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A novel culture system for modulating single cell geometry in 3D

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ABSTRACT

Dedifferentiation of chondrocytes during *in vitro* expansion remains an unsolved challenge for repairing serious articular cartilage defects. In this study, a novel culture system was developed to modulate single cell geometry in 3D and investigate its effects on the chondrocyte phenotype. The approach uses 2D micropatterns followed by *in situ* hydrogel formation to constrain single cell shape and spreading. This enables independent control of cell geometry and extracellular matrix. Using collagen I matrix, we demonstrated the formation of a biomimetic collagenous "basket" enveloping individual chondrocytes cells. By quantitatively monitoring the production by single cells of chondrogenic matrix (e.g. collagen II and aggrecan) during 21-day cultures, we found that if the cell's volume decreases, then so does its cell resistance to dedifferentiation (even if the cells remain spherical). Conversely, if the volume of spherical cells remains constant (after an initial decrease), then not only do the cells retain their differentiated status, but previously de-differentiated redifferentiate and regain a chondrocyte phenotype. The approach described here can be readily applied to pluripotent cells, offering a versatile platform in the search for niches toward either self-renewal or targeted differentiation.

1. Introduction

Cartilage injury remains a major challenge in orthopedic treatments due to a very limited self-repairing potential of articular cartilage [1]. Recently, autologous chondrocyte implantation (ACI) has emerged as a promising clinical procedure for repairing large or seriously damaged articular cartilage [2–4]. Although a number of satisfactory clinical cases have been demonstrated using ACI treatment [4,5], a number of unsolved challenges remain [6]. The success of ACI depends on the availability of a sufficient number of chondrocytes. Conventionally, this is done through *in vitro* cell expansion in monolayer culture. However, chondrocyte dedifferentiation (i.e. loss of their differentiated chondrocyte phenotype) often occurs, resulting in the formation of inferior cell types and consequently treatment failure [7–10].

To address this challenge, various strategies have been developed to prevent chondrocyte dedifferentiation or to regenerate the chondrogenic phenotype. These can be broadly classified as follows: (1) employing scaffolds for 3D culture, such as collagen scaffolds [11–14] and biodegradable polylactide polymers [15,16]; (2) Using stimulating factors in culture medium, such as cytoskeletal inhibitors [17], growth factors [18], and other stimuli [19]; and (3) employing suspension [20], aggregated [21] or pelleted cultures [22]. A common theme in these approaches is to maintain or restore spherical cell shape, which is believed to promote the adoption of a chondrocytic phenotype.

However, conventional cell handling methods have limited control over factors such as microenvironment, culture conditions and cell–cell distance. As a consequence, many findings are open to debate [7,22–24]. For example, chondrocytes embedded in a 3D alginate gel have a spherical shape, but can either maintain a chondrocytic phenotype or undergo dedifferentiation [7,23]. Variations of cell seeding density and pellet sizes in pellet cultures have all been shown to lead to significantly different outcomes on chondrocyte phenotypes [14,25,26]. Methods that can precisely control the microenvironments of chondrocytes are needed for in-depth understanding of the effects of cell geometry on the chondrocyte phenotype.

In the last decade, micropatterning has become a versatile tool for the study of geometrical influences on cell function at the single cell level in 2D culture [27–32]. Using single cell patterning, various studies show that cell shape and area both control cell growth, death [28,31,33], and regulation of stem cell differentiation [27,29]. Surprisingly, studies concerning single chondrocytes are limited [30]. However, how cell geometry in 3D affects cell function remains illusive. One major reason for this lack of understanding is rooted in the difficulties of controlling cell morphology in a complex 3D setting and for long periods of culture [34].

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In this study, we have developed a novel approach to investigate the effects of cell shape and cell spreading on chondrocyte dedifferentiation and re-differentiation at the single cell level. We developed a 3D single cell culture system that exploits single cell patterning with *in situ* matrix gelation, to create well-defined microenvironments around individual cells. Synthesis of cartilage-specific matrix, i.e., collagen type II (Col II) and chondroitin-sulfate containing proteoglycan (CSPG) by individual chondrocytes was quantitatively evaluated. During periods of 21 days in culture, the influence of cell shape and spreading on both retaining and regaining the chondrocyte phenotype in 3D cultures was revealed.

2. Experimental section

2.1. Fabrication of micropatterned glass substrates

Micropatterned glass surfaces with two different chemistries were fabricated as previously reported [35]. Briefly, a thoroughly cleaned glass substrate was first functionalized with 5% of (3-aminopropenyl)trimethoxyl silane (97%, Sigma–Aldrich Co) in ethanol to generate an amino-terminated surface. Thereafter it was spin coated with S1805™ G2 photoresist (Rohm and Hass Co.) followed by a standard photolithographic procedure to form a photoresist pattern on the substrate. Oxygen plasma etching was then employed to remove amino-groups on the exposed surface. A second surface modification of the exposed area was conducted in 1% 2-(methoxy(polyethyleneoxy)propyl)trichlorosilane (PEG-silane, 90%, Gelest Inc., Morrisville, PA) in dry toluene under vacuum. Finally the photoresist layer was removed using sonication in DMSO.

2.2. Contact angle

To monitor each step of surface modification on a substrate, contact angles of the substrate were measured using a contact angle goniometer (Easy Drop, Kruss GmbH) and DMS software (based on the Young–Laplace equation). At least three randomly selected positions on each sample were measured using a 1 μl drop of distilled water. Contact angle value is given as mean ± standard deviation from triplicate samples.

2.3. Cell culture

Bovine chondrocytes were isolated from the proximal side of the metacarpal phalangeal and monolayer cultured in Dulbecco's Modified Engle Medium (1× DMEM, containing 4.5 g/l D-glucose, Gibco-Invitrogen, Life Technologies Ltd., UK) with 10% heat-inactivated fetal bovine serum (FBS, Gibco-Invitrogen), 2% antibiotic/antimicrotic (Gibco-Invitrogen), and 55 mg/l ascorbic acid. When the cell layer became confluent in 3 days, it was trypsinized using 0.05% trypsin/1.0 mM EDTA (Sigma), frozen as aliquots and stored at −80 °C as stock (i.e. passage 2). The cells were thawed and monolayer cultured likewise (passage 3), or repeatedly re-plated in a T75 flask with 1 × 106 cells to get a confluence of 2×10^6 cells (i.e. one population doubling) for each passage from 3 to 7 (denoted as P3 or P7).

2.4. Formation of single cell patterns of chondrocytes

Micropatterned substrates were sterilized by immersion in ethanol for 30 s followed by washing with Dulbecco's phosphate buffered saline (PBS) solution in a biological safety laminar hood. A confluent layer of chondrocytes either at P3 or P7 were trypsinized and suspended in culture medium. The cell suspension was then adjusted to desirable seeding densities for different sized patterns, namely 2×10^5, 0.5×10^5 and 0.2×10^5 cells/ml for 20, 40, and 80 μm micropatterns respectively. A patterned substrate was then immersed in a cell suspension of appropriate density for 1–2 h, followed by removal of cell suspension and a gentle rinse with fresh medium to remove loosely attached cells. The substrate with attached cells was then cultured at 37 °C in a humidified atmosphere with 5% CO_2 and with medium changed every 2 days. The unpatterned substrates were used as a control.

2.5. Formation of single cell 3D culture system

In situ gelation of collagen I solution was employed to form a 3D matrix around individual cells on patterns. Single-cell patterns were first cultured for 5 h prior to the gel formation. This was to allow cells to attach firmly to the surface and adapt their shape to the underlying patterns. To prepare collagen I solution for gelation, 80 μl of acidic collagen I solution (3 mg/ml, Sigma–Aldrich Co.) was neutralized to pH 7–8 with 20 μl of a mixture of PBS (10×) and 0.1 N NaOH aqueous solution at 1:1 ratio. The neutralized collagen I solution was pipetted onto the substrate (about 10 μl per cm^2) and allowed to spread evenly over single cell patterns. The gelation occurred at 37 °C and was allowed to proceed for 2–3 h. The whole system was then immersed in fresh medium for a specific period of culture at 37 °C in a humidified atmosphere with 5% CO_2 with medium changed every 2 days. Seeded chondrocytes on unpatterned aminosilane substrates were used as a control.

2.6. Immunofluorescence staining

Single cell patterns of chondrocytes were rinsed with PBS, fixed with 4% formaldehyde (containing 0.06 M sucrose) for 15 min at 37 °C and quenched in 0.05 M NH_4Cl for 15 min. For the single cell 3D culture systems, collagen I gel was mechanically removed before fixation since the presence of collagen I contributes to background fluorescence signals. Permeabilization of cells was performed in a permeabilizing buffer (containing 0.5% Triton X-100, 0.3 M sucrose, 0.05 M NaCl, 6.3 mM $MgCl_2.6H_2O$, and 0.02 M Hepes, pH7.2) for 5 min at 4 °C. The fixed cells were stained with Alex Fluor 488 Phalloidin (Life Technologies Ltd.) in 1% bovine serum albumin in PBS buffer (BSA solution) for 30 min at 37 °C, followed by blocking treatment with 1% BSA solution for 1 h at room temperature.

Primary antibody (1:200 dilution in 1% BSA solution) and fluorescence labeled secondary antibody (1:400 dilution in 1% BSA solution) staining was carried out at room temperature for 1 h successively. Each step was washed three times with 0.1% triton x-100 in PBS for 5 min to eliminate non-specific adsorption. Finally, a cover slip was mounted onto the stained samples using a Vectashield mounting medium with DAPI (Vector Laboratories Inc.). All samples were stored in darkness at 4 °C. Anti-collagen II polyclonal antibody from rabbit (ab300, Abcam, UK) and Alex Fluor 546 goat anti-rabbit lgG (H + L) (Life Technologies Ltd.) were used as primary and secondary antibodies respectively for collagen II staining. Anti-chondroitin sulfate monoclonal antibody from mouse (ab11570, Abcam) and Alex Fluor 647 donkey anti-mouse lgG (H + L) (Life Technologies Ltd.) were used for chondroitin-sulfate containing proteoglycan staining.

2.7. Qualitative and quantitative imaging

Phase contrast and fluorescence images were obtained using an inverted fluorescence microscope (Observer Z1, Zeiss), equipped with an ORCA-ER camera (HAMAMATSU ORCA-ER C4742–80, Hamamatsu photonics K. K.). A long working distance objective,

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LD plan-Neofluar 20×/0.4, was used to image live cells in 3D culture. To obtain high-resolution fluorescence images of the immunostained cells, EC Plan-Neofluar 20×/0.5 and 40×/0.75 objectives were used. Optical filter sets of 475AF40, 525AF45, 630AF50 and 365WB50 (Omega) were employed for detection of Alex Fluor 488 Phalloidin, Alex Fluor 546, Alex Fluor 647, and DAPI respectively.

For quantification of the amount of collagen II and CSPG produced by individual cells, fluorescence images of different samples were obtained with a low NA objective (EC Plan-Neofluar 10×/0.3) in order to capture signals from the whole cell and its periphery. The same acquisition settings were applied to all of the experiments. Prior to each experiment, the microscope and light source (e.g. incident light intensity) was adjusted so that a standard 10 μM fluorescein solution gave a predefined fluorescence intensity, thus enabling relative quantification of fluorescence intensities of samples between different experiments.

2.8. Data analysis

The total amount of collagen II and CSPG matrix produced by individual chondrocytes (denoted as F_{CSPG} and F_{col-II} respectively) was evaluated based on their immunofluorescence intensity in the pattern where cells were confined. The total value of fluorescence intensity per defined area (i.e. the area of a single circle in each of the three micropatterns) was calculated from immunofluorescence (using EC Plan-Neofluar 10×/0.3 objective) using *Image J* software and with background subtraction. This is to take into account both intracellular synthesis and the deposition of extracellular matrix by cells. For cells on the unpatterned substrates, a circular area of 80 μm was used as the defined area during image analysis. To take into account the difference in 2D and 3D cultures, the quantitative comparison is either among cells on different patterns without collagen I coating (i.e. 2D system) or among cells on different patterns with collagen I coating (i.e. 3D system). For each condition, independent experiments ($n = 3$) were conducted, and 50–80 randomly chosen cells were measured from each experiment. Unless noted, average values and standard deviation from triplicate are given. All statistic analysis was performed using Prism software. A difference was regarded as significant when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) following a two-way ANOVA with post hoc testing unless otherwise denoted.

3. Results

3.1. Fabrication and characterization of chemical micropatterns

Fig. 1 illustrates the micropatterned glass substrates with two chemistries on the surface, namely amino- and Polyethyleneglycol (PEG)-groups respectively. Three circular patterns with diameters of 20, 40, and 80 μm were prepared. The distance between two adjacent circles was either 80 or 100 μm. Fabrication of the patterned substrates was carried out as previously reported [35], and monitored at each step. After the first step of amino-silanisation of a whole glass substrate, contact angle of the substrate increased from <5° to 39.9° ± 0.8, indicating successful generation of an amino-terminated surface. The second step involved photopatterning of a photoresist layer to protect the amino-terminated surface underneath. This was followed by oxygen plasma etching to remove amino-groups on the exposed area and a second silanisation with PEG-silane. As shown in Fig. 1 (top), the photoresist patterns remained unaffected after these processes. This allowed transferring of pre-designed dimensions precisely to the protected amino-terminated surface underneath. After the removal of the photoresist layer, a 2D micropatterned surface was generated that comprised precisely defined amino-terminated circles surrounded by a non-adhesive PEG-terminated space.

3.2. Controlling cell shape and spreading of chondrocytes on 2D micropatterns

Within about two hours of culture, chondrocytes adhered exclusively onto the pattern areas, due to the adhesiveness of the amino-terminated surface [36] and the 'non-stick' nature of the surrounding inert PEG layer. Since the pattern areas spanned from 314 μm^2 (i.e. 20 μm patterns), to 1256 μm^2 (i.e. 40 μm patterns), and to 5024 μm^2 (i.e. 80 μm patterns), cell-seeding density was optimized for different dimensions to achieve one cell per pattern. As shown in Fig. 2(A), patterns with no more than one cell per element have been obtained for each pattern size. After 5 h in culture, 100% of cells on the 20 μm patterns adopted a "3D" like spherical shape without spreading. >81% of cells on 40 μm patterns spread over the underlying patterns and took on a round shape. >70% cells on 80 μm patterns were elongated, although most of these had a crescent-like body that followed the circumference of the underlying circular patterns. In contrast, >88% cells on the unpatterned aminosilane surface (used as a control) were fully extended with random polygonal shapes.

However, after one day in 2D culture, cells started to migrate outside the patterns. Taking an example of cells on the 20 μm patterns (Fig. 2(B)), by day 2 in 2D culture, neighboring cells started to form a bridge between adjacent pattern elements. The migrating cells were fully extended, taking on fibroblastic morphologies. This limitation of 2D patterns in confining the cells probably resulted from the loss of non- adhesiveness from the surrounding PEG region due to the extracellular matrix deposited by cells [35] or the adsorbed serum proteins from culture medium, covering the PEG motifs.

3.3. Developing a biomimetic single cell culture system in 3D

In vivo, chondrocytes are surrounded by their own matrix, they do not form cell-to-cell contacts, and individuals have a collagenous "basket" enveloping their pericellular matrix [37]. To mimic this microenvironment, *in situ* formation of collagen I matrix was employed to form a collagenous "basket" around individual cells. This was done by simply pouring a collagen I matrix solution over cell patterns formed after 5 h in 2D culture (Fig. 3(A)). Importantly, it was found that the matrix gel remained attached during the extended periods of culture (up to 21 days) despite regular medium exchange, indicating reasonable affinity between the collagen I matrix and the amino-terminated surface. This novel 3D culture system provided a well-defined enclosure to modulate cell shape as well as preserve secreted matrix.

Fig. 3(B) shows cell morphologies within the confined 3D microenvironments during the course of a 21 day culture. All the cells remained on patterns for the whole period of the experiments. An obvious reduction in cell dimension was observed for all the cells (Table 1). At day 2, cells on 40 μm patterns showed the largest percentage reduction, ~40% of their initial size at 5 h in culture. Interestingly, following this initial reduction no further size changes were observed during the 21 day culture. In contrast, a continual reduction was observed for cells on the other patterned substrates (Table 1). For example, at day 21, the dimension of cells on 20 μm and 80 μm patterns had reduced to 67% and 68% of their size at 2 days, but during the same period, the size of cells on the 40 μm patterns only changed by 2%. All cells were viable at day 21 and proliferated upon removal of the collagen I matrix (data not shown).

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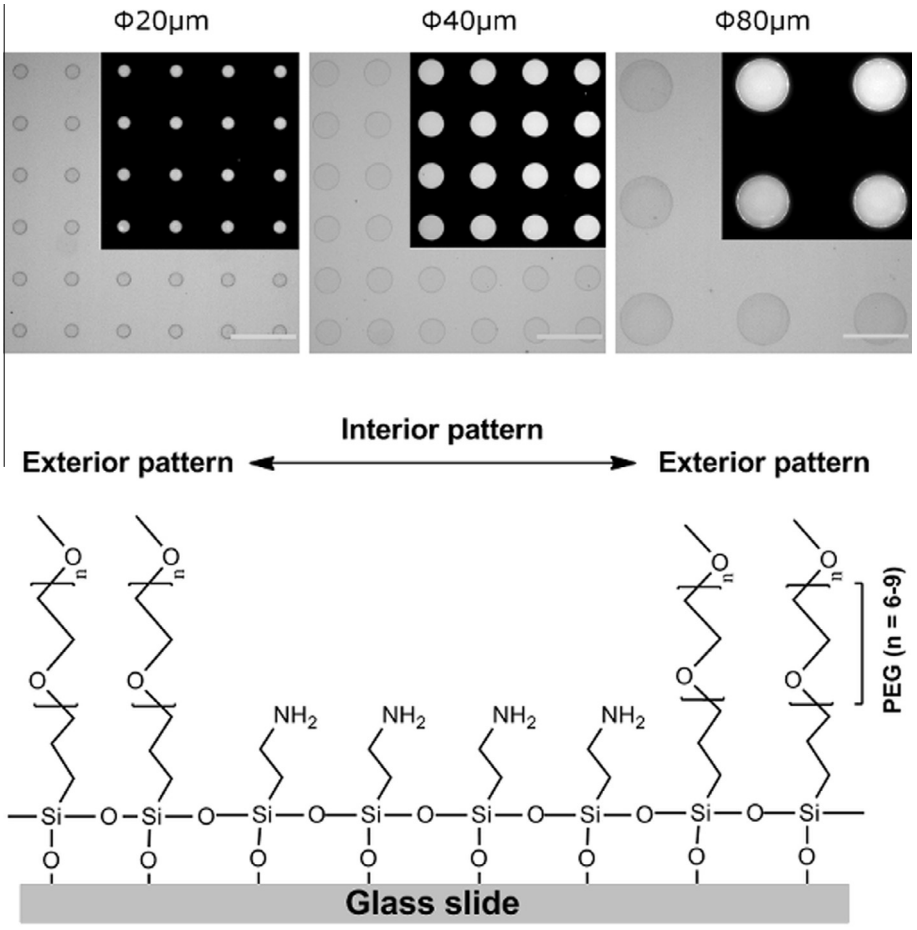

Fig. 1. (Top) Phase images of different photoresist patterns on glass surface. Inserts show enlarged fluorescent images. Scale bars are 100 μm. (Bottom) Chemical structure of the amino-terminated surface.

3.4. Effects of cell shape and spreading on retaining chondrocyte phenotype

3.4.1. Short-term effects in 2D cultures

To investigate this effect, passage 3 chondrocytes were used as they have a certain degree of differentiated phenotype [7,10]. The immunofluorescence images of chondrocytes on 2D patterned substrates at 5 h in culture are shown in Fig. 4 and bright field images in Fig. S1 (Supporting Information). Cells on 20 μm and 40 μm patterns revealed a ring-like F-actin network concentrated at the cell periphery, whereas elongated stress fibers were shown on some cells on 80 μm patterns and the majority of cells on the unpatterned surface.

CSPG is one of the major components of aggrecan in articular cartilage [37,38]. Production of extracellular CSPG is one of the features of chondrocytes. As shown in Fig. 4(A), punctate patterns of CSPG were observed for all cells. Co-localization of F-actin and the CSPG pattern revealed the deposition of CSPG in the extracellular space for cells on 40 μm, 80 μm patterns and the unpatterned surface. Although the presence of CSPG at the periphery of cells on 20 μm patterns was not obvious, it was found on the cell membrane via z-scan imaging (Fig. S2, Supporting Information).

Synthesis and extracellular deposition of collagen II is a key phenotypic marker of differentiated chondrocytes [39,40]. At 5 h, collagen II was observed in all cells (Fig. 4(A)) but with different distribution patterns. For cells on patterns, collagen II immunofluorescence was concentrated in cytoplasm around the nucleus of the cell. In contrast, cells on unpatterned surfaces have scattered patches within and around the cell body. No obvious extracellular deposition of collagen II was shown at the cell periphery, suggesting that the synthesis of collagen II was mainly intracellular at this time point.

For quantitative comparison of the matrix production by individual cells, the total immunofluorescence intensity of CSPG or collagen II staining per cell (F_{CSPG} and $F_{col\ II}$) was measured. F_{CSPG} of cells on patterns are either comparable to (20 μm and 80 μm patterns) or higher than (40 μm patterns) that on unpatterned surface (Fig. 4(B)). In the case of $F_{col\text{-}II}$, no significant difference was observed between cells on 40 μm, 80 μm and unpatterned surfaces, although the values were lower for cells on 20 μm patterns (1.5-fold, $p < 0.0001$). However, considering $F_{col\text{-}II}$ and F_{CSPG} of cells on the 20 μm and 40 μm patterns were obtained from substantially smaller areas (i.e. 314 μm^2 and 1256 μm^2 respectively) compared to that for cells on unpatterned surfaces (i.e. 5024 μm^2 of a circular area of 80 μm), it is clear that the spherical shape is beneficial for the production of CSPG and collagen II.

After 2 days in 2D culture, most cells had migrated outside the pattern and adopted polygonal shapes, similar to that of those on unpatterned substrate. A strong fibrous F-actin structure was present on the majority of cells, which was accompanied by disappearance of both CSPG and collagen II (Fig. S3, Supplementary Information), indicating the loss of differentiated phenotype.

3.4.2. Long-term effects in 3D culture

The biomimetic 3D culture system confined cells on patterns for weeks thereby enabling the investigation of the long-term effects

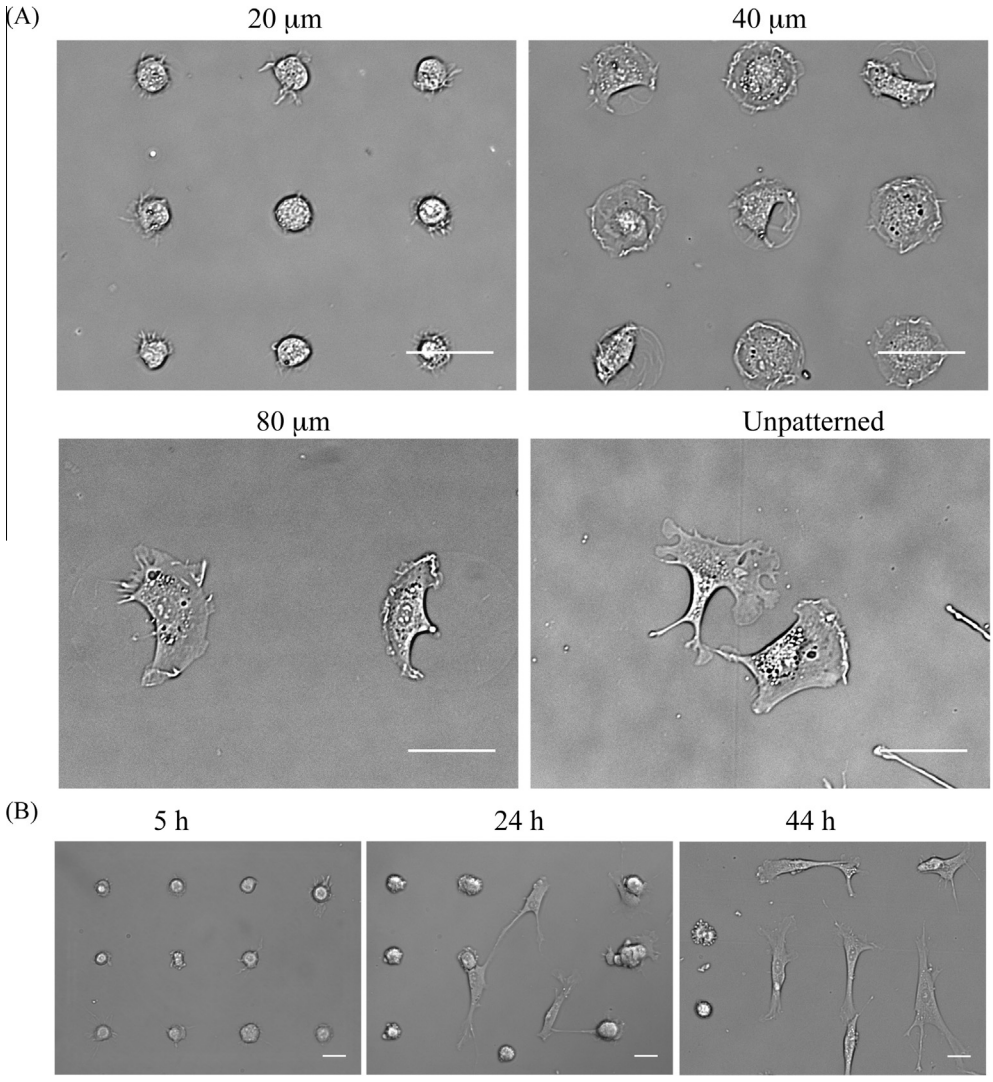

Fig. 2. (A) Bright field images of single chondrocytes on various patterns and unpatterned surface after 5 h in 2D culture. Scale bars are 50 μm. (B) Bright field images of chondrocytes on 20 μm patterns at different times in 2D culture. Scale bars are 20 μm.

of cell geometries on chondrocyte dedifferentiation. At day 2, all cells showed CSPG immunofluorescence within their main body (Fig. 5(A) and Fig. S4, Supporting Information). There was no significant difference in F_{CSPG} between them (Fig. 5(B)). At day 10, substantial extracellular deposition of CSPG was only observed for cells on 40 μm patterns (Fig. 5(A)). In addition, F_{CSPG} of cells on 20 μm and 40 μm patterns increased by 3.3 and 4.3-fold respectively compared to that at day 2 (Fig. 5(B), $p < 0.0001$). No significant difference was found for cells on 80 μm patterns and unpatterned substrates (Fig. 5B). In addition, F_{CSPG} of cells on 40 μm patterns was significantly higher than the others ($p < 0.0001$). Unfortunately, at day 21, cells on all the substrates showed substantially reduced CSPG. However, this is probably due to the loss of CSPG upon mechanical removal of the collagen I coating. As illustrated previously [39], CSPG reaches farther out into the extracellular space than does type II collagen. At day 21, collagen I gel appeared to be more strongly adhered to the substrate. It is likely that a portion of the outmost CSPG attached to the gel and was removed with the collagen I gel.

Similarly, at day 2, all cells showed strong collagen II immunofluorescence (Fig. 6(A)), indicating the existence of a certain degree of differentiated phenotype. Filament-like collagen II was shown in cells on patterns and was mainly restricted to within the cell body (Fig. 6(A), right column). In contrast, punctate collagen II was scattered within the cell body and nearby surface for cells on unpatterned substrates. At day 10, a thick ring-like collagen II layer had developed at the periphery of cells on 40 μm patterns, and was accompanied by a slight increase in $F_{col\text{-}II}$ (Fig. 6(B), $p < 0.01$) – an indication of regeneration of chondrogenic phenotype. In contrast, collagen II was mainly located within the cell body for cells on the other substrates, which showed significant decrease in $F_{col\text{-}II}$ ($p < 0.0001$).

At day 21, $F_{col\text{-}II}$ of cells on 20 μm patterns was further reduced (Fig. 6(B), $p < 0.001$). For cells on 40 μm patterns, extracellular collagen II deposition was still shown at the cell periphery. Although their $F_{col\text{-}II}$ values were slightly reduced, they were still comparable to those seen on day 2 ($p > 0.05$). No significant changes were shown for cells on 80 μm patterns and unpatterned substrates ($p > 0.05$). It should be noted, $F_{col\text{-}II}$ of cells on 40 μm patterns was higher than on other patterns during the whole period of culture.

Taken together, all these observations clearly indicate that cells on 40 μm patterns have the strongest resistance to dedifferentiation, as discussed further below.

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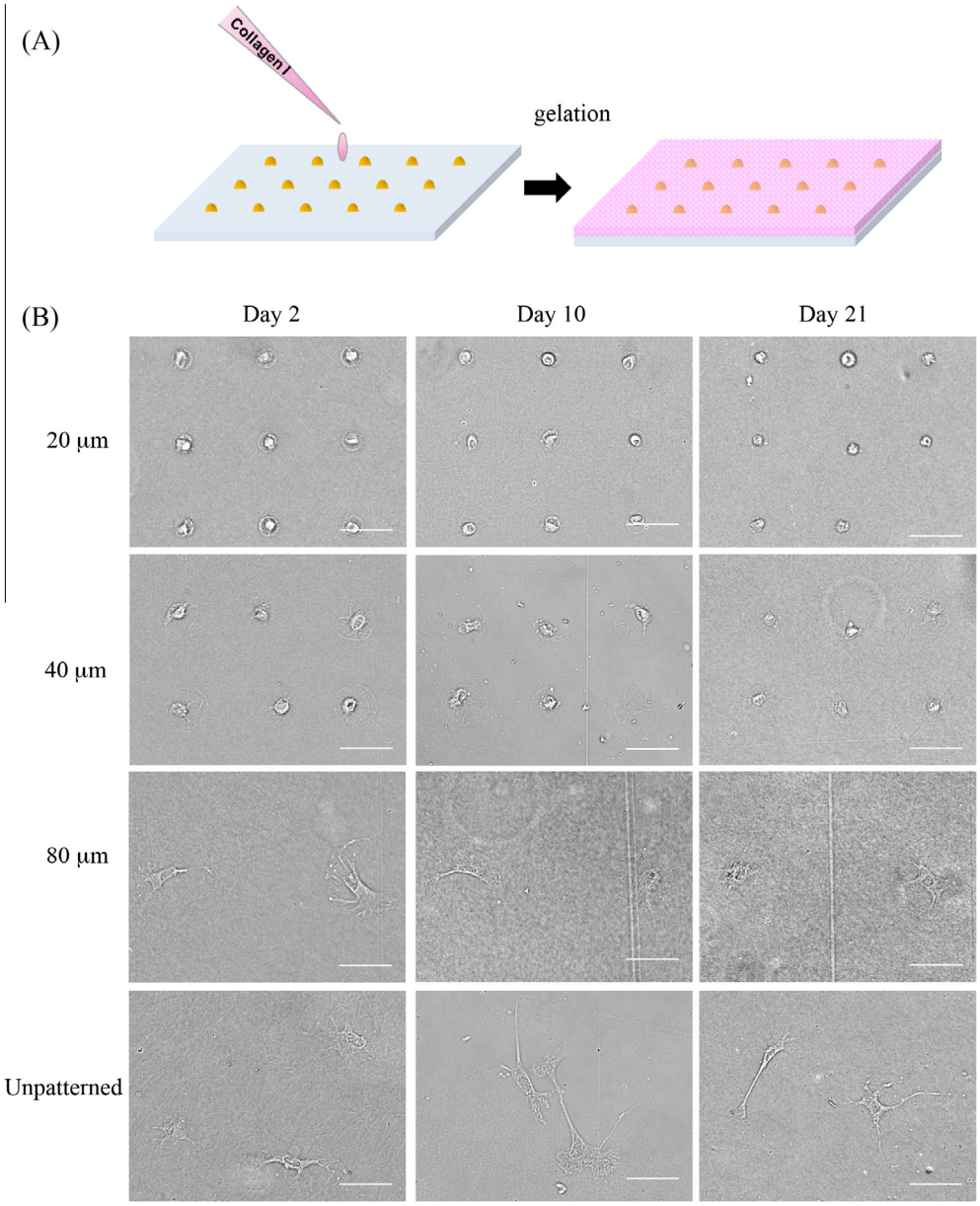

Fig. 3. (A) Schematic drawing of the formation of single cell culture in 3D. (B) Bright field images of single chondrocytes on various patterned and unpatterned surface in 3D culture for a period of 21 days. Scale bars are 50 μm. It should be noted, the blurred background in the images is mainly from the collagen gel matrix.

Table 1
Variation of cell area on different patterns during the period of culture.*

Pattern diameter (μm)	5 h in 2D (μm²)	Day 2 in 3D (μm²)	Day 10 in 3D (μm²)	Day 21 in 3D (μm²)
20	377 ± 90	320 ± 80	208 ± 57	216 ± 34
40	835 ± 296	335 ± 101	363 ± 151	341 ± 106
80	1294 ± 630	991 ± 460	952 ± 399	677 ± 347
Unpatterned	1591 ± 803	972 ± 714	964 ± 429	987 ± 404

* Cell areas were determined from phase images using Image J software.

3.5. Effects of cell shape and spreading on regeneration of differentiated phenotype

The apparent benefit with regard to maintaining a differentiated phenotype, of controlling cell shape and restricting spreading in long-term culture raises a question; whether such confinement can also promote the regaining of a chondrocytic phenotype after cells have become dedifferentiated? To examine this potential, we used monolayer expanded passage 7 (P7) chondrocytes which are commonly considered as "irreversibly" dedifferentiated [12].

P7 chondrocytes were cultured on the 2D patterned substrates in the same condition as P3 cells. To maintain the majority of cells in a spherical form, only 20 μm and 40 μm patterns were used. After 5 h in culture, P7 chondrocytes developed similar geometries to the P3 chondrocytes on patterned and unpatterned substrates shown in Fig. 2(A). However, a much lower level of collagen II and CSPG immunofluorescence was observed for all P7 cells in comparison to P3 cells (Fig. S5, Supporting Information), indicating they were dedifferentiated.

After formation of the confined 3D culture system and following 2 days in culture, CSPG were present in all cells (Fig. 7(A)). At day 10, F_{CSPG} of cells on 20 μm and 40 μm patterns increased (Fig. 7(B), $p < 0.05$ and $p < 0.0001$ respectively). However, no significant difference was observed for cells on unpatterned substrates. Similarly, at day 2, clear collagen II fluorescence was shown in cells

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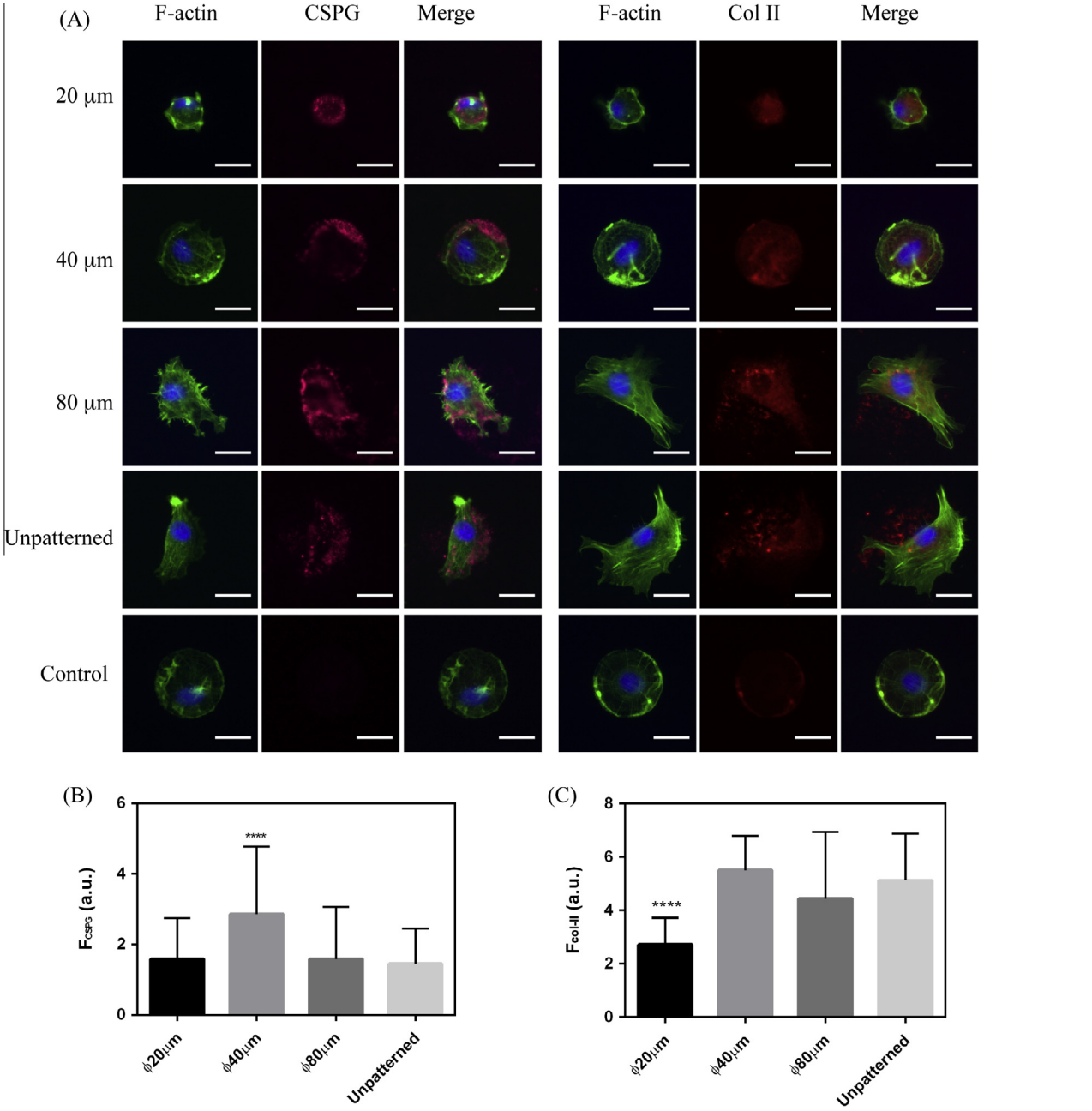

Fig. 4. (A) Immunofluorescence images of P3 chondrocytes on various substrates at 5 h in culture. Pseudo-colors are used to show different stains, namely blue – nuclei, green – F-actin, pink – CSPG and red – collagen II. Scale bars are 20 μm. The absence of the primary antibodies was used as a control. The observed negligible fluorescence signal indicates that non-specific adsorption of the secondary fluorescence-labeled antibodies is low. The absence of the primary anti-CSPG antibodies in each case was used as a control. Representative images of cells on the 40 μm patterns are shown here. (B) Mean fluorescence intensity per cell of CSPG (F_{CSPG}) and (C) of collagen II (F_{col-II}) by individual chondrocytes on different substrates after 5 h in culture. Data were analyzed using a one-way ANOVA with Turkey's test. Standalone asterisks denote that the condition is compared to all the others in the figure.

on both 20 μm and 40 μm patterns in comparison to weak signals in cells on unpatterned substrates (Fig. 8(A)). At day 10, F_{col-II} of cells on 20 μm and 40 μm patterns increased (Fig. 8(B), $p < 0.05$ and $p < 0.0001$ respectively) whereas cells on unpatterned substrates showed no significant difference. However, only the cells on 40 μm patterns deposited substantial extracellular collagen II which surrounded the cell periphery as a thick ring. Furthermore, the F_{col-II} of cells on 40 μm patterns was significantly higher than the others ($p < 0.005$). These results suggest that initially dedifferentiated P7 chondrocytes on 40 μm patterns regained their chondrocytic phenotype by day 10 in this confined 3D culture environment.

4. Discussion

By using micropatterned substrates to control the adhesion of single cells, many studies have shown that cell shape and area play important roles on cell fate (e.g. differentiation, apoptosis and growth) [28,31,41]. Despite the abundance of information from 2D culture, understanding the role of patterning in 3D is limited. The novel culture system used in the present work is capable of independent control of individual cell geometries and extracellular matrix. For the first time, dedifferentiation and redifferentiation of chondrocyte phenotypes were investigated here in biomimetic 3D microenvironments at the single cell level.

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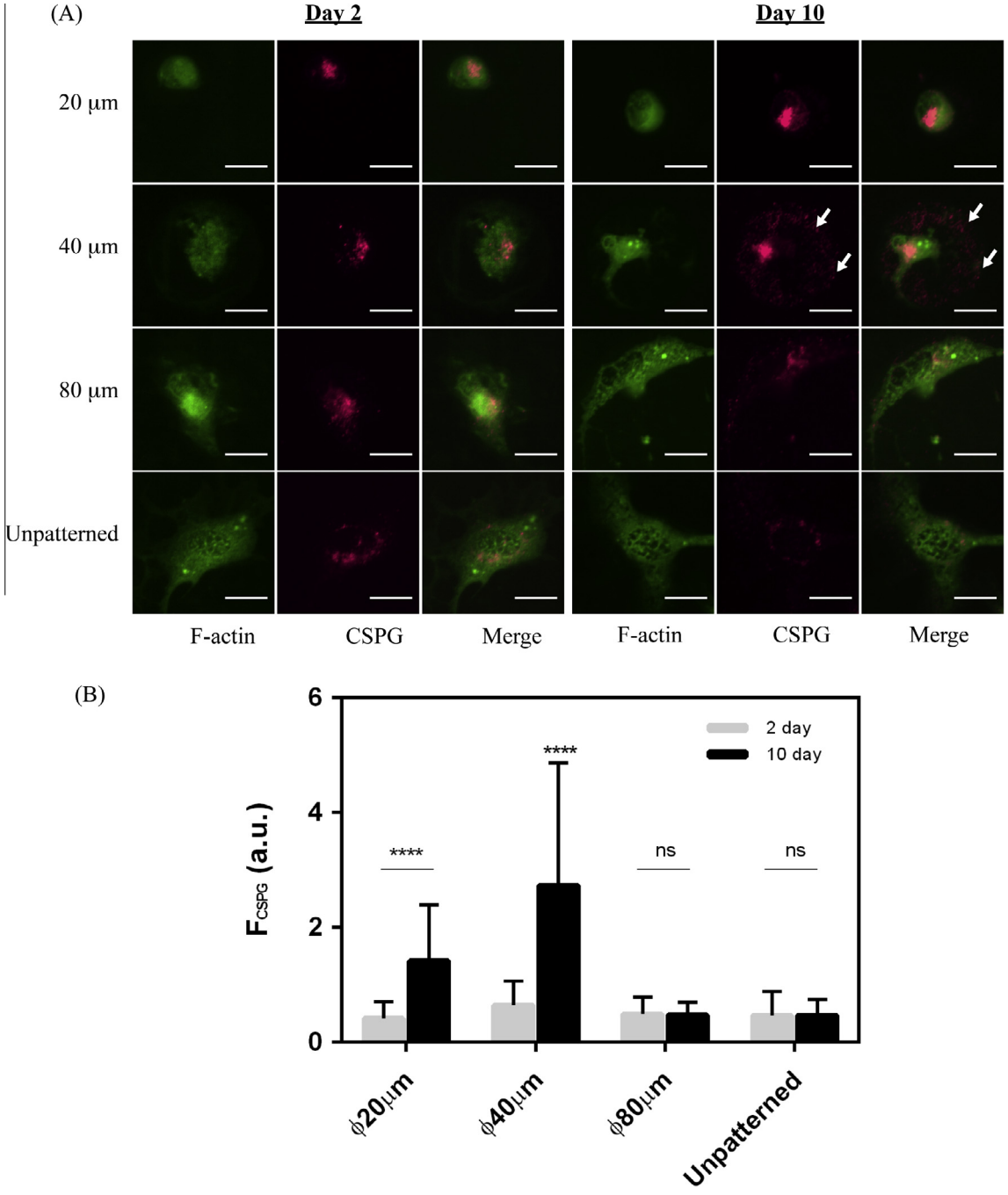

Fig. 5. (A) Representative immunofluorescence images of CSPG staining of single chondrocytes (P3) in 3D culture. Pseudo-colors represent different staining: blue – nuclei, green – F-actin, and pink – CSPG. Scale bars are 20 μm. White arrows indicate the deposited CSPG. (B) F_{CSPG} of P3 chondrocytes on different substrates during the course of culture.

4.1. Dedifferentiation depends on the interplay of cell geometries and cell-matrix interactions in 3D

To overcome chondrocyte dedifferentiation during *in vitro* expansion, substantial efforts focused on maintaining a spherical cell shape. Despite various positive effects being illustrated, contradictory findings from similar conditions have also been reported [7,18,23,25,26,42,43]. These raise some very basic questions, for instance, whether the spherical shape is the dominant factor in preventing dedifferentiation, and how "spherical" a cell should be? How does cell geometry evolve in a long-term culture where cells are under constant regulation of a range of cues (e.g. cell-to-matrix interactions and physical stress)?

Through using a single cell approach, we aimed to address these questions by progressively moving from a simple 2D environment to a more complex 3D setting. As illustrated, the use of 2D adhesive micropatterns was effective in controlling both cell shape and spreading over a short period of time. Different percentages of spherical cells (Table S1, Supporting Information) can be easily tuned with geometric constrains. Immunofluorescence staining has been well-established for high-sensitive and specific analysis of chondrogenic CSPG and collagen II matrix [39,44]. In combination

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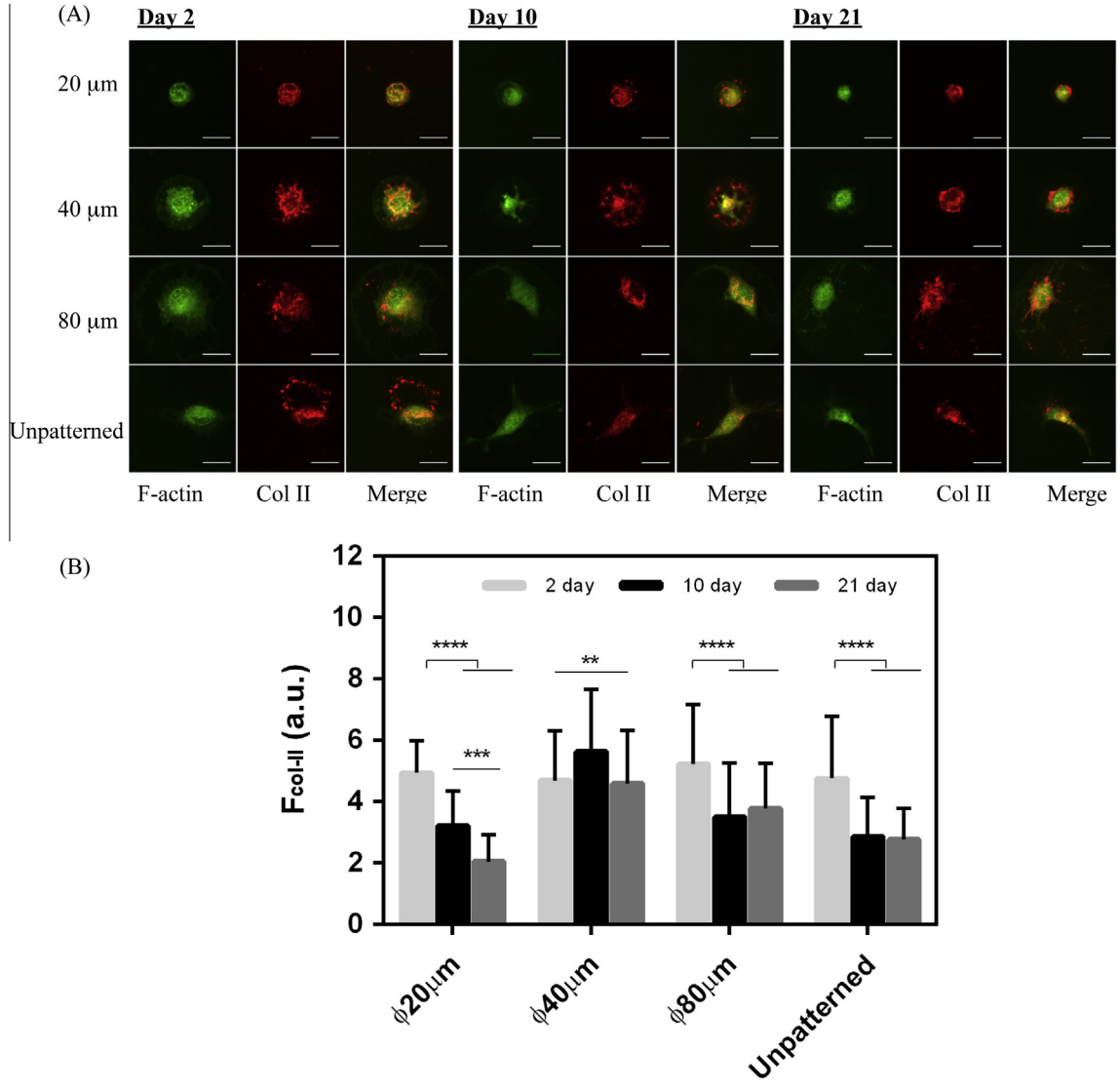

Fig. 6. (A) Representative immunofluorescence images of collagen II staining of single chondrocytes (P3) in 3D culture. Pseudo-colors represent different staining: blue – nuclei, green – F-actin, and red – Collagen II. Scale bars are 20 μm. (B) (F_{col-II}) of P3 chondrocytes on different substrates during the course of culture.

with quantitative imaging, this allowed relative quantitation of fluorescence intensity per pattern and thereby objective comparison of the total production of chondrogentic matrix by individual cells. This approach revealed that a spherical cell shape is beneficial for the production of CSPG and collagen II, and importantly, this influence could occur on a 2D substrate, at the very early stages of culture.

However, in 2D, maintaining the spherical cell shape alone was not efficient with respect to inhibiting dedifferentiation in long-term culture, as reported by recent work where single chondrocytes were cultured on round RGD patterns (from 10 μm to 30 μm diameter) for up to 10 days [30]. Dedifferentiation occurred for cells on all patterns, and the probability of dedifferentiation increased for larger patterns (e.g. ~65% on 30 μm dots) [30]. The present work, as discussed below, illustrates that this trend is different in 3D settings.

In conventional 3D culture, variations of material composite and culture conditions often lead to drastically different microenvironments around individual cells. In contrast, this single cell 3D approach offers identical extracellular microenvironments to cells. Cell shape was precisely controlled on a 2D pattern, and was tracked before and after the formation of the extracellular matrix. As a result, the initial physical stress of the matrix on the reduction of cell dimension were revealed and quantified (Table 1). The round but more spread cells on 40 μm patterns seem to have the least resistance to the physical stress in comparison to well-elongated cells and tightly round cells on 20 μm patterns, indicating their relatively flexible cytoskeleton. This flexible cytoskeleton appears to be critical in maintaining constant cell volume during the 21 day culture, and encouraged redifferentiation. This phenomenon highlights the critical influences on cell fate due to the variations in seeding density and cell-matrix holding time [13,45], since they all affect initial cell morphology in a matrix.

Compared to the majority of 3D culture systems, the current single cell 3D culture eliminates cell-to-cell contacts and is thus similar to the environment of mature chondrocytes in cartilage [37]. Over a short period of time, we have found that this restriction enhances cell resistance to dedifferentiation. All cells at day 2 in 3D culture, either spherical or elongated, clearly produced CSPG and collagen II in comparable amounts (Figs. 5 and 6), while their counterparts in 2D culture showed little or no production (Fig. S2, Supporting Information). This finding agrees well with earlier studies where elimination of cell–cell interaction (e.g. via

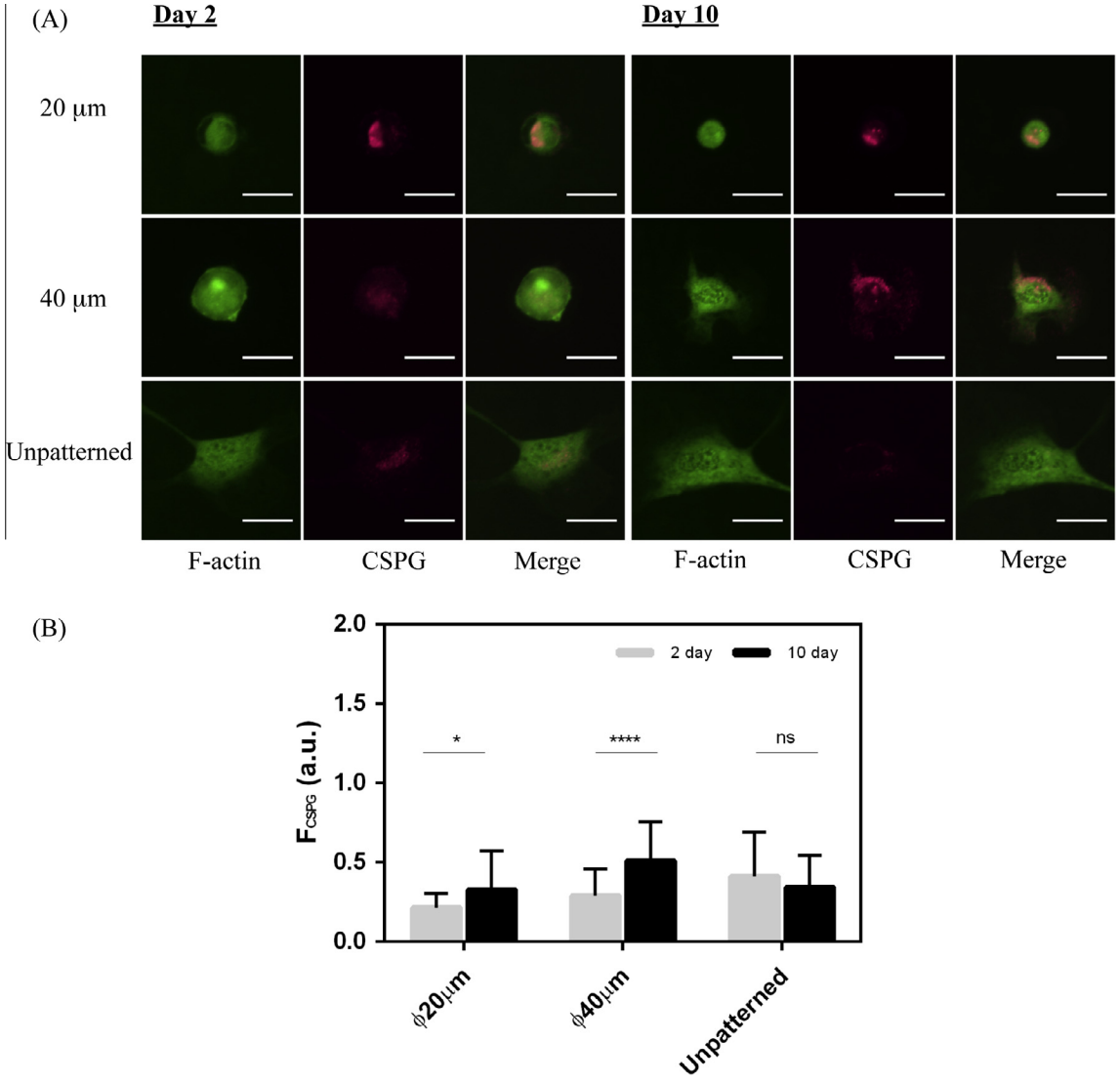

Fig. 7. Redifferentiation of P7 chondrocytes on different substrates. (A) Representative immunofluorescence images of CSPG staining of single chondrocytes at day 2 and day 10 in 3D culture. Pseudo-colors represent different staining: blue – nuclei, green – F-actin, and pink - CSPG. Scale bars are 20 μm. (B) F_{CSPG} of P7 chondrocytes during the course of culture.

silencing Ob-cadherin expression) in cultured chondrocytes can reduce dedifferentiation [14,46].

Over a long period of culture, variations in cell shape and spreading in 3D gave distinctively different effects compared to those observed with 2D culture [30]. The extent of dedifferentiation increased with a decrease in cell volume for cells of perfect roundness (i.e. cells on 20 μm patterns with aspect ratio close to 1). In contrast, the less spherical and more spread cells (i.e. cells on 40 μm patterns) not only showed a higher resistance to dedifferentiation, but also the capability of regenerating the differentiated chondrocyte phenotype (Fig. 7). Concomitant with this is the presence of an extended peri-cellular F-actin network in cells on 40 μm patterns but not in cells on 20 μm patterns. Since chondrocytes interact with both collagen I and amino-terminated surfaces, this difference suggests in the case of cells on 40 μm patterns, both interactions might play equal roles, whereas in the case of cells on 20 μm patterns, the interaction with the collagen I matrix might dominate. This shows that in 3D not only cell shape but also cell-to-matrix interactions play a critical role.

The interplay of cell shape and cell-matrix interaction on cells can be also derived from cell volume changes during long-term culture. The constant shrinkage of cells on 20 μm patterns is a clear indication of the combined influence from both cell-matrix interaction and the mechanical contraction of the gel [34,47,48]. Alterations in cell volume were shown to be involved in a series of cell functions, including metabolic activities and gene expressions [49], and therefore may account for their tendency toward dedifferentiation. The capability of cells on 40 μm patterns to maintain a constant cell volume is likely to be due to their flexible cytoskeleton, which may be able to modulate the matrix tension toward chondrogensis [50]. Since the deposited collagen II matrix was accumulated locally, this created more physiological-like microenvironments and can further enhance the retaining and regaining of differentiated phenotype [18].

4.2. The implications of the 3D single cell approach in ACI therapies

Currently, re-differentiation of chondrocyte monolayer expansion often involves the use of soluble additives, which may cause potential complications for ACI therapies [6]. The current biomimetic 3D culture system facilitates retaining and regaining differentiated chondrocytes. Its capability of transforming advanced,

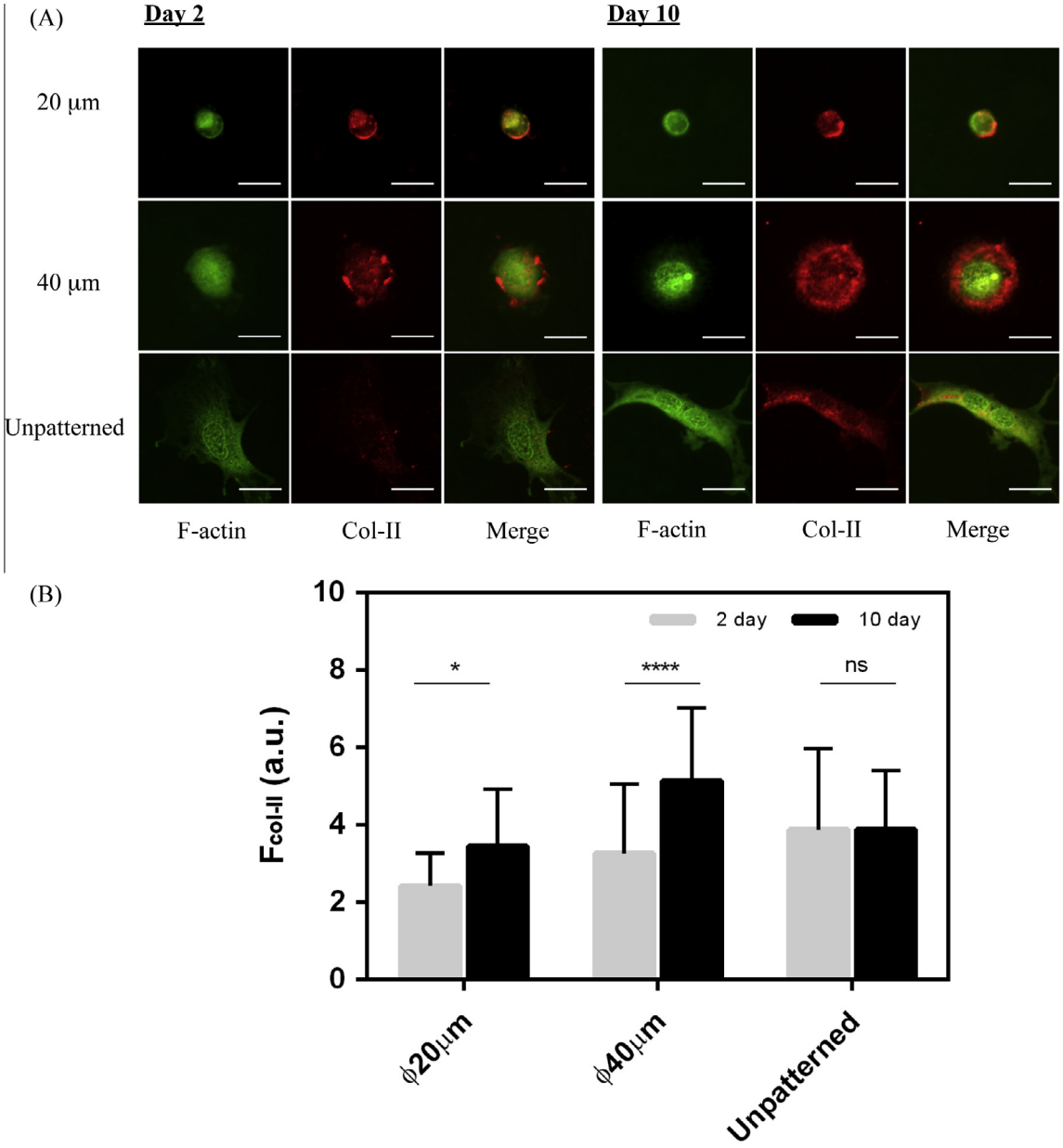

Fig. 8. Redifferentiation of P7 chondrocytes on different substrates. (A) Representative immunofluorescence images of collagen II staining of single chondrocytes at day 2 and day 10 in 3D culture. Pseudo-colors represent different staining: blue – nuclei, green – F-actin, and red – collagen II. Scale bars are 20 μm. (B) F_{col-II} of P7 chondrocytes during the course of culture.

dedifferentiated P7 chondrocytes into differentiated cells without any soluble additives illustrates a new avenue for re-differentiation of expanded chondrocytes. In addition, individual cells were under almost identical microenvironments, thereby reducing re-differentiation variations due to environmental factors.

Currently, the use of 3D matrix (e.g. matrix-assisted chondrocytes implantation) has emerged as promising approaches for regenerating large cartilage defects [6]. With the 3D single cell approach presented here, a multitude of interconnected parameters influencing redifferentiation can be investigated in parallel and in well-controlled conditions. For example, in future studies, different 3D matrix materials and mixtures of materials could be readily employed in place of collagen in the present system, and thus factors, such as physical and chemical composition of matrix as well as a wide range of environmental influences (e.g. pH, O_2 level), can be rapidly screened in a highly comparable way. As a result, rapid identification of optimal microenvironments that can be reproduced in matrix-assisted chondrocyte implantation is possible. In the context of generating chondrocytes via mesenchymal stem cell chondrogenesis, the approach described here offers a versatile platform in the search for niches that offer targeted differentiation [51–53].

5. Conclusions

We have developed a novel, single cell culture system allowing independent control of cell geometry and extracellular matrix. Using this approach, we have shown chondrocyte dedifferentiation is modulated by the interplay of 3D cell geometries and multiple factors of the matrix. It was found that a balanced between cell spreading area and spherical shape effectively retains and regains a differentiated phenotype. In contrast, a decreasing cell volume in 3D can advance differentiation even though the cells remain

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spherical. These results highlight the importance of controlling and understanding the variations in 3D culture conditions.

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 3–8, are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2015.06.008.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.06.008.

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