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Chondrogenic capacity and alterations in hyaluronan synthesis of cultured human osteoarthritic chondrocytes

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ABSTRACT

During osteoarthritis there is a disruption and loss of the extracellular matrix of joint cartilage, composed primarily of type II collagen, aggrecan and hyaluronan. In young patients, autologous chondrocyte implantation can be used to repair cartilage defects. However, for more elderly patients with osteoarthritis, such a repair approach is contraindicated because the procedure requires a large expansion of autologous chondrocytes *in vitro* leading a rapid, perhaps irreversible, loss of the chondrocyte phenotype. This study investigates whether osteoarthritic chondrocytes obtained from older patients can be expanded *in vitro* and moreover, induced to re-activate their chondrocyte phenotype. A decrease in chondrocyte phenotype markers, collagen II, aggrecan and SOX9 mRNA was observed with successive expansion of cells in monolayer culture. However, chondrogenic induction in three-dimensional pellet culture successfully rescued the expression of all three marker genes to native levels, even with 4th passage cells–cells representing an approximate 625-fold expansion in cell number. This data supports the use of osteoarthritic cells for autologous implantation repair. In addition, another set of gene products were explored as useful markers of the chondrocyte phenotype. Differentiated primary chondrocytes exhibited a common pattern of hyaluronan synthase isoforms that changed upon cell expansion *in vitro* and, reverted back to the original pattern following pellet culture. Moreover, the change in isoform pattern correlated with changes in the molecular size of synthesized hyaluronan.

1. Introduction

Hyaline articular cartilage is a highly specialized avascular tissue characterized by unique biomechanical properties. The viscoelastic properties that make cartilage firm-yet-pliable, arise from the composition of its extracellular matrix (ECM) composed primarily of type II collagen (COL2) and the large proteoglycan termed aggrecan (ACAN) [1,2]. Aggrecan is retained in cartilage by binding to long filaments of another glycosaminoglycan, hyaluronan (HA) [1,3,4]. Disruption and loss of this ECM is associated with degenerative changes in cartilage such as osteoarthritis (OA) [1,2]. The synthesis of this cartilage-specific ECM requires the expression of genes associated with the differentiated chondrocyte phenotype—a phenotype controlled by the transcription factor SOX9 [5].

HA is synthesized at the plasma membrane by an enzyme called hyaluronan synthase (HAS), of which there are three isoforms (HAS1-3). It has been suggested that the different isoforms synthesize HA of varying lengths [6]. Despite the critical role of HA in retaining aggrecan, little is known about the regulation of HAS activity, differential usage of the three HAS isoforms by human chondrocytes or, differences that may occur in the size of produced HA by chondrocytes, especially upon passage in monolayer. Thus, changes in HAS levels and isoforms represent an uncharted area of regulation that support the chondrocyte phenotype.

Autologous chondrocyte implantation (ACI) is a promising technique for the treatment of focal cartilage defects that occur in damaged but otherwise young, healthy joints [7,8]. This technique is based on the isolation of a limited number of chondrocytes from a nonload-bearing area of the patient's knee and expansion of these cells in monolayer [7,9]. However, monolayer passage of chondrocytes results in dramatic changes in cell shape and loss

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of the chondrocyte phenotype, a loss that includes a reduction of COL2 and increase in type I collagen [4,10]. It is expected that these passaged, ''de-differentiated cells'' will regain chondrogenic properties (re-differentiate) once re-implanted in the joints. This expectation has contraindicated the use of ACI for cartilage replacement in older OA patients as, unlike cells from healthy, young cartilage, the capacity of autologous OA cells to re-differentiate is not well documented [8,9,11].

The objectives of this study are to determine: (1) the effects of monolayer expansion on the phenotype of OA chondrocytes derived from more elderly patients; (2) the re-differentiation capacity of OA chondrocytes and if there is a cell expansion limit to this capacity and; (3) whether changes occur in the expression pattern of the three HAS isoforms and whether these changes affect the molecular size of the HA synthesized.

2. Materials and methods

2.1. Tissue harvest

Human articular cartilage was obtained from femorotibial joints of twenty nine patients (6 males, 23 females; mean age of 71.0 (56–86) years) undergoing knee joint replacement surgery for OA at Nagoya University Hospital (Nagoya, Japan) or Vidant Medical Center (Greenville, NC). For some experiments, RNA was isolated directly from slices of intact cartilage at time of dissection and termed ''native cartilage''. The acquisition of human OA cartilage was approved by both Institutional Review Boards.

2.2. Cell isolation and passage

Cartilage slices were digested with 3 mg/ml collagenase XI (Sigma, St. Louis, MO) overnight at 37 °C at Nagoya University or, by sequential digestion with Pronase (EMD Millipore, Billerica, MA) and collagenase P (Roche, Indianapolis, IN) at East Carolina University [12]. Isolated chondrocytes were resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Belgium) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Piscataway, NJ or Hyclone, Logan, UT). After isolation, cells were plated in tissue culture flasks at a density of 1×10^4 cells/cm^2. Monolayers of these freshly isolated cells were defined as ''primary'' cells. The medium of all monolayer cultures was changed twice a week. When cells in monolayer reached confluence, one plate would be lysed directly for RNA extraction and another detached by treatment with trypsin–EDTA (0.25% trypsin/2.21 mM EDTA, Sigma). The released cells would then be replated as a monolayer at 1×10^4 cells/cm^2 or, tested for phenotype recovery as a pellet culture. The term passage number was defined as the number of times chondrocytes were trypsinized and replated as monolayers (P1–P4).

2.3. Re-differentiation culture

To test the capacity of OA cells to re-differentiation, P1–P4 cells, released from monolayers were cultured next as pellets at 2.5×10^5 cells/pellet [13]. Pellets were cultured in 500 μl of chondrogenic medium containing hMSC Chondrogenic differentiation BulletKit with SingleQuots (dexamethasone, ascorbate-2-phosphate, proline, pyruvate, and ITS+ Premix; Lonza, Allendale, NJ) supplemented with 10 ng/ml transforming growth factor-β3 (TGF-β3; Peprotech, Rocky Hill, NJ) and 250 ng/ml bone morphogenetic protein-2 (BMP-2; GenScript, Piscataway, NJ). The medium was replaced every 3–4 days for 10 days and then harvested for subsequent analysis. In another experiment, a P2 monolayer was sub-cultured for 2 weeks in alginate beads as described previously [4], released back into monolayer and then analyzed for changes in HA size.

2.4. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Cartilage fragments were freeze pulverized in liquid nitrogen followed by direct RNA extraction with RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Total RNA was also extracted from monolayers by direct addition of the lysis buffer. The same protocol was used for pellet cultures at different passages, following a digestion with 3 mg/ml collagenase XI for 3 h at 37 °C. From the total RNA extracted from each sample, cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

PCR reactions were performed using TaqMan® Universal PCR Master Mix and TaqMan® Gene Expression Assays for COL2 (Hs00264051) and GAPDH (Hs99999905), or using the LightCycler480 SYBR Green I Master (Roche, Mannheim, Germany). The primers pair sequences (Nihon Gene Research Laboratories, Sendai, Japan); forward and reverse; are as follows: ACAN, 5′-CCAGGAGGTATGTGAGGA-3′, 5′-CGATCCACTGGTAGTCTTG-3′; SOX9, 5′-CTGGGCAAGCTCTGGAGA-3′, 5′-ATGTGCGTCTGCTCCGTG-3′; HAS1, 5′-CAGACCCACTGCGATGAGAC-3′, 5′-CCACCAGGTGCGCTGAAA-3′; HAS-2, 5′-TCAGAGCACTGGGACGAAG-3′, 5′-CCCAACACCTCCAACCAT-3′; HAS3, 5′-CAGCAACTTCCATGAGGC-3′, 5′-CACAGTGTCAGAGTCGCA-3′. The copy number of each gene was determined by absolute quantification method with standard curves using Exor4 software (Roche).

2.5. Histology and immunohistochemistry

After culturing in chondrogenic medium, pellets were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. Sections were stained with Safranin O for the detection of proteoglycans. For immunohistochemistry, sections were deparaffinized and blocked endogenous peroxidase in 3% H_2O_2/MeOH followed by blocking with 1% goat serum albumin. Rabbit anti-human monoclonal antibody for COL2 (Cosmo Bio, Carlsbad, CA) diluted 1:200 into 1% goat serum albumin was then applied for 2 h at room temperature. The primary antibody was detected by the avidin biotin conjugate method, which was applied according to manufacturer's instructions using Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Peroxidase activity was detected using Histofine DAB kit (Nichirei) and counterstained with hematoxylin. Sections for negative controls were incubated with 1% goat serum albumin instead of the first antibody. Sections of the pellets were stained for HA by biotinylated HA-binding protein (HABP) probe following the procedure described previously [3] using Histofine SAB-PO kit and DAB kit as was done for COL2 staining. Sections for negative controls were incubated with 1% goat serum albumin instead of the HABP probe.

2.6. Dimethylmethylene blue (DMMB) assay

To evaluate sulfated glycosaminoglycan content of collected media from monolayer cultures, DMMB assay was performed following the previously reported procedure [14]. After isolation of primary cells, or at each passage for monolayer culture, cells were plated in wells at a density of 1×10^6 cells/cm^2 and allowed to recover for one day in DMEM with 10 % FBS. The medium was changed to serum free DMEM (750 μl/1×10^6 cells) and collected after 24 h. The medium samples were concentrated 5-fold using Ultracel 10 k centrifugal filter units (EMD Millipore), and equal volumes were analyzed by this assay.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To evaluate HA content of collected media from monolayer cultures, HA ELISA was performed using DuoSet® ELISA Development Systems (R&D Systems, Minneapolis, MN) following manufacturer's instructions. The same medium samples analyzed by the DMMB assay were used for HA ELISA but with a 1:10 dilution with water.

2.8. Size determination of HA

The molecular size of HA secreted into the media of monolayer cultures was determined using an agarose gel elecrophoresis assay as previously described [15]. For all cells, the culture medium was changed to serum free DMEM and collected 24 h later. The medium samples were concentrated 20-fold and 15 μl aliquots were loaded into a 0.75% agarose gel, and then electrophoresed at 150 V in Tris–acetate–EDTA buffer. Gels were fixed in 25% isopropanol, stained overnight in Stains-All (Sigma), destained in 10% ethanol and 7.5% acetic acid, and imaged on a light box.

2.9. Transient transfection

For overexpression of human HAS isoforms, P1 chondrocytes (2.0×10^6 cells) were released from monolayer culture using trypsin–EDTA and mixed with Amaxa™ human chondrocyte solution (Lonza Cologne GmbH, Köln, Germany) and 5 μg of human HAS1 or HAS2 pCR3.1 plasmid (gifts from Dr. Tim Bowen, Cardiff University, UK). Cells were then transfected by nucleofection using an Amaxa Nucleofector® device, re-plated and allowed to recover for one day in DMEM with 20% FBS, and used for HA size determination assay described above.

2.10. Statistical analysis

All data are presented as mean ± standard deviation (SD). For the mRNA expression, the final numeric value was calculated as the ratio of the gene to GAPDH, and then used directly for HAS isoforms expression pattern (Fig. 4A), or expressed by setting native cartilage as 1 in the graph of each gene (Figs. 1A–C and 3A–C). The data of each passage group were then compared with Native cartilage, and differences between monolayer and pellet culture of each passage were analyzed, by an unpaired Student's *t*-test. *P*-values <0.05 were considered significant.

3. Results

3.1. Chondrogenic markers

3.1.1. Native cartilage tissue versus primary chondrocytes

There were no significant changes observed in the mean mRNA expression level of three critical chondrocytes genes, COL2, ACAN and SOX9 between native intact OA cartilage and primary chondrocytes (Fig. 1A–C). This indicates that the collagenase-based cell isolation procedure alone did not induce an alteration of the chondrocyte phenotype. Moreover, these results also suggest that freshly isolated cells, although derived from OA cartilage, are not already de-differentiated *in vivo*.

3.1.2. Expansion of OA chondrocytes in monolayer culture

In the first expansion of chondrocytes from primary to P1 passage, mRNA expression of COL2 dramatically decreased by over 1000-fold (Fig. 1A). The expression of ACAN and SOX9 also decreased significantly by approximately 10-fold and 40-fold, respectively (Fig. 1B and C). With successive passage, the expression of

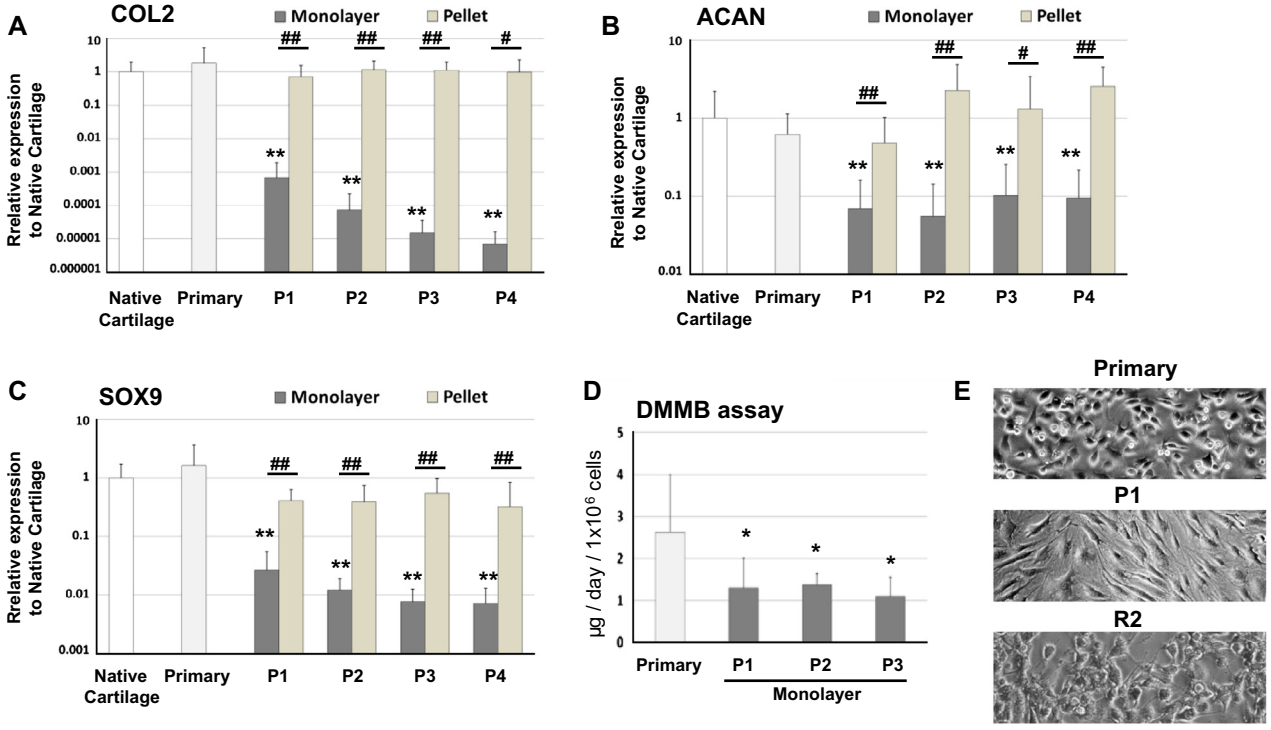

Fig. 1. Expression of chondrogenic marker genes and glycosaminoglycan release by human OA chondrocytes. The mRNA expression of COL2 (A), ACAN (B) and SOX9 (C) was quantified from total RNA isolated from native intact OA cartilage (white bars), primary chondrocytes (light grey bars) or passaged monolayers (dark grey bars) as well as P1–P4 re-differentiated in pellet cultures (gold bars). Samples were derived from OA patient material ($n = 20$, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ Monolayer versus Pellet, $^{*}P < 0.05$; $^{**}P < 0.01$ versus Native Cartilage). (D) Glycosaminoglycan released into the medium of primary, P1–P3 cultures was analyzed by DMMB assay ($n = 7$, $^{*}P < 0.05$ versus Primary). Values are mean ± SD (error bars). (E) Microscopic images of primary, P1 cells and P2 cells sub-cultured in alginate beads (R2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

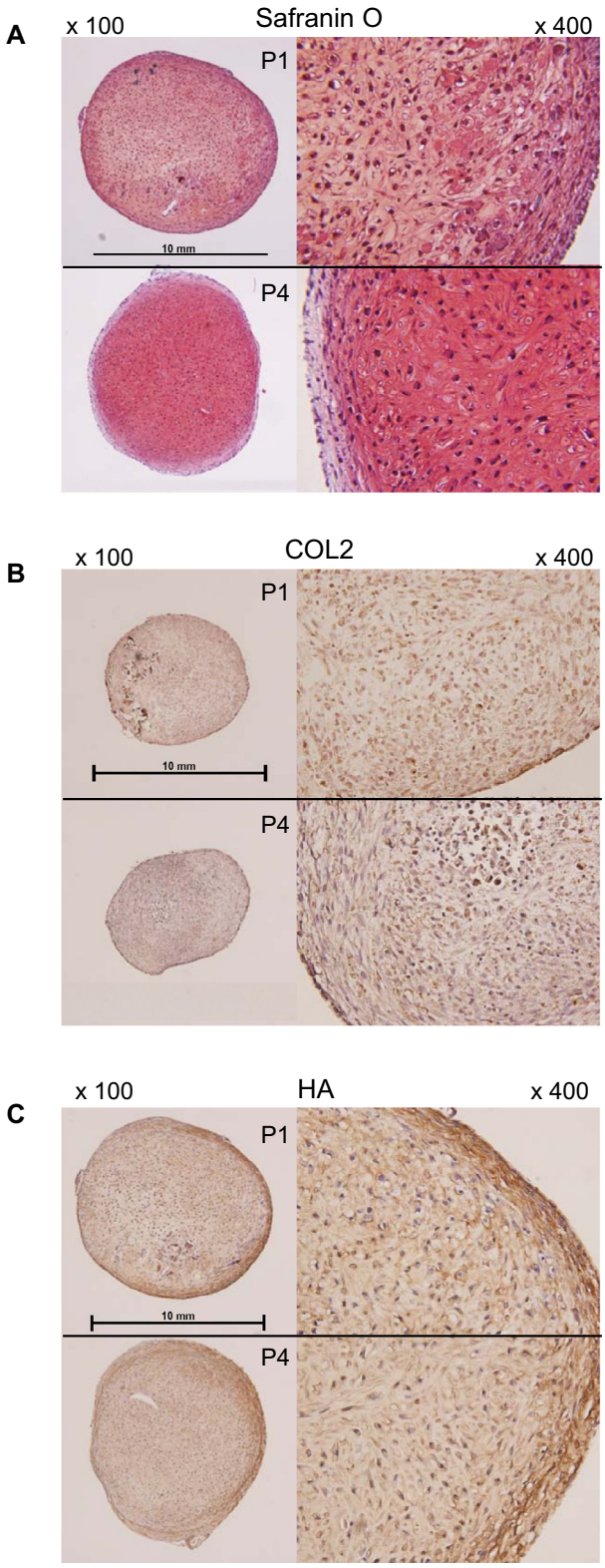

Fig. 2. Histological analysis of the pellet neocartilages from human OA chondrocytes. Representative sections of pellet neocartilages were stained with (A) Safranin O, (B) Immunohistochemistry staining for COL2 and, (C) HABP probe staining for HA. Comparisons were made between pellet-recovered P1 and P4 cultures isolated from an identical patient.

COL2 and SOX9 progressively decreased. Interestingly, ACAN mRNA levels, although significantly lower than primary cells, did not display a progressive diminution in expression with passage number (Fig. 1B). Additionally, the amount of glycosaminoglycan released into the medium of monolayer cultures, indicative of ACAN protein levels (Fig. 1D) remained constant after an initial drop from primary to first passage cells. Cell morphologies changed dramatically from as cells went from primary to P1, in parallel with the changes in mRNA (Fig. 1E).

3.1.3. Induced chondrogenic re-differentiation

To evaluate the re-differentiation potential of expanded cultures of OA chondrocytes, released cells were grown in a three-dimensional pellet culturing system that mimics *in vitro*, the *in vivo* ACI environment. Using cells from all stages of expansion, the size of the neocartilage pellets increased after 10 days of culture, indicating the robust production of extracellular matrix (not shown). When analyzed for marker expression, COL2, SOX9 and ACAN mRNA of P1-P4 cells (Fig. 1A–C) all returned to values equivalent to primary chondrocytes and intact native cartilage. In pellet cultures derived from P4 cells, the COL2 expression was increased by more than 100,000-fold (Fig. 1A). As shown in Fig. 1E, the morphology of P2 cells returned to that of primaries when they were cultured in alginate beads (described as R2).

3.1.4. Safranin O staining and immunohistochemistry for COL2

Since the re-differentiation capacity assay gives rise to a neocartilage, the quality of re-differentiation could also be assessed using standard histological methods for tissues. As shown in Fig. 2A, after 10 days of pellet culture, rich Safranin O staining was detected throughout the entire pellet indicative of the high proteoglycan deposition (ACAN) levels unique to cartilage. No difference in Safranin O staining was observed between rescued P1 and P4 cells from the same representative patient (Fig. 2A). At higher magnification, most cells exhibited a chondrocyte-like rounded morphology. Similar to proteoglycan staining patterns, positive staining for COL2 protein deposition was observed, principally within the pericellular matrix at this time point (Fig. 2B). Pellets derived from P1 and P4 cells displayed near equivalent uniformity and intensity of COL2 staining. Negative controls did not show significant staining (not shown).

3.2. HA synthesis

3.2.1. HAS isoform expression pattern and staining for HA

The HAS isoform expression pattern was investigated as a possible new chondrocyte marker. All three isoforms, HAS1, HAS2 and HAS3 were expressed in roughly equivalent copy numbers by cells within intact native OA cartilage and primary chondrocytes (Figs. 3 and 4A). Upon first passage, HAS1 and HAS2 displayed a significant decrease (Fig. 3A and B). Similar to ACAN, the initial decrease in HAS2 did not change with passage after the initial drop. Interestingly, HAS3 did not exhibit a decrease but rather a slight increase with successive passage (Fig. 3C) such that in P1–P4 cells, HAS3 copy number predominates. Additionally, HA glycosaminoglycan synthesized by these HAS isoforms remains constant with passage number (Fig. 3D). Upon re-differentiation in pellet cultures, the HAS expression patterns reverse, returning to the pattern present in native cartilage (Figs. 3 and 4A). Robust staining for HA using HABP was also visualized within sections of the pellet neocartilages, from pellets derived from P1 and P4 monolayers. Like the COL2 staining, HA accumulation at this stage of pellet culture was principally pericellular.

3.2.2. Size determination of HA

Given the change in HAS isoform with passage, the size of newly synthesized HA was assessed using agarose gel electrophoresis. This procedure provides only a rough size estimate of the extreme size of this highly heterogeneous glycosaminoglycan but, one that

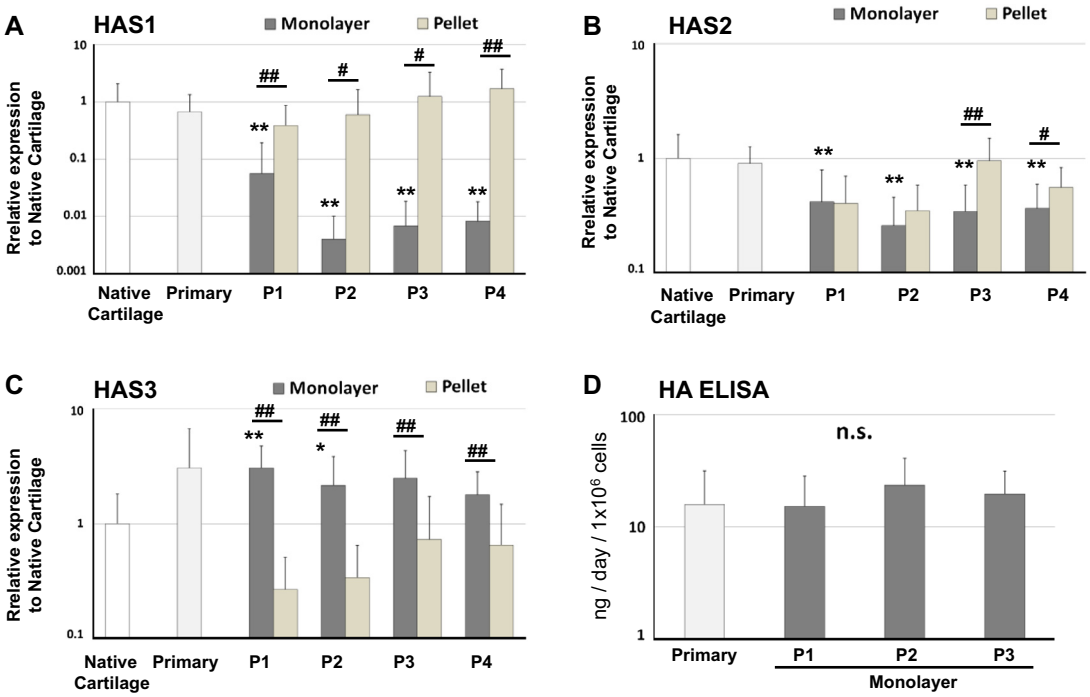

Fig. 3. Expression of HAS isoforms and HA release by human OA chondrocytes. The mRNA expression of HAS1 (A), HAS2 (B) and HAS3 (C) was quantified from total RNA isolated from native intact OA cartilage (white bars), primary chondrocytes (light grey bars) or passaged monolayers (dark grey bars) as well as P1–P4 re-differentiated in pellet cultures (gold bars). Samples were derived from OA patient material ($n = 20$, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ Monolayer versus Pellet, $^{*}P < 0.05$; $^{**}P < 0.01$ versus Native Cartilage). (D) HA released into the medium of primary, P1–P3 cultures was analyzed by HA ELISA ($n = 7$, n.s. = not significant). Values are mean ± SD (error bars). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

closely reflects the biological state. As shown in Fig. 4B and C, HA derived from primary chondrocytes does not substantially enter the gel, comparable to HA standards $>1 \times 10^6$ Da. Interestingly, the HA present in the media of P1–P4 cells all exhibited sizes comparable to middle molecular weight HA standards that were $<1 \times 10^6$ Da (Fig. 4B). When P1 cells were transfected with human pHAS1 or pHAS2, clear bands for HA were now observed migrating at high molecular weight (Fig. 4C). In addition, when P2 cells were cultured in alginate beads to effect a rescue of phenotype, the HA again migrated at higher molecular size (Fig. 4C).

4. Discussion

Pathological alterations of OA cartilage represent a major clinical problem and recent interest has been targeted at ACI as a potential therapeutic approach for OA joints [9,11,16]. In this study, we focused on alterations in the expression and synthesis of ECM components, such as collagen, aggrecan, and HA, all macromolecules that are essential for cartilage homeostasis.

The gene expression profile of chondrocytes dramatically shifts following sequential or extended monolayer culture of chondrocytes, a process that has been termed "de-differentiation". However, the more important question is whether expanded cells retain a capacity to recover their chondrogenic phenotype when cultured in an *in vivo*-like cartilage environment as would occur with ACI. The current results demonstrate that OA chondrocytes obtained from 29 patients, aged 56–86, each retained the capacity for chondrocyte phenotype re-activation up to at least 4th passage, representing an approximate 625-fold expansion in cell number. These results support the eventual use of ACI for OA in elderly patients [9].

One limitation of this study is that we could not include a control of normal articular cartilage from young subjects due to the limitation of donors. At issue is the fact that some investigators have reported irreversible changes in phenotype between chondrocytes isolated from OA cartilage versus those of young healthy joints [8,17,18]. Other reports indicate comparable proliferation or differentiation potential of OA chondrocytes [9,11,16]. Our current findings did not directly compare the differentiation/re-differentiation capacity of chondrocytes from young healthy donors to OA cells. However, similar changes in SOX9, COL2, ACAN and HAS2 were observed with serial passage and rescue of bovine metacarpophalangeal articular chondrocytes, derived from cattle slaughtered as 18–24 months, representative of young, healthy animals [4].

Although there are several reports that have shown the expression pattern or functions of HAS isoforms in cell lines, synovial cells or chondrocytes [3,6,19–22], there seems no consensus as to how isoform usage affects the synthesis or function of HA in human joints. The present study demonstrates that all three HAS isoforms are expressed in nearly equal copy numbers by the cells of intact OA cartilage and primary chondrocyte cultures with a slight preponderance of HAS1 in the native cartilage. This pattern is one example wherein OA chondrocytes may differ from cells derived from non-OA human donor cartilage. Earlier studies using competitive RT-PCR demonstrated HAS2 copy numbers more than 100-fold higher than HAS1 or HAS3 [4]. However, these results are difficult to compare directly due to differences in RT-PCR approach and the samples were derived from ankle cartilage as opposed to knee OA cartilage used in this study.

Successive monolayer culture of chondrocytes resulted in HAS3 becoming the predominant isoform expressed. It is difficult to determine whether the changes in isoform usage have a biological impact except that the HAS1 and HAS3 consistently display an inverse switching of copy numbers upon de-differentiation and re-differentiation. Although the HAS isoform pattern alters, no remarkable changes in the overall HA glycosaminoglycan levels were observed. This left the possibility that changes in HA size may be occurring. Previous studies using recombinant HAS3 expression in cell lines demonstrated that this HAS isoform

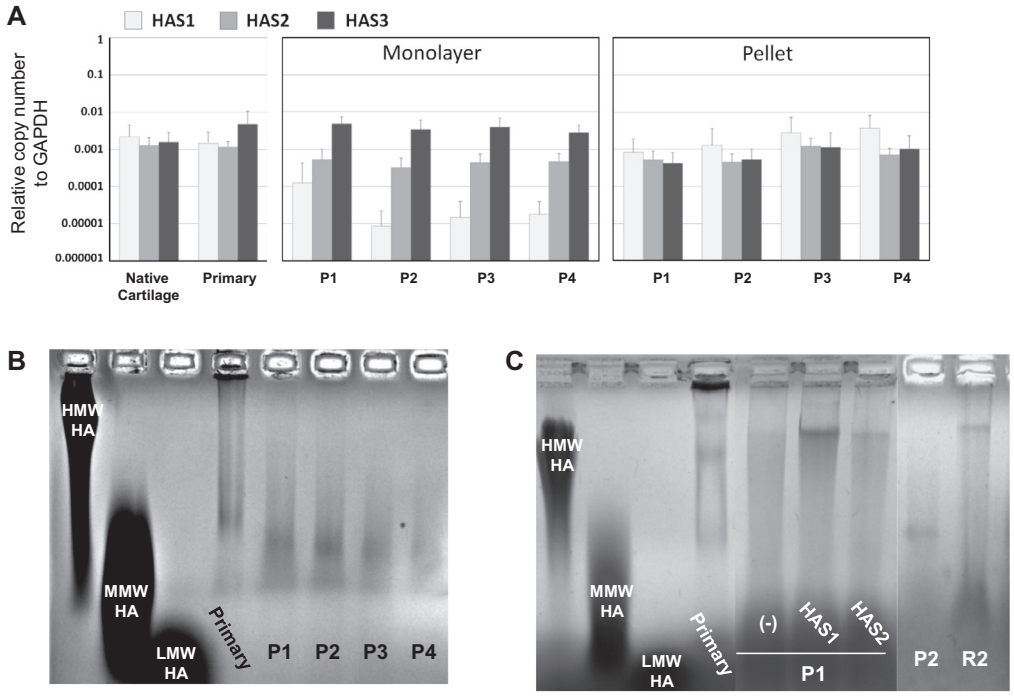

Fig. 4. Changes in HAS isoform expression pattern and the size determination of HA. (A) Summary of HAS isoform mRNA expression data taken from Fig. 3 for native intact OA cartilage and primary chondrocytes (left panel), monolayers (middle panel) and re-differentiated cells in pellet cultures (right panel). (B) Concentrated media from primary chondrocytes and P1–P4 expanded monolayers, derived from an identical patient, were run on agarose gels and stained for HA to determine the size distribution. (C) The same assay was performed using the media from the chondrocytes of another patient to determine the effects of human HAS1 or HAS2 overexpression on P1 monolayers, or sub-culture of P2 cells in alginate beads (R2). HA standards shown in the left hand lanes were from a commercial source (Lifecore Biomedical, Chaska, MN) with sizes ranging from 1200–1800 kDa, 180–350 kDa, and <5 kDa (termed high, middle, and low molecular weight (HMW, MMW, and LMW)-HA, respectively).

synthesize HA of middle molecule size (10^5–10^6 Da) whereas recombinant HAS1 and HAS2 synthesized larger sized HA molecules (>2×10^6 Da) [6]. Differing HA sizes in these ranges are readily distinguished by agarose gel electrophoresis. In this study, large molecular size HA was only observed in the media of primary chondrocytes. The HA present in the media of P1–P4 cultures–cultures in which the HAS3 isoform predominates, all exhibited sizes in the range of 10^5–10^6 Da. Interestingly, an overexpression of human HAS1 or HAS2, or a re-differentiation culture in alginate beads appeared to increase the size of HA that de-differentiated chondrocytes could produce. Thus, the data is consistent with a change in HA isoform giving rise to a change in HA size.

In conclusion, OA chondrocytes expanded and allowed to de-differentiate *in vitro* retain the capacity to re-activate their expression of chondrogenic marker genes and moreover, generate a cartilage-like tissue *in vitro*. The observation of this capacity validates the use of OA tissue from elderly patients as a potential cell source for tissue engineering in clinical settings. In addition, the expression pattern of HAS isoforms in OA chondrocytes become altered upon de-differentiation and re-differentiation suggesting their usefulness as chondrogenic markers. Changes in HAS isoforms also hint at critical effects these changes may have on cartilage function. By regulating the molecular size of produced HA, the capacity of HA to retain aggrecan within the tissue may be altered.

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