defect sites. S1 (SZ) cells were labelled with PKH26 (red) and S2/S3 (MZ/DZ) cells were labelled with CFDA cell tracer (green). Scale bars: 100 μm. Arrows indicate the surface of implanted tissue construct. (C) Alcian blue, Type I and II collagen immunohistochemical analysis of osteochondral defects of rat knee implanted with fibrin hydrogel only (Control, n = 4), Bi-layered (n = 6) or FT (n = 6) chondrocytes. Samples are representative of each group. Scale bars: 500 μm. Region between the two arrows indicates the defect site in each image. (D) Qualification of the repaired cartilage by Modified O’Driscoll score. (E) Compressional stiffness of the repaired tissues. n = 6 per group. One-way ANOVA showed significant difference among different chondrocyte implantation methods. * denotes significant difference between Bi-layered and Control groups, and # denotes significant difference between Bi-layered and FT groups with post hoc Scheffe’s test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
biochemical property of the size-sorted subpopulations and the derivative tissues. Significantly higher expression of aggrecan, Col 2 and middle/deep zone-specific Col 9 (as well as COMP, data not shown) was found in S2 and S3, relative to S1 (Fig. 4B). In contrast, the superficial zone PRG4 was found to be specifically expressed in high level in S1 (Fig. 4B). The ability of the size-sorted chondrocytes subpopulations to reform cartilage tissues of differential levels of ECM molecules (sGAG, Col 2 and Col 9; Fig. 4D and E) and the compressional modulus (Fig. 4F) was demonstrated. S2 and S3 chondrocytes produced more than 2 folds of the sGAG, Col 2 and Col 9 proteins than S1 chondrocytes, corresponding to more than 2 folds increase in compressional modulus. Analysis of PRG4-positive cell in the reformed tissue constructs found high percentage (47.9%) of cells synthesising this lubricating molecule in the S1 construct compared to ≤10% in the S2 and S3 constructs (Table 2). The levels of ECM molecules and mechanical strength of the tissues formed from S1, S2 and S3 correlated well with those found in native zonal cartilage [7,17,41], indicating that S1, S2 and S3 were well-representation of the enriched populations of SZ, MZ and DZ chondrocytes, respectively. Notably, the compressional strength of the cartilage constructs generated by S2 (MZ) and S3 (DZ) chondrocytes was significantly higher than the construct generated from unsorted FT chondrocytes (Fig. 4F), indicating a distinctive advantage of using enriched populations of zonal chondrocytes to generate functionally superior cartilage. We next validated the efficacy of delivering layered zonal chondrocytes in repairing cartilage defect using a rat osteochondral defect model, in comparison to delivery of heterogeneous mixture of FT chondrocytes. Given the technical difficulty in delivery three zonal layers of chondrocytes in a rat osteochondral defect of 1.5 × 1.5 mm in diameter and depth, and the similar in sGAG, Col 2 and Col 9 production profile of the S2 and S3 subpopulations, a bi-layered zonal chondrocytes delivery, with sequential implantation of fibrin hydrogel of mixed S2/3 (MZ/DZ) chondrocytes overlaid with S1 (SZ) chondrocytes, was attempted instead (Fig. 5A). Despite the high variation of repaired cartilage within each experimental group (Supplement Fig. S2), we detected more cases of hyaline cartilage tissue restoration to the similar tissue height as the native cartilage in the Bi-layered group, compared to the, at best, partial cartilage tissue formation in the FT group (Fig. 5C and Supplement Fig. S2). Histological scoring showed that implantation of size-sorted zonal chondrocytes as bi-layered constructs yielded cartilage of better quality, with significant improvement compared with implantation of unsorted FT chondrocytes or cell-free fibrin hydrogel (Fig. 5D), which was further correlated to the higher compressional strength of the regenerated tissues (Fig. 5E). Implantation of stratified cartilaginous constructs seeded sequentially with superficial and middle chondrocyte subpopulations has been previously attempted in mini-pigs using a scaffold free system, which showed signs of early repair with defects after one week of implantation [24]. However, no follow-up of longer term outcomes was reported. The study also reported that the implanted stratified cellular organization was, by and large, not maintained after one week in vivo. In contrast to the carrier-free delivery in the Chawla
et al. study [24], the two subpopulations of chondrocytes in our study were sequentially delivered encapsulated in fibrin hydrogels. The two distinct layers were preserved at the cartilage defect site two weeks post implantation (Fig. 5B). The positioning of a layer of PRG4-producing S1 (SZ) chondrocytes at the articular surface, for at least two weeks post-implantation in the bi-layered group could have contributed to the immediate in situ lubrication of the surface of the implanted constructs [9,10], and helped in preventing neotissue disruption from shearing motion of the articular joint. Further, cross-communication between the SZ and the other zonal chondrocytes in the bi-layered constructs might also benefit the proliferative and biosynthetic activities of the MZ/DZ chondrocytes, and vice versa, PRG-4 secretion by SZ chondrocytes [18,20], resulting in overall improved biochemical and mechanical properties of the regenerated cartilage. Articular cartilage with its superficial region removed has been shown to significantly reduce the dynamic modulus of the remaining tissue [42]. The improved mechanical compressive modulus of the bi-layered implantation in this study could also be the result of the presence of the superficial region acting as a low permeability barrier to maintain fluid load support of the lower cartilage zones [42]. The challenge in the controllability of delivering multi-layered hydrogel using the rodent osteochondral defect model has limited the significance of current study. Further follow-up in vivo study using a larger animal model with larger defect size should improve the reproducibility of the bi-layer fibrin hydrogel delivery. A more manageable defect model would yield better significance of layered zonal chondrocytes delivery in repairing cartilage, and would also allow for the analysis of the lubricating and shearing properties on the surface of the repaired cartilage.

For autologous application of patient-derived chondrocytes, in order to yield clinically relevant cell numbers, extended expansion of chondrocytes is inevitable. To this end, we have subjected the size-sorted subpopulations of chondrocytes to two passages of expansion in the presence of growth factors. Cell underwent 15–20 population doublings (variation dependent on the age of the porcine) in a period of three weeks. Although the expanded cells underwent de-differentiation during expansion, when subjected to 3D chondrogenic culture, they were able to reform cartilage and recapitulate the phenotypic characteristics. Quantitation of ECM formation (Fig. 6B), histological analysis (Fig. 6C) and mechanical compression analysis (Fig. 6D) of the repaired cartilage tissue indicated that the relative levels of the ECM macromolecules and the compressional modulus of the tissue constructs were similar to that of non-expanded size-sorted cells. These comparable biochemical and mechanical characters of the cartilage generated from expanded and non-expanded chondrocytes subpopulations were in accordance to previous in vitro studies performed with chondrocyte subpopulations that demonstrated the ability of the zonal chondrocytes to maintain different biosynthetic activities over weeks of culture [17]. Interestingly, we detected stronger expression of PRG4 and increased PRG4-positive cells in all constructs of expanded cells when compared to their respective non-expanded counterparts (Table 2). Almost all cells in the expanded S1 constructs were intensely PRG4-positive, increased from the roughly 48% in the unexpanded S1 constructs, while the expanded FT, S2 and S3 construct had their PRG4-positive cells increased substantially. Chondrocytes, regardless of zonal derivation, adopted a fibroblastic morphology during expansion, resembling the fibroblastic morphology of the superficial zone chondrocytes of articular cartilage. Mesenchymal stem cells when forced into fibroblastic morphology and stress F-actin cytoskeletal structure, undergone chondrogenic differentiation and developed into PRG4-expressing superficial zone-like chondrocytes [43,44]. Cytoskeletal alteration in SZ chondrocytes has also been associated to PRG4 formation [45].

The increase in PRG4 could also be induced by TGFβ1 in the growth factor supplement in the expansion media [45,46]. On the other hand, the increase in PRG4-positive cells could derived from the cartilage derived progenitor cells (CDPC), which was first identified to reside on the surface of articular cartilage [45,46]. Prolong two-dimensional expansion was critical for the emergence and proliferation enhancement of CDPCs [47], which was found to highly express PRG4 [48]. Residue CDPCs in the S2 and S3 subpopulations might preferentially proliferated during expansion and accounted for the increased PRG4-positive cells. The presence of CDPCs in each of the enriched zonal subpopulations and their influence to the generation of stratified cartilage tissue will warrant further investigation.

PRG4 expression could increase with expansion period and inevitably result in alteration of phenotypes of the middle/deep zonal (S2/S3) cells. Indeed, we observed more pronounced loss in zonal characteristics when we compared cells yield from expansion without growth factors (Supplement Fig. 5S). In the absence of growth factors, population doubling was much slower (11–17 population doubling in >28 days), and the re-differentiated chondrocytes produced substantially lower amount of Col 2 and sGAG, while expressing significantly higher PRG4, across all zonal sub-populations. Consequently, the compressional modulus of the FT, MZ (S2) and DZ (S3) constructs were significantly reduced. For clinical application, in which tissue derived chondrocytes are usually low in quantity, while substantially larger quantity of expanded cells is required for critical defect repair, appropriate expansion condition for the zonal chondrocytes will have to be designed in order to retain the phenotypic characteristics of the extensively expanded zonal chondrocytes. In addition, given the limited available patient tissue in autologous chondrocyte implantation procedure, application of the spiral microchannel sorting on the small number of freshly isolated chondrocytes could be challenging. It is therefore essential to establish the practicality of employing our microchannel cell-sorting strategy to the expanded chondrocytes. Extended expansion of full thickness chondrocytes could result in loss of their original size-zone specificity relationship. The employment of the sorting technique to the expanded full thickness chondrocytes will likely require coupling with appropriate manipulation during cell expansion to retain both cellular biophysical and biochemical phenotypes. To this end, our highly effective cell-sorting strategy will be able to provide enriched zonal subpopulations required for such investigation.

The spiral microchannel cell-sorting technique applied in this study is label-free and high-throughput. One million chondrocytes can be processed within approximately 30–45 s with the protocol described. The advantage of the described sized-based microfluidic separation method over conventional separation method (such as centrifugal elutriation) and other microfluidic separation method (electrical, magnetic, acoustic, inertial, etc.) is its capacity of maintaining close-to-micron level separation resolution at millions of cells per minute processing rate. The high-throughput of this microfluidic sorting method can be achieved at low cell concentration (lower than million/mL to millions/mL), large volume (tens of mL) and high flow rate (mL/min), unlike many other microfluidic techniques where high cell concentration (tens of millions to hundreds of millions/mL), small volume (μL) and low flow rate (μL to tens of μL per min) are required. Thus, our technology is suitable for processing large numbers of cells without the requirement of long processing time that could affect cell viability. Previous work suggested that the stimulation to the cells raised by the spiral microchannel sorting is similar to the differential centrifugation process widely used in cell separation [30]. In our study, the sorted chondrocytes were viable and healthy, and able to proliferate when cultured, and reform cartilage tissue in vitro and in vivo. Comparing
to the conventional manual segregation method employed thus far to derive zonal chondrocytes [17,24], our method is not only efficient and labour-saving, but also in principle, applicable for processing tissue of any size and shape, such as debrided cartilage derived from trauma-induced cartilage lesions, where clear demarcation of zonal layers to tissue depth is not available, thus cannot be segregated by conventional manual methods.

5. Conclusion

In summary, we have established a protocol to efficiently separate, and enrich, superficial, middle and deep zone chondrocytes from full thickness articular cartilage based on cell size differences using a spiral microchannel device. The size-sorted cells showed maintainable zone-specific characteristics after two passages of culture expansion, formed zone-specific cartilage tissue in hydrogel constructs in vitro, and facilitated better cartilage regeneration in vivo when delivered in bi-layered hydrogel constructs in rodent cartilage defect sites, compared to conventional chondrocyte implantation approach. This study provides an effective approach to obtain zonal chondrocytes in large number, and provides the proof-of-concept of delivery stratified zonal chondrocyte implantation for the clinical repair of critical size articular cartilage defects that can cater to defects of variable shapes and sizes.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiomaterials.2018.02.050.

References


