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Probing the microenvironmental conditions for induction of superficial zone protein expression

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SUMMARY

Objective: To determine the *in vitro* conditions which promote expression of *superficial zone protein (SZP)*.
Methods: Chondrocytes from 6-month-old calves were expanded in monolayer culture and the expression of *SZP* in alginate bead and monolayer culture was quantified with quantitative real time-polymerase chain reaction (qRT-PCR) and immunostaining. The effect of oxygen tension on *SZP* expression was determined by qRT-PRC analysis of cells cultured in two dimension (2D) and three dimension (3D) under hypoxic (1% pO_2) or normoxic (21% pO_2) conditions. Finally, to examine the effect of cyclic tensile strain on expression of *SZP* in 2D and 3D cultures, chondrocytes encapsulated in alginate beams or seeded on type I collagen coated polydimethylsiloxane (PDMS) chambers were subjected to 5% strain at 1 Hz, 2 h/day for 4 days or 2 h at the fourth day of culture and mRNA levels were quantified.
Results: Bovine chondrocytes in monolayer showed a drastic decrease in SZP expression, similar in trend to the commonly reported downregulation of *type II collagen (Col2)*. Chondrocytes embedded in alginate beads for 4 days re-expressed *SZP* but not *Col2*. *SZP* expression was higher under normoxic conditions whereas *Col2* was upregulated only in alginate beads under hypoxic conditions. Cyclic mechanical strain showed a tendency to upregulate mRNA levels of *SZP*.
Conclusions: A microenvironment encompassing a soft encapsulation material and 21% oxygen is sufficient for fibroblastic chondrocytes to re-express *SZP*. These results serve as a guideline for the design of stratified engineered articular cartilage and suggest that microenvironmental cues (oxygen tension level) strongly influence the pattern of *SZP* expression *in vivo*.

Introduction

The development of replacement tissue for healing cartilage injuries poses a major scientific challenge. The avascular nature, limited proliferation and restricted access to nutrients and regenerative cells prevent the spontaneous regeneration of cartilage defects[1,2]. Furthermore, the dense matrix that surrounds cartilage cells (chondrocytes) inhibits their migration to the defect site[3,4]. Articular cartilage is organized into the deep, middle and superficial zones[5,6]. The superficial zone in cartilage is believed to be crucial for long term stability and function of the tissue by protecting the deeper layers and providing lubricants that limit friction and prevent wear[7]. *Superficial zone protein (SZP)*, a splice variant of the gene proteoglycan 4 (PRG4), is mainly produced by the chondrocytes of the superficial zone and is a key molecule involved in cartilage lubrication[8–11].

Chondrocytes have been shown to dedifferentiate when cultured on two-dimensional (2D) tissue culture plastic (TCP) where they assume a fibroblastic phenotype[12,13]. Culturing chondrocytes in alginate[14–16] (Alg) or agarose[17] hydrogels has been shown to maintain the cartilage phenotype. However, most studies address the re-expression of *type II collagen (Col2)* and *aggrecan* which are present throughout cartilage tissue but are synthesized in lower amounts by superficial zone cells compared to cells of the deeper layers[5]. Only a few studies have investigated the effects of dedifferentiation on the superficial zone phenotype and particularly the expression of *SZP*[8].

An engineered cell-based cartilage replacement that recapitulates the stratified cartilage structure is desirable. In the current study we focused on microenvironmental factors which regulate the expression of *SZP*. Synthesis of *SZP* has been shown to be affected by growth factors such as bone morphogenic protein-7 (BMP-7)[18] and transforming growth factor beta (TGFβ)[19] as well as

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cytokines such as interleukin-1 beta (IL-1β)[20]. Gene expression and synthesis of *SZP* is also modulated by mechanical stimulation such as shear[21], surface motion[22] and mechanical stress[23]. Finally, differentiation under hypoxic conditions stimulated matrix production by middle/deep chondrocytes and *PRG4* by superficial zone chondrocytes[24].

We hypothesized that an *in vitro* system that most closely resembles the *in vivo* cartilage superficial zone environment would allow dedifferentiated chondrocytes to re-express markers of the superficial zone. To test this hypothesis we designed experiments to address the effect of three-dimensional (3D) cultures, oxygen tension and cyclic tensile strain on the expression of *SZP*. We studied the expression of *SZP* at ambient oxygen levels as well as under hypoxic conditions and compared its expression profile to that of *Col2*. We also designed a mechanical loading chamber for application of homogeneous 3D strain compatible with the commercially-available STREX machine. We then cultured chondrocytes on 2D substrates and 3D hydrogels in the presence and absence of cyclic mechanical strain and quantified the expression of *SZP*.

Materials and methods

Materials

Phosphate buffer saline (PBS), fetal bovine serum (FBS), cell culture media [Dulbecco's modified eagle medium (DMEM)-Glutamax], antibiotic–antimycotic (Anti–Anti), trypsin/ethylenediaminetetraacetic acid (EDTA) and Alexa-488 goat anti-mouse conjugated IgG IgM (H + L) were from Invitrogen AG, Basel, Switzerland. Dow Corning Sylgard 184 Silicone Elastomer Kit was obtained from OMYA AG, Switzerland. Atelocollagen bovine type I collagen (Col1) (Koken) was from Holzel Diagnostika, Koln, Germany. Cell strainers were from Becton Dickson AG Allschwill, Switzerland. Formaldehyde, Immunoglobin G (IgG), Fluoroshield™ with 4′,6-diamidino-2-phenylindole (DAPI), pronase E from *Streptomyces griseus*, bovine serum albumin (BSA), monensin, [D-(+)-glucono-δ-lactone (GDL)] and collagenase type II from *Clostridium histolyticum* were purchased from Sigma Aldrich Chemie GmbH, Buchs, Switzerland. L-Ascorbic Acid Phosphate Magnesium Salt was obtained from Wako (IG instrumenten-Gesellschaft AG, Zurich). $CaCO_3$ particles of 5 μm size were obtained from Plasma-Chem GmbH, Berlin. Col2 antibody II-II6B3 was from Developmental Studies Hybridoma Bank, University of Iowa. Monoclonal antibody against native bovine lubricin (3A4) was from MD Bioproducts, Zurich, Switzerland.

Cell isolation

Full thickness cartilage tissue was harvested from the medial and lateral femoral condyles of 6-month-old calves obtained from the local abattoir[25]. Immature cartilage tissue containing blood vessels was excluded. Cartilage tissue slices were minced with a sterile blade and the tissue was treated with 0.2% pronase in DMEM supplemented with 1% Anti–Anti for 2 h at 37°C, 7% CO_2 with gentle stirring. The pronase digestion was followed by 6 h of digestion in 0.03% collagenase in DMEM supplemented with 1% Anti–Anti at 37°C, 7% CO_2 with gentle stirring. The digested tissue was filtered through a 100 μm cell strainer followed by a 40 μm cell strainer. Cells were counted and viability was consistently above 90% for all the performed isolations as determined using trypan blue exclusion with an automated cell counter (Countess™ Automated Cell Counter, Invitrogen AG, Basel, Switzerland). Cells were seeded at 10000/cm^2 in DMEM supplemented with 1% Anti–Anti, 10% FBS and 50 μg/mL L-ascorbic acid and passaged at 80–90% confluency with trypsin/EDTA. Cells of subsequent passages were seeded at 5000/cm^2. The above media formulation was used in all experiments and media was changed every 3 days.

Assessment of chondrocyte dedifferentiation

Cartilage tissue was minced then placed in 350 μL RLT Plus Buffer (Qiagen AG, Zurich, Switzerland) and homogenized using a rotor stator homogenizer (TH 220 tissue homogenizer, Omni International, LabForce, Nunningen, Switzerland). A volume of 540 μL RNA free water and 10 μL Protease K solution (Qiagen) were added and incubated at 55°C for 10 min with shaking at 1000 rpm. RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations and total RNA was quantified using a spectrophotometer (Nanodrop ND-1000). Chondrocytes isolated from at least three different calves were seeded at 10000 cells/cm^2, these cells were designated as passage 0 (P0). After 4 days of culture, part of the P0 cells seeded in a 25 cm^2 tissue culture plastic (TCP) flask were washed in PBS and lysed for mRNA analysis. At 80% confluence after 7 ± 1 days, cells were trypsinized with 0.25% Tripsin/EDTA for 5 min, counted and seeded at 5000 cells/cm^2. Cells were passaged every 4 ± 1 days or lysed by adding 350 μL RLT Plus Buffer after washing once with PBS for mRNA quantification. This was repeated till passage 4 (P4). The 260/280 ratio measured by the spectrophotometer in all samples was consistently 2.0 ± 0.1. Total RNA was reverse transcribed starting with 500 ng RNA and gene expression of *SZP* and *Col2* was determined using quantitative real time-polymerase chain reaction [(qRT-PCR) StepOnePlus, Applied Biosystems]. *Ribosomal protein L13 (RPL13)* was used as a housekeeping gene. Analysis of qRT-PCR data was performed following the method of Livak and Schmittgen[26] and the 95% confidence intervals (CIs) were calculated according to Willems *et al.*[27] All primers used in this study are given in Table I.

Restoration of the cartilage phenotype

Chondrocytes at passage 3 or 4 were seeded on TCP at 5000 cells/cm^2 or embedded in alginate beads at 6 × 10^6 cells/mL. To prepare alginate beads, a cell pellet was suspended in 1.2% (w/v) alginate (Pronova UPLVG, Novamatrix) and collected in a syringe connected to a 21-gauge needle. All alginate solutions were sterilized by filtering through a 0.2 μm sterile Millipore filter. The alginate/cell mixture was dispensed in a 102 mM $CaCl_2$ mixture under continuous gentle stirring for 10 min. Beads were washed 3× 2 min each with PBS and cultured in a six-well plate containing 10–11 beads/well, 3 mL media/well. Cells in beads or TCP were incubated for 3–4 days at 37°C in a humidified incubator (Binder CB 53, Germany) which allows automatic control of CO_2 and O_2 levels. The incubator controls O_2 levels with an O_2 sensor and maintains the set level by varying the N_2 supply. The CO_2 levels were maintained at 5% while O_2 levels were varied between 21% for normoxia and 1% for hypoxia. At the end of the culture period, beads were washed with PBS once then collected in 1.5 mL Eppendorf tubes and

Table I
Primers used in qRT-PCR

Gene	Accession number	Primer sequence (5′–3′)	Product size (BP)
RPL13	NM_001076998.1	F - GCCAAGATCCACTATCGGAAA R - AGGACCTCTGTGAATTTGCC	98
Col2	NM_001113224.1	F - GGC CAG CGT CCC CAA GAA R - AGCAGGCGC AGG AAG GTC AT	160
SZP	NM_001206633.1	F - CACCTAGAGTGAGAAAACCAA AGA CTA C R - CGG TTC AGG CAT TGC TGA T	71

incubated in alginate dissolving buffer (55 mM sodium citrate, 30 mM EDTA and 150 mM NaCl, pH 6.8) for 10 min at 37°C, 1000 rpm shaking. Cells were centrifuged for 2 min at 10000 rpm, washed with PBS (this step ensures removal of remaining alginate molecules that may impair the mRNA purity) and centrifuged for 2 min at 14500 rpm then lysed with 350 μL RLT Plus Buffer for mRNA quantification. Cells seeded on TCP were washed once with PBS then lysed with 350 μL RLT Plus Buffer.

Chamber for application of strain and assessment of homogeneity

The STREX device (ST-140-10, B-Bridge International) was used to apply mechanical strain to cells seeded in 2D and 3D. Silicon oil was well mixed 1:10 (w/w) with its crosslinker, then degassed PDMS was poured in chamber molds (32 × 32 mm) and allowed to cure at 80°C for 6 h. For the application of mechanical strain in 3D, STREX PDMS chambers were modified to contain a series of support ridges to hold the gel as it is stretched. The homogeneity of the applied strain was assessed by measuring the displacement of 10–30 μm glass beads embedded in an alginate gel before and after strain. Assessment of homogeneity was performed with 10% strain on 18 different areas within the strained gel with four images taken in each area ($n = 3$).

Application of mechanical strain

For the application of strain in 2D, PDMS chambers were plasma treated for 2 min in air. The chambers were then incubated with 0.1 mg/mL Col1 (Koken CosmoBio) in 150 mM NaCl, 1 mM HCl for 30 min to coat the chambers with a thin Col1 layer[25]. Chambers were washed once with 150 mM NaCl and once with cell culture media followed by seeding passage 3 chondrocytes at 10000 cells/cm^2. 5% tensile strain at 1 Hz was applied for 2 h, 24 h post-seeding for 4 days or once after 4 days of culture. Chondrocytes were seeded on non-strained chambers and in 25 cm^2 TCP flasks as controls. For application of 3D strain, 0.75 mL of 2% alginate (w/v) + chondrocytes was injected in modified PDMS chambers. Alginate was prepared using $CaCO_3$ as a source for calcium ions which results in uniform, slow-gelation of the material which can be injected[28,29]. The gel was prepared by mixing 886 μL 2.25% alginate in 150 mM NaCl, 41 μL of 100 mg/mL $CaCO_3$ in ultra-pure water, 73 μL of 200 mg/mL freshly prepared GDL in ultra-pure water and 6 × 10^6 cells. The solution was allowed to gel for 15–30 min at 37°C. The hydrogels were covered with 5 mL of cell culture media after gelation and cultured for 24 h. Mechanical strain was applied at 5.8% magnitude and 1 Hz for 2 h, 24 h post-seeding for 4 days or once after 4 days of culture. Samples were lysed 2 h after the final strain was applied. Expression of *SZP* and *Col2* was determined by qRT-PCR using *RPL13* as a housekeeping gene.

Immunostaining

Alginate beads and TCP seeded cells were treated with 0.1 μM monensin 24 h post-seeding in order to entrap synthesized proteins within the cell cytoplasm[30,31]. At the end of the culture time, beads or TCP seeded cells were washed once with PBS, fixed with 4% paraformaldehyde, 0.2% Triton X-100 for 15 min then washed once with PBS and stored in PBS. Alginate beads were stored in PBS containing 10 mM $CaCl_2$ to prevent gel dissolution. Beads were embedded in Optimum Cutting Temperature (O.C.T) compound and frozen on a dry ice block for 2 min and 6 μm-thick slices were cut using a microtome (CryoStar NX70, ThermoScientific). The slices were dipped in ethanol then blocked with 5% BSA in PBS for 1 h, washed 3× with PBS, then incubated with 1:100 of 3A4[10] antibody overnight at 4°C. Samples were washed 3× in PBS and incubated with 1:400 Alexa-488 anti-mouse secondary antibody for 1 h at room temperature. For the cartilage tissue, 10 μm-thick slices were incubated with the 3A4 antibody 1:200 or with Col2 antibody (II-II6B3) 1:50 overnight at 4°C, and the process was followed as above after which samples were mounted with Fluoroshield™ with DAPI. Samples were imaged using a confocal microscope (Carl Zeiss AG/LSM 510, equipped with a 40 × 0.6 NA objective).

Statistical analysis

All quantitative data were obtained from at least three independent donors and expressed as the 95% CI. Statistical evaluation for pairs of datasets was performed using the paired student's *t* test while analysis for multiple samples was carried out by analysis of variance (ANOVA) and *post-hoc* Tukey's tests where *P*-values <0.05 were considered significant. Statistical analysis was performed using OriginPro v8.1.

Results

Both SZP and Col2 undergo dedifferentiation during serial passaging

Serially passaged chondrocytes exhibited a massive downregulation in *Col2* expression [Fig. 1(A)] similar to what has been reported in previous studies. The downregulation was already significant in passage 1 cells (95% CI, 0.0022–0.013) when compared to cartilage tissue (95% CI, 0.048–20.61, $P = 0.00055$). The expression continued to be downregulated reaching a minimum at passages 3 and 4. The expression of *SZP* decreased with passaging, exhibiting a similar trend to that reported for *Col2* [Fig. 1(B)]. The downregulation was significant in passage 1 cells (95% CI, 0.038–0.115) when compared to cartilage tissue (95% CI, 0.128–7.782, $P = 0.0011$). Cells at passage 2 exhibited a significantly lower gene expression (95% CI, 0.015–0.087, $P = 8.6E\text{-}6$) compared to cartilage tissue but not significantly lower than passage 4 cells.

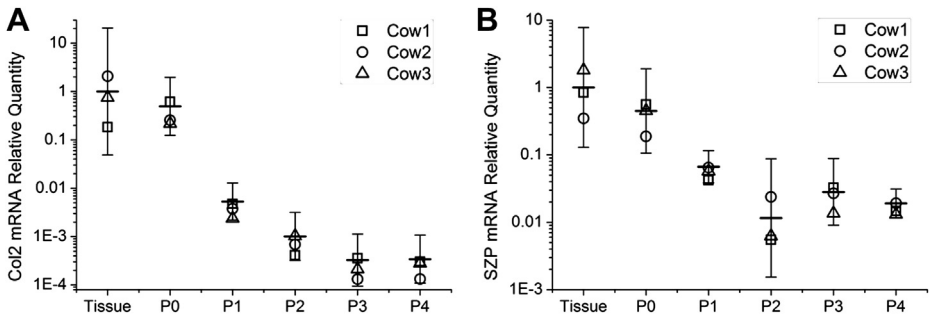

Fig. 1. Chondrocytes were harvested from cartilage tissue and serially passaged by culturing on tissue culture plastic for 4 days from passage 0 to passage 4. Analysis of *Col2* mRNA levels (A) and *SZP* (B) showed a decrease in expression with passaging. Gene expression was normalized to expression in cartilage tissue ($n = 3$).

Redifferentiation of serially passaged chondrocytes

Chondrocytes encapsulated in alginate beads for 4 days re-expressed *SZP* but not *Col2* (Fig. 2). The expression of *SZP* was upregulated 52.3-fold (95% CI, 25.9–105.6) in alginate beads compared to chondrocytes cultured on TCP (95% CI, 0.49–2.02, $P = 0.00013$) [Fig. 2(B)]. This fold change is comparable to the fold change between cartilage tissue and passage 3 cells (50.6-fold, Fig. 1) indicating that cells could to a large degree restore the expression of *SZP* by 3D culture. The expression of *Col2* showed no significant change ($P = 0.09$) after 4 days in alginate culture compared to TCP [Fig. 2(A)]. In order to confirm the expression of *SZP* at the protein level, immunostaining for *SZP* was performed on alginate beads and TCP samples. Strong staining for *SZP* was found in alginate encapsulated cells but not in cells cultured on TCP [Fig. 2(C)].

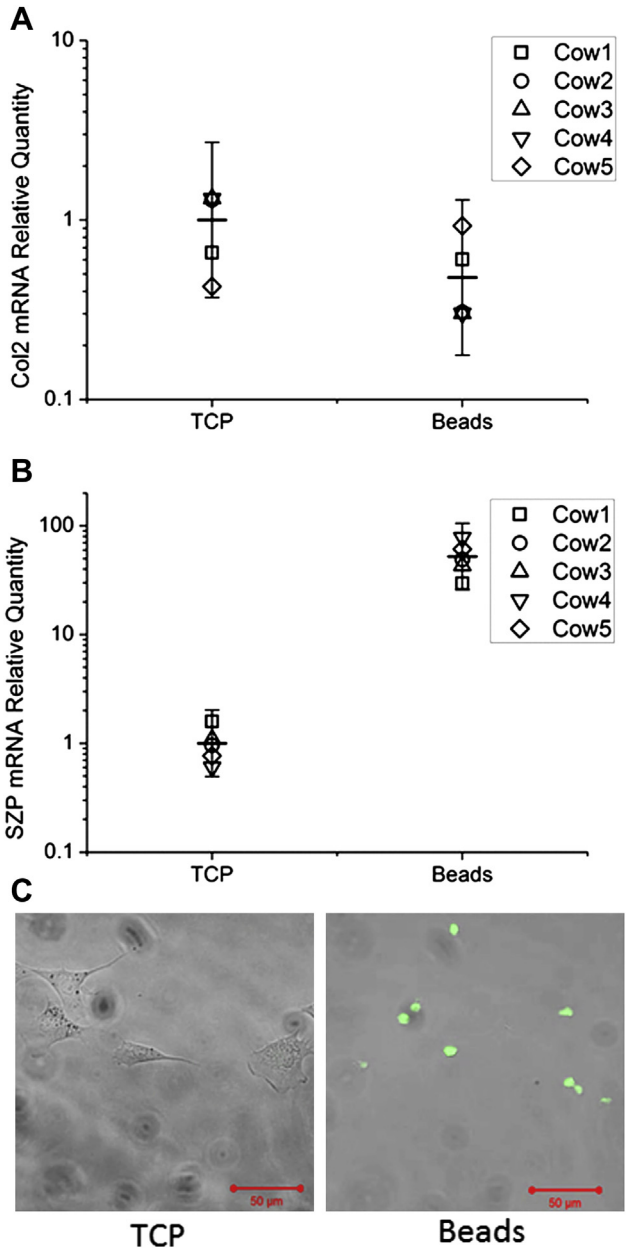

Fig. 2. Passage 3 chondrocytes were cultured on tissue culture plastic (TCP) for 4 days or encapsulated in alginate beads (Beads) for the same period to determine if expression of *SZP* and *Col2* could be restored. qRT-PCR mRNA levels for (A) *Col2* and (B) *SZP* on TCP and within alginate beads normalized to TCP gene expression ($n = 5$) and (C) immunostaining of *SZP* in cells cultured on TCP and in alginate beads, scale bar 50 μm.

Effect of oxygen tension on expression of SZP

Under hypoxic conditions (1% pO_2) *Col2* was upregulated while *SZP* was downregulated. Cells encapsulated in alginate beads exhibited a 49.5-fold upregulation in *Col2* gene expression under hypoxic conditions (95% CI, 7.47–17.12) compared to normoxia (95% CI, 0.12–0.45, $P = 0.016$) [Fig. 3(B)]. The expression of *SZP* on the other hand was downregulated 3-fold on average in hypoxia (95% CI, 1.6–6.54) compared to normoxia (95% CI, 3.46–32.54, $P = 0.042$) [Fig. 3(A)]. This observation is in accordance with the oxygen distribution in cartilage tissue where the superficial zone oxygen levels are estimated to be between 7% and 10% [Fig. 3(C and D)]. In the deep and middle zones, where oxygen levels are less than 1%, *SZP* is not present and *Col2* is highly synthesized [Fig. 3(C and D)]. The expression of *SZP* was also downregulated 7.7-fold on average in TCP cultures under hypoxia (95% CI, 0.04–0.51) compared to normoxia (95% CI, 0.17–5.82). The downregulation in *SZP* for TCP substrates had a *P* value of 0.059, and *Col2* levels did not vary significantly. The effect of normoxia independent of substrate (TCP or alginate) on *SZP* and *Col2* expression as determined by a two-way ANOVA was significant ($P = 0.046$ and $P = 0.026$) respectively. The effect of substrate was significant for *SZP* ($P = 0.016$) but not for *Col2* ($P = 0.33$). On the protein level, *Col2* staining was limited with no differences observed comparing the various studied conditions which may be due to the limited amount of *Col2* synthesized within the 4 days of culture. On the other hand, synthesis of *SZP* was clearly higher in alginate beads under normoxic conditions compared to hypoxic conditions [Fig. 3(C)]. In general, no differences in staining could be observed between the surface and the center of the beads which indicates that the overall oxygen tension induced in the incubator had a more potent influence on protein synthesis compared to the differences induced by the depth within the bead itself.

Application of cyclic mechanical strain to 2D and 3D constructs

The strain within the hydrogel was found to be fairly uniform across the width and length of the specimen [Fig. 4(B)]. Application of 10% tensile strain in the STREX machine [Fig. 4(A)] resulted in a tensile strain of 7.5 ± 0.6% while the same compressive strain resulted in 8 ± 0.5% within the hydrogel [Fig. 4(C)]. To determine the homogeneity of the strain field, the displacement of glass beads [Fig. 4(D)] was measured before and after application of 10% compressive/tensile strains in areas designated in Fig. 4(E). The difference in displacement of glass beads among these different locations was found to be non-significant [Fig. 4(F)]. The area between the two small round pillars [marked with arrows, Fig. 4(E)] was used thereafter for analysis of strain effects.

Effect of mechanical strain on SZP and Col2 expression

Gene expression of *SZP* was upregulated in 2D and 3D cultures by mechanical strain of 2 h/day (Fig. 5). A single application of mechanical strain at day 4 of culture resulted in 1.5-fold upregulation (95% CI, 0.92–2.56) in *SZP* while continuous strain for 2 h/day for 4 days resulted in 2.4-fold upregulation (95% CI, 1.53–3.91) in 3D cultures [Fig. 5(B)]. Cells seeded on collagen coated PDMS did not show a response to the single strain treatment while a strain of 2 h/day resulted in 1.7-fold upregulation (95% CI, 0.95–3.16) [Fig. 5(A)]. Although no statistical significant differences were

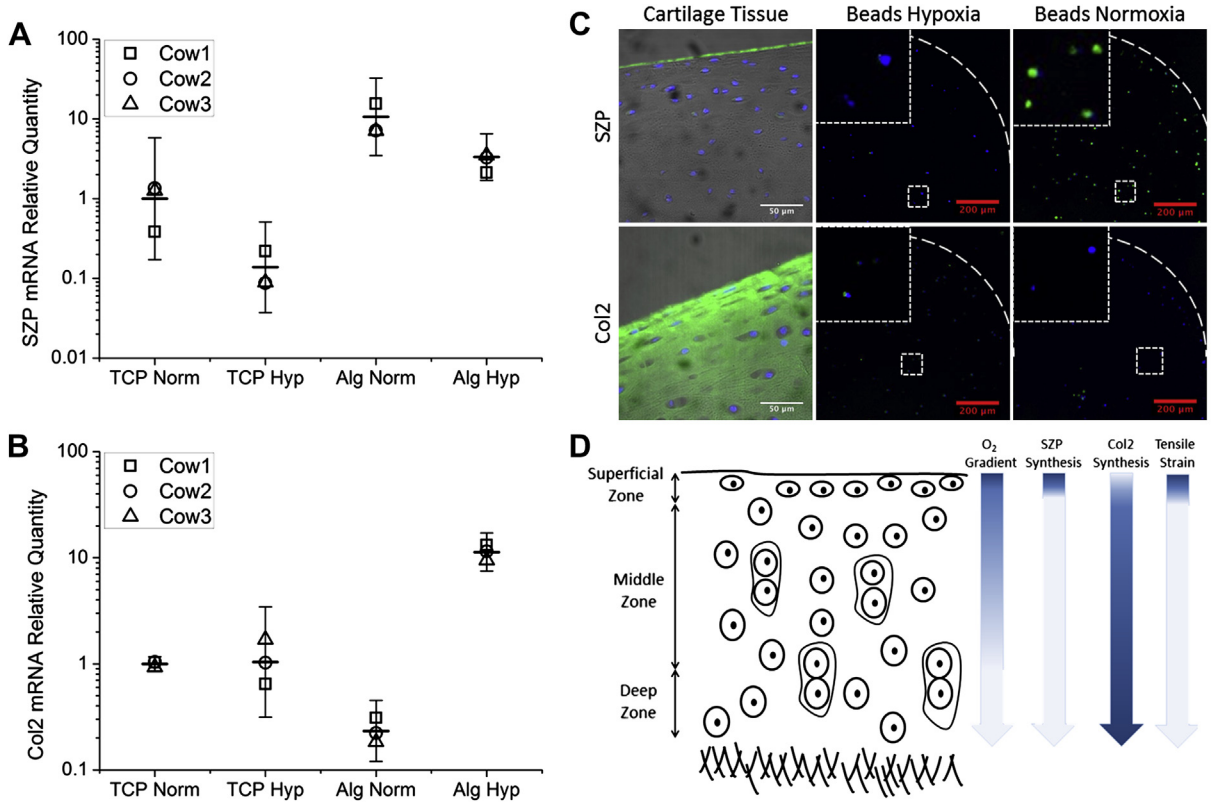

Fig. 3. The effect of oxygen tension on *SZP* and *Col2* mRNA levels was quantified by qRT-PCR. (A) mRNA levels of *SZP* and (B) *Col2* for cells seeded on TCP or encapsulated in alginate beads in normoxic or hypoxic conditions normalized to expression in TCP normoxic conditions ($n = 3$). (C) Immunostaining of *SZP* and *Col2* in cartilage tissue and alginate beads under normoxic and hypoxic conditions. The curved dashed line marks the edge of the alginate bead and the dashed box is shown at a higher magnification in the insert (scale bar 50 μm for cartilage samples and 200 μm for alginate samples). (D) Schematic representing cartilage tissue and its different layers as well as the gradients of oxygen and tensile strain and the corresponding *SZP* and *Col2* synthesis by cells of the different layers.

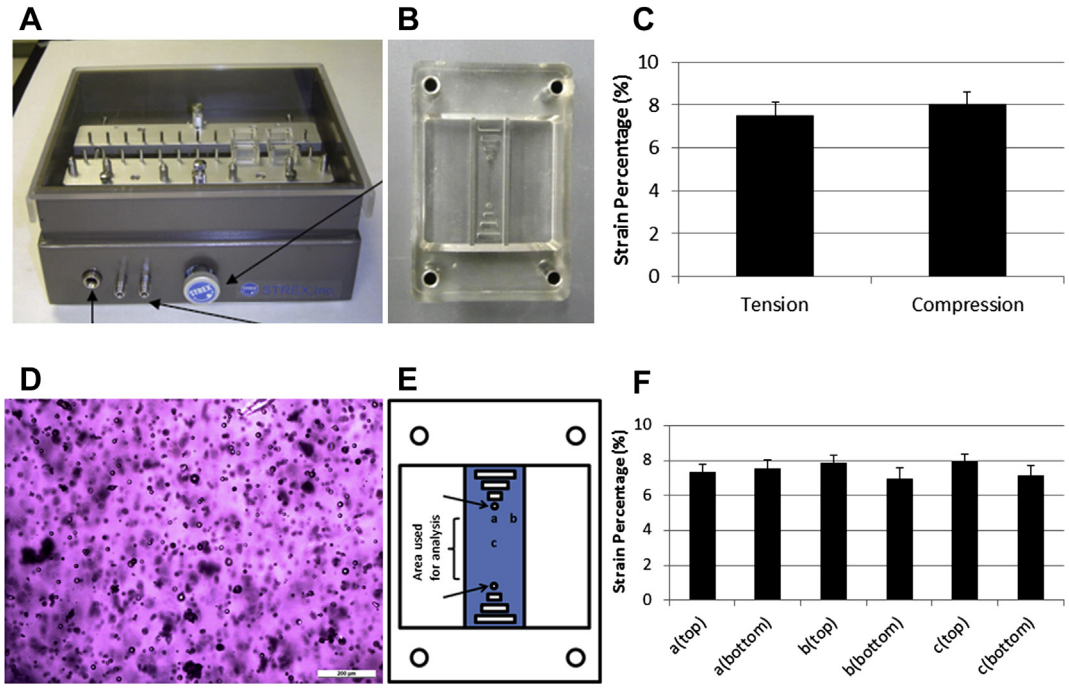

Fig. 4. Design for application of 3D cyclic strain. (A) STREX device. (B) New chamber designed for 3D loading. Metal tubes were inserted in the holes connecting the chamber to the machine to minimize non-uniform deformations around the holes. (C) Strain measured within a hydrogel in response to 10% compressive/tensile strain set by the instrument. (D) Glass beads embedded in an alginate gel (scale bar 200 μm). (E) Schematic representation of the chamber: The gel was injected in the blue area, letters (a, b and c) mark areas where images of glass beads were taken. Three images were taken 1 mm away from the small round pillar marked with an arrow (a), three images at the same level closer to the wall (b) and three images were taken in the center middle (c) to cover the whole gel in one plane. To assess variation in the *z*-axis, images were taken at two different depths resulting in a total of 18 images per gel. In each image four measurements of displacement were performed. (F) The homogeneity was assessed by measuring the displacement before/after compression/tension of 10–30 μm glass beads. Displacement was found to have no significant difference between the various locations ($n = 3$, $P > 0.05$).

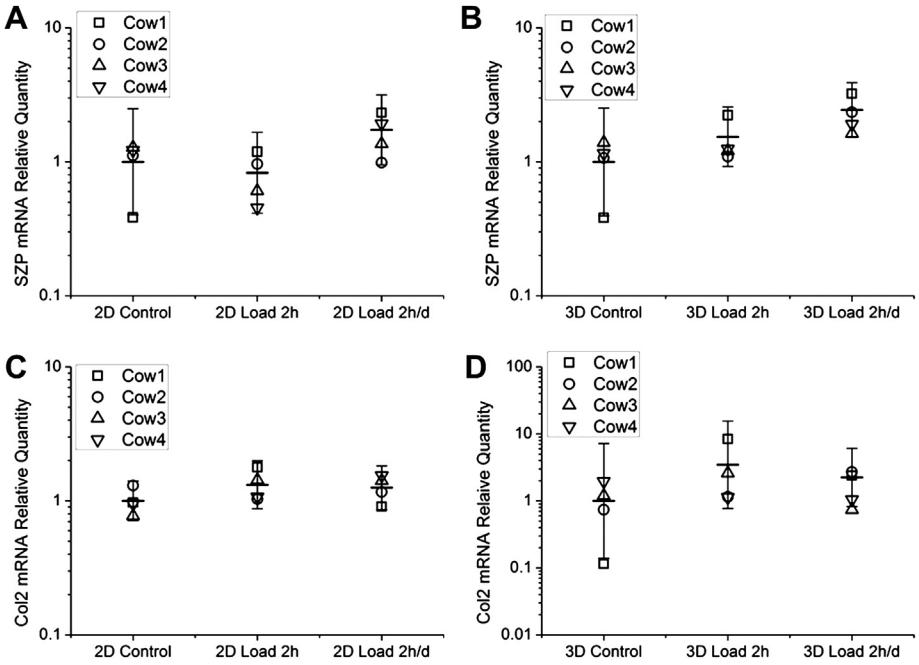

Fig. 5. The effect of mechanical strain on *SZP* and *Col2* mRNA levels was measured by qRT-PCR. (A) *SZP* mRNA levels and (C) *Col2* mRNA levels for cells seeded on collagen coated PDMS in response to 2 h strain applied 4 days post-seeding or applied every day for 2 h throughout the culture period (total of four times). (B) *SZP* mRNA levels and (D) *Col2* mRNA levels for cells in response to the same strain regime when encapsulated in alginate gels ($n = 4$). Non-strained samples were used as reference sample and RPL13 was used as a housekeeping gene.

obtained between group means as calculated by two-way ANOVA ($P = 0.31$), the data indicate an upregulation trend especially with application of 2 h/day strain. The response of *Col2* to mechanical strain was highly inconsistent among studied donors and differences between group means were insignificant as determined by a two-way ANOVA ($P = 0.58$). Expression of *Col2* was on average not affected in 2D loaded cultures [Fig. 5(C)]. Loading in 3D caused on average an upregulation of 3.46-fold (95% CI, 0.77–15.58) and 2.25-fold (95% CI, 0.82–6.12) in *Col2* expression for samples loaded for 2 h and 2 h/day respectively when compared to non-strained controls [95% CI, 0.14–7.18, Fig. 5(D)].

Discussion

In the current study we investigated the effect of serial passaging on the expression of *SZP* and *Col2* by bovine chondrocytes. Chondrocyte dedifferentiation is a common phenomenon observed in 2D culture and we show here that the reduction in *SZP* expression along with the precipitous drop in *Col2* expression can be now considered a part of the dedifferentiation phenomenon associated with monolayer culture. Dedifferentiation and redifferentiation studies have often focused on the expression of *Col2, Col1 and aggrecan* while little attention has been given to the behavior of the main superficial zone marker, *SZP*. We observed that *SZP* is downregulated with passaging similar to *Col2* and its expression can be restored within 4 days of encapsulation in alginate beads. On the other hand, the expression of *Col2* did not increase during the 4 days of alginate bead culture and partial restoration of the expression could only be achieved under hypoxic conditions. This is an interesting observation which indicates that 3D culture is not sufficient for re-expression of the cartilage phenotype. Although the common consensus is that encapsulation in alginate allows recovery of *Col2* expression[14–16], some studies have shown this is not always the case[8].

The avascular nature of cartilage leads to low oxygen levels compared to vascularized tissues. Several studies have investigated the influence of oxygen tension on chondrocyte metabolic and catabolic activities[24,32–36]. These studies showed an increased *Col2* and *aggrecan* expression[24,32–35] and improved glycosaminoglycan (GAG) deposition[36] in hypoxic compared to normoxic conditions. The effects of oxygen tension on the expression of superficial zone markers are less investigated. Schrobback *et al.*[24] found increased *SZP* mRNA levels in hypoxic compared to normoxic conditions. However, this study used 5% oxygen as a hypoxic condition and 20% for normoxic conditions. The oxygen level in cartilage is believed to be between 7% and 10% at the surface and near 0.1% adjacent to the subchondral bone[24,32]. Therefore, using 5% oxygen as a hypoxia model might not induce different responses in *SZP* expression. In our study 1% oxygen level was used as a hypoxic environment. We hypothesized that simulation of the physiological cartilage conditions would direct *in vitro* cultured chondrocytes to express zonal cartilage phenotypes. This hypothesis was confirmed by the results which showed that normoxic conditions were required for *SZP* expression while hypoxic conditions induced *Col2* re-expression and downregulated *SZP* expression. Cells of the superficial zone synthesize a matrix that is high in collagen and low in proteoglycans however these cells synthesize less collagen than deeper layers and are the only cells in cartilage that synthesize SZP [Fig. 3(C and D)]. Fig. 3(C) shows high immunoreactivity for *Col2* in the superficial zone of cartilage however, the utmost layer of cartilage stains strongly for *SZP* and not *Col2*.

Chondrocytes of the superficial zone have a more flat morphology compared to those of deeper layers which suggest that these cells might show a better response to culture conditions that induce a flat morphology such as 2D cultures. It has been reported that passage 0 cells have a higher expression of *SZP* in 2D compared to 3D which is in contradiction with our results[37]. The use of passage 0 cells in the previous report, however, may explain their results as freshly isolated cells are weakened by the enzymatic digestion process and may be less active when embedded in a 3D matrix. In preliminary studies, we observed that cells encapsulated in matrix metalloproteinase (MMP) sensitive polyethylene glycol

(PEG) gels modified with arginine-glycine-aspartic acid (RGD) peptides exhibited a spread morphology and *SZP* was downregulated compared to the non-modified PEG gel. This indicates that the spread morphology in 3D does not necessarily correlate with *SZP* upregulation (Supplementary data, Fig. S4).

Physiologic mechanical loading has been shown to stimulate gene expression and synthesis of chondrogenic markers[38–43]. The mechanism through which chondrocytes sense mechanical load is not completely understood but may involve stretch-activated ion channels and integrin signaling[41]. To further augment the expression of *SZP*, we used the commercially-available STREX device to apply cyclic mechanical strain to chondrocytes cultured in 2D and 3D[44,45]. Tensile strains are expected to be highest in the superficial zone [Fig. 3(D)] and thus were expected to induce expression of superficial zone markers[46]. Limited studies have employed the STREX device for application of 3D strain[45]. These studies did not verify uniformity of strains within the strained hydrogel and in our experience could result in non-homogeneous strains throughout the gel. In this study the strain within the gel appeared to be lower than the strain applied to the chamber where a 20% applied strain corresponded to approximately 14% strain in the hydrogel (Supplementary data, Fig. S1 and Table S1). The magnitude and duration of load were chosen based on preliminary experiments of which some are presented in the supplementary data (Fig. S2). We showed using this system that mechanically strained cells tended to have higher *SZP* expression. Mechanical stimulation also caused on average an upregulation in *Col2* expression, however this response was inconsistent among studied donors (Fig. 5). Application of mechanical strain under hypoxic conditions was also tested to achieve a further augmentation of *Col2* and rescue the *SZP* downregulation induced by hypoxic conditions. However, application of load under hypoxia caused in general a downregulation of both *SZP* and *Col2* (Fig. S3). The system developed in the current study can be used for application of homogeneous 3D strain to cells embedded in a hydrogel and may be used for several biomedical applications including but not limited to tendon, muscle and bone tissue engineering.

The current study like most of today's research in cartilage engineering uses immature bovine chondrocytes due to their greater synthetic potential compared to mature chondrocytes and unavailability of normal human adult chondrocytes. Aged chondrocytes have lower proliferation rates, synthesize less collagen and proteoglycans and may be less sensitive to growth factor stimulation[47–49]. Since *SZP* appears to be an earlier, more responsive marker of differentiated chondrocytes, we speculate that the *SZP* expression of aged chondrocytes will respond to 3D culture and oxygen in a similar way as young bovine cells, though the magnitude of change will likely be lower.

Cartilage lubrication is crucial for maintaining the function of articular cartilage and preventing its degeneration. Understanding the mechanisms that regulate the expression of *SZP* will allow the design of engineered tissue with optimal lubrication properties. We have shown that *SZP* expression in dedifferentiated chondrocytes can be upregulated to cartilage tissue levels when cultured in alginate at normoxic conditions. Moreover, we designed a chamber compatible with the STREX device that allows application of homogeneous strain in 3D to any hydrogel. The results of the current study provide insights into the regulation of *SZP* and tools to control its expression *in vitro*.

Author contributions

Rami Mhanna contributed to acquisition of data and analysis of all data, writing the manuscript and conception and design. Ece Öztürk contributed to acquisition of data and critical revision of the article. Philippe Schlink contributed in acquisition of data and critical revision of the article. Marcy Zenobi-Wong contributed to the conception and design of the study, analysis and interpretation of the data and drafting of the article. All authors approved the final version of the article.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2013.08.017.

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