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Pentosan Polysulfate Sodium Restores the Phenotype of Dedifferentiated Monolayer Canine Articular Chondrocytes Cultured in Alginate Beads

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Abstract

Autologous chondrocyte transplantation is a promising option for the repair of isolated osteoarthritic cartilage lesions that requires isolation and expansion of chondrocytes from a small cartilage biopsy prior to implantation. However, when cultured *in vitro*, chondrocytes lose their stable phenotype and dedifferentiate to fibroblastic-like cells. The study investigated the potential of pentosan polysulfate (PPS) sodium to restore the phenotype of dedifferentiated monolayer articular chondrocytes. Canine articular chondrocytes isolated from four cartilage samples were culture expanded to establish primary culture. First passage chondrocytes were cultured as alginate beads for 18 days under normoxia in PPS concentrations of 0, 1, 5, 15 and 40 µg/mL in 20% DMEM. Effect of PPS on type I, II and X collagen, aggrecan and Runx2 gene expression were evaluated by real-time PCR. Runx2, HIF-1α and HIF-2α protein expression were evaluated by Western blot and proteoglycan deposition was determined by Alcian blue stain. Dedifferentiated chondrocytes fully retained their phenotype as evidenced by increased synthesis of cartilage-specific genes, type II collagen and aggrecan mRNA with complete suppression of type I and X collagen at PPS concentrations of 15 and 40 µg/mL. Compared to the control, type II collagen and aggrecan mRNA were significantly upregulated ($P<0.05$) at 5, 15 and 40 µg/mL and 5 and 15 µg/mL PPS, respectively. PPS significantly enhanced proteoglycan with peak deposition at 5 µg/mL compared to control. HIF-1α and HIF-2α proteins were detectable at protein level for the first time under normoxia condition in alginate culture. The study demonstrates for the first time the restoration of dedifferentiated canine articular chondrocytes phenotype by combining alginate encapsulation with culture in PPS without the addition of known chondrocytic growth factors. The study confirms PPS as novel chondroinductive factor with potential to offer a solution to the major challenges that exist in cartilage tissue engineering.

Keywords: Osteoarthritis; Pentosan polysulfate sodium; Chondrocyte dedifferentiation; Chondrocyte redifferentiation; Chondrocyte phenotype; Autologous chondrocyte transplantation

Introduction

Autologous chondrocyte transplantation (ACT) which involves *in vitro* monolayer expansion of chondrocytes prior to implantation is a promising alternative strategy for the repair of isolated osteoarthritic (OA) cartilage lesions. However, when monolayer expanded *in vitro*, articular chondrocytes lose their phenotype and dedifferentiate to fibroblastic-like cells synthesizing fibrocartilage proteins instead of hyaline cartilage proteins [1-4]. Fibrocartilage is undesirable for ACT as it does not possess the biochemical composition or structural organization to provide proper mechanical function within the joint environment and degrades with time due to insufficient load-bearing capacity [3,5,6].

In an attempt to restore the phenotype of dedifferentiated monolayer chondrocytes, three-dimensional (3D) hydrogel cultures have been employed. In general, 3D hydrogels provide a suitable condition for redifferentiating of chondrocytes because of their ability to mimic *in vivo* environment and support a spherical cell morphology which plays a major role in gene expression [3,7]. Some previous studies have reported phenotype restoration in long-term 3D encapsulation cultures marked by complete suppression of type I collagen and re-expression of type II collagen and aggrecan to levels similar to primary cultures (P0) [8-11]. In contrast, other studies have demonstrated drastically higher type I collagen expression with reduced level of expression of type II collagen and aggrecan when expanded chondrocytes are encapsulated in alginate beads [3,12]. To optimize the redifferentiation of chondrocytes, other studies have combined alginate encapsulation with culture in established chondrocytic growth factors [12-15] and reduced oxygen tension to physiological level [16,17]. While these studies demonstrated restoration of key chondrocyte phenotype markers, the undesirable type I collagen was still detectable and even reported higher in alginate than monolayer chondrocytes cultured in medium supplemented with chondrogenic growth factors (1% insulin-transferrin-sodium selenite medium supplement (ITS), 1% L-ascorbic acid-2-phosphate and 10 ng/ml transforming growth factor beta 3) [12] whereas hypoxia showed no effect on type I collagen [16,17]. Taken together, these findings provoke the desire to improve the current culture strategies or develop novel culture conditions if implantable phenotypically stable chondrocytes are to be established for successful cartilage tissue regeneration and ACT for OA treatment.

One strategy that requires further research involves combining alginate encapsulation with culture in drugs capable of modulating chondrocytes gene expression. Pentosan polysulfate (PPS), a semi-synthetic sulfated polysaccharide derived from wood of beech plant (*Fagus sylvatica*) was recently shown to promote proliferation and

chondrogenesis of human bone marrow-derived mesenchymal precursor cells (MPC) while suppressing osteogenic expression and bone formation in micromass cultures (MMC) [18]. However, follow-up studies are currently lacking to clarify whether these chondrogenic effects may aid in restoring the phenotype of dedifferentiated monolayer expanded articular chondrocytes in 3D hydrogel cultures for cartilage tissue engineering purpose. PPS is a proposed disease modifying OA drug (DMOAD) which has also been previously shown to improve synovial and subchondral blood flow, limit cartilage matrix degeneration and stimulate hyaluronic acid (HA) and proteoglycan (PG)/aggrecan synthesis [19-23]. Recently, our laboratory demonstrated its involvement in the prevention of inflammatory intracellular responses induced by interleukin 1-beta (IL-1β) through inhibition of phosphorylation of certain MAPKs, p38 and ERK [24].

Therefore, the purpose of the present study was to investigate the potential of PPS to completely restore the phenotype of dedifferentiated monolayer articular chondrocytes *in vitro* since successful ACT and cartilage tissue regeneration therapy mandatorily depend on a cell population with a stable chondrocyte phenotype. The present study confirmed that PPS can completely restore the phenotype of dedifferentiated monolayer expanded chondrocytes marked by enhanced cartilage-specific genes, type II collagen and aggrecan, with indiscernible type I and X collagen.

Materials and Methods

Canine articular chondrocytes (CAC) source and expansion culture

Canine articular chondrocytes (CAC) were isolated from four cartilage samples of three dogs; a client-owned 10 months old dog undergoing femoral head and neck ostectomy due to Legg-Calve-Perthes disease (LCPD) and, a 3 year old and a 10 year old experimental dog. All procedures involving the use of experimental dogs were in accordance with Hokkaido University Institutional Animal Care and Use Committee guidelines (approval#:12-0059). Chondrocytes from the 3 year dog were isolated from both humeral head cartilages representing two independent samples. Primary cultures (P0) were established as previously described elsewhere [2]. Briefly, the cartilage was sliced into small pieces in a 100 mm polystyrene culture plate (Corning, Lowell, MA, U.S.A.) containing 10 mL of 0.05% trypsin solution and incubated with moderate magnetic agitation for 25 min at room temperature (RT). After rinsing, cartilage slices were digested in 20 mL 0.3% collagenase (Wako Pure Chemical Industries Ltd, Osaka, Japan) by incubating for 24 h at 37ºC in an incubator with 5% CO_2. Cell suspension was mixed homogeneously by repeated pipetting and sieved through a 70 µm nylon filter. The filtrate was centrifuged at 400 xg for 10 min and resulting cell pellet resuspended in 20 mL Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL, Grand Island, NY, U.S.A.) supplemented with of 10% fetal bovine serum (FBS) (Nichirei Biosciences Inc., Tokyo, Japan, Batch#:83300104), 100 IU/ml of penicillin and 0.1 mg/ml of streptomycin. Cells were manually counted using haemocytometer with 0.5% Trypan blue stain. Chondrocytes were plated at 1×10^4 cells/cm^2 and cultured to ~85-90% confluence in 10% DMEM in 100 mm polystyrene culture plates at 37°C in 5% CO_2. Medium was replenished every 72 h and at confluence, P0 monolayer cells were detached using TryPleTM Select Enzyme (1x) (GIBCO BRL). First passage (P1) cells were used in all alginate experiments. As control, monolayer cells were subcultured up to passage 3 (P3).

Alginate encapsulation of chondrocytes

Chondrocytes (P1) at 1.1×10^7 cells/mL seeding density were encapsulated in alginate beads (1.25% (w/v) sodium alginate solution, Wako Pure Chemical Industries Ltd., Osaka, Japan, Product code: 192-09951) as described elsewhere in 12-well plates (Corning) [2]. After washing the beads thrice with 0.15 M NaCL solution, then twice with 10% DMEM, specific treatment with PPS (Cartrophen Vet® injection (NaPPS - 100 mg/mL; Biopharm Australia, NSW, Australia) concentrations of 0, 1, 5, 15 and 40 µg/mL supplemented in 20% DMEM was assigned. We used 20% FBS based on previous studies that showed that 20% FBS [25] and human serum (HS) [13] support redifferentiation of human chondrocytes. The beads were incubated and cultured under normoxia condition at 37°C in a humidified atmosphere of 5% CO_2 for 18 days. Each culture well contained 75 beads equivalent to 1.1×10^7 cells and medium was changed every 72 h.

Reverse transcriptase-PCR gel electrophoresis and quantitative real-time PCR

Following recovery of cells from alginate beads, total RNA and protein was extracted using TRIZol® Kit (Invitrogen, Life Technologies, Carlsbad, CA, U.S.A), according to the manufacturer's instructions. As control, RNA was extracted from 1.0×10^6 monolayer chondrocytes at P0-P3 and probed for gene expression to demonstrate the differentiation status of the cells in monolayer culture. 500 ng RNA was reverse transcribed into cDNA using M-MLV RT kit (Invitrogen) and amplified using PCR (TakaRa Bio, Tokyo, Japan) according to manufacturer's recommended procedures to probe for Sox-9, type I, II and X collagen, aggrecan, runt-related transcription factor (Runx2), hypoxia inducible factor-alpha isoforms (HIF-1α and HIF-2α) and parathyroid hormone related protein (PTHrP) mRNA expression levels. The PCR conditions were an initial denaturation of 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s and then a finishing step of 72°C for 1 min. Amplified products were analysed by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. PCR products were identified and verified based on the expected product size relative to the standard ladder. Quantitative real-time PCR (qPCR) was performed with KAPA SYBR® FAST qPCR kit (KAPA biosystems, Woburn, MA, USA) to compare the relative mRNA expression between the treatments by the two step method. The qPCR conditions were an initial denaturation of 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 20 s then a pre-melt condition of 60°C for 90 s followed by a final melt step. The standard curve method was used to determine the relative mRNA expression. All qPCR reactions were validated by the presence of a single peak in the melt curve analysis. All mRNA expressions were normalised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the control group was used as the calibrator. Primer sequences for all genes were designed using data published on the National Center for Biotechnology Information (NCBI) website using NCBI's standard and pairwise BLAST programs (Table 1).

Name of gene	Domain	[a]Primer	[b]Fragment	Accession
GAPDH	664-683	5'-CTGAACGGGAAGCTCACTGG-3'	129 bp	NM_001003142.1
	773-792	5'-CGATGCCTGCTTCACTACCT-3'		
Sox-9	565-583	5'-GCCGAGGAGGCCACCGAACA-3'	179 bp	NM_001002978.1
	724-743	5'-CCCGGCTGCACGTCGGTTTT-3'		
Type I Collagen	150-169	5'-GTGGATACGCGGACTTTGTT-3'	164 bp	NM_001003187.1
	294-313	5'-GGGATACCATCGTCACCATC-3'		
Type II Collagen	4127-4146	5'-CACTGCCAACGTCCAGATGA-3'	215 bp	NM_001006951.1
	4322-4341	5'-GTTTCGTGCAGCCATCCTTC-3'		
Type X Collagen	1782-1801	5'-TTTCTCCTACCACGTGCATG-3'	117 bp	XM_003639401.1
	1879-1898	5'-GAAGCCTGATCCAGGTAGCC-3'		
Aggrecan	6569-6588	5'-ACTTCCGCTGGTCAGATGGA-3'	111 bp	NM_001113455.1
	6660-6679	5'-TCTCGTGCCAGATCATCACC-3'		
HIF-1α	861-880	5'-GTACTTCACTGCACAGGCCA-3'	102 bp	NM_001287163.1
	943-962	5'-ACAAATCAGCACCAAGCACG-3'		
HIF-2α	1248-1267	5'-TGCAAAGCACGGGGGCTACG-3'	72 bp	XM_531807.3
	1300-1319	5'-GGCTGCAGGTTGCGAGGGTT-3'		
Runx2	1168-1187	5'-TTACTTACACCCCGCCAGTC-3'	139 bp	XM_014118252.1
	1287-1306	5'-TATGGAGTGCTGCTGGTCTG-3'		
PTHrP	82-103	5'-GACTGCTGAGAAGATCCCCTTC-3'	73 bp	NM_001003303.1
	135-154	5'-TCGCGGGACCTTTTCCTTGA-3'		

[a]Primers for forward and reverse sense are presented in a 5' to 3' orientation, [b]The expected fragment size

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HIF-: Hypoxia Inducible Factor-; Runx2: Runt-Related Transcription Factor; PTHrP: Parathyroid Hormone Related Protein

Table 1: Sequence of primers used for PCR to evaluate gene expression by chondrocytes in alginate culture.

Alcian blue stain for PG deposition analysis

Alginate beads were fixed in 4% formalin for 30 min. After fixation, the beads were sectioned and rinsed thrice with 1x PBS then stained for 30 min with 1% Alcian blue solution prepared in 0.1 N HCL. Thereafter, the beads were thoroughly rinsed with 0.1 N HCL before adding distilled water to neutralize the acidity. The stained sections were visualized under light microscope and images captured for PG deposition analysis. PG deposition was semi-quantified using a semi-automatic grading technique previously established by Gutiérrez et al [26] using Image J analysis.

Western blotting analysis

Total protein was quantified by Bradford protein assay using the Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, NanoDrop products, Wilmington, U.S.A.). Briefly, 20 µg of protein was separated on 12% (w/v) Sodium Dodecyl Sulfate-polyacrylamide gel (SDS-PAGE) and electroblotted to nitrocellulose membranes (Whatman, Dassel, Germany) at 60 V (constant) for 2 h 30 min at 4°C in 25 mM Tris, 190 mM Glycine, 20% methanol and 0.1% SDS (pH 8.3). The membranes were blocked with Tris-buffered saline-Tween buffer (TBS-T, 20 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) Tween-20) containing 5% (w/v) skim milk for 1 hr at RT. The primary antibodies were Runx2 (C-19) goat polyclonal antibody (Santa Cruz Biotechnology, Dallas, USA; Cat. #:sc-8566), mouse monoclonal Anti-HIF-1α Clone H1α67 (Sigma Aldrich, St. Louis, U.S.A; H6536) and EPAS-1 (HIF-2α) (C-16) goat polyclonal antibody (Santa Cruz Biotechnology; Cat. #:sc-8712) (1/200 dilution) and F-Actin rabbit polyclonal antibody (Bioss Antibodies, Massachusetts, USA, Cat. #: 1571R) (1/1000 dilution). Secondary antibodies (1/5,000 dilution) for F-Actin and HIF-1α primary antibodies were Pierce® Goat anti-Rabbit Poly-HRP (Pierce Biotechnology, Rockford, U.S.A; Cat. #:32260, Lot number – QG217308) and Zymed® Rabbit anti-mouse IgG-HRP conjugate (Invitrogen Corporation, Zymed Laboratories, Inc. California, U.S.A, Cat. #:81-6720), respectively. Anti-IgG goat Rabbit-Poly-HRP (R&D Systems, Minneapolis, U.S.A, Cat. #:HAF0017) was used for Runx2 and HIF-2α. The membranes were washed thrice (at 5 min interval) with adequate TBST between each antibody incubation.

The blots were developed using Western Blot Ultra-Sensitive HRP substrate (Takara Bio Inc., Otsu, Japan; Cat. #:T7104A, Lot#:AF3P025). The protein-antibody reaction were visualized for chemiluminescent signal using FUJIFILM Luminescent Image Analyzer LAS-3000 (Fujifilm Life Science, LTD, Tokyo, Japan) according to the instrument manual for the imaging system. All immunoreactive Western blots were quantified by densitometric analysis using Image J analysis software (NIH) as per the request of the Image J developers following software calibration. Densitometric results are presented as intensities relative to F-Actin (normalizer).

Immunocytochemistry: colocalization of Runx2 with PPS

P1 chondrocytes (1×10^4 cells) were plated on 8-well Permanox® slides (Thermo sciencetific nunc, New York, U.S.A.) in 10% FBS DMEM for 24 h. Cells were gently washed and incubated overnight in serum free DMEM with or without 20 µg/ml tetramethyl-rhodamine isothiocyanate labeled-PPS (TRITC-PPS). Cells were gently washed with cold 1x PBS (2-8°C), fixed with cold methanol (-20°C) for 5 min at RT then washed thrice at 5 min interval with cold 1x PBS containing 0.1% tween 20 (PBST). Non-specific antibody binding was blocked by incubating the fixed cells for 1 h at RT in 5% bovine serum albumin (BSA) (Sigma-Aldrich; Lot#019K1144) prepared in PBST. Cells were incubated with anti-human Runx2 (C-19) (1/100 dilution) overnight at 4°C then washed as previously. Rabbit anti-goat IgG-fluorescein isothiocyanate (FITC)-conjugated (Santa Cruz Biotechnology; SC-2777) (1:1000 dilution) in 1% BSA was used to visualize antigen signal (1 h, RT). Prolong® Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Eugene, Oregon, U.S.A) was used to stain the nucleus and mount the slides according to manufacturer's instructions. Slides were viewed using Zeiss LSM 700 confocal laser microscope (Zeiss, Urbana, Illinois, U.S.A).

Statistical analysis

Quantitative data analysis was performed using SPSS version 16.0. Analysis of variance (ANOVA) was used to compare the effects of PPS on gene expression between the treatments with equal variances assumed. Where significant difference was observed, post hoc multiple comparisons were performed using Fisher's Least Significant Difference (LSD). All quantitative data, unless specified is summarized as mean values (± 95% confidence interval (CI)) for four (4) independent experiments. Significant difference was defined as P-value less than 0.05.

Results

Culture of CAC in monolayer leads to dedifferentiation

The culture of CAC as monolayer resulted in dedifferentiation as early as P0 up to P3 as demonstrated by the concomitant expression of cartilage-specific genes; type II collagen and aggrecan and types I and X collagen mRNA bands. The passaged chondrocytes also distinctly expressed HIF-2α mRNA band (Figure 1A). In addition, the expression of Sox-9, Runx2 and PTHrP were also evaluated specifically at P1 monolayer since this was the selected passage for the alginate beads culture experiments. Sox-9, Runx2 and PTHrP mRNA were all distinctly expressed in all P1 CAC evaluated except in one experiment were PTHrP mRNA band intensity was reduced (Figure 1B).

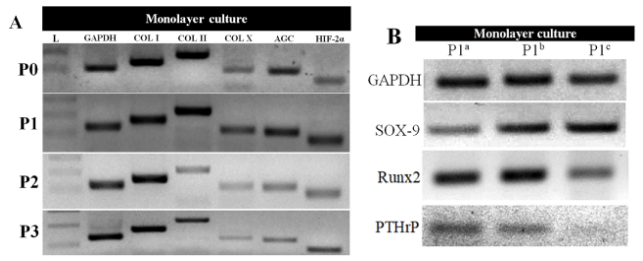

Figure 1: Canine articular chondrocytes (CAC) undergo dedifferentiation in passaged monolayer cultures. RNA was collected from monolayer expansion cultures of canine articular chondrocytes at every passage from P0 to P3 to detect the expression of chondrogenic specific-genes, type II collagen (CII) and aggrecan (AG), dedifferentiation gene marker, type I collagen (CI), chondrocyte hypertrophy gene marker, type X collagen (CX) and hypoxia inducible factor-2alpha (HIF-2α). In addition, Sox-9 (S9), Runx2 and PTHrP were evaluated at P1 by RT-PCR gel electrophoresis. Results confirm that the culture of CAC as monolayer results in dedifferentiation as early as P0 as demonstrated by the concomitant expression of cartilage-specific genes (type II collagen and aggrecan), dedifferentiation-specific gene (types I collagen) and hypertrophy gene (type X collagen) (A). Furthermore, P1 CAC also distinctly expresses Sox-9 (S9), Runx2 and PTHrP (B). Results represent one (1) experiment of four (4) independent experiments for each passage evaluated.

Alginate suppresses Sox-9 mRNA expression in CAC; PPS selectively promotes a chondrocyte phenotype while suppressing chondrocyte hypertrophy and dedifferentiation

As expected, following encapsulation of monolayer expanded chondrocytes in alginate beads the cells assumed a spherical morphology and maintained the shape throughout the culture period. Chondrocytes in all treatments distinctly expressed type II collagen and aggrecan bands. Surprisingly, the key positive cartilage regulator, Sox-9 mRNA was undetectable in all alginate cultures (Figures 2A-2C). Type I and X collagen mRNA bands were inhibited by PPS in dose-dependent pattern with both mRNA bands completely suppressed and undetectable at 15 and 40 µg/mL (Figures 2A-2C). Although Runx2 mRNA was expressed in all the treatments, its expression was evidently reduced in all the treatments compared to the results in monolayer culture. Interestingly, PTHrP mRNA was undetectable (data not shown) in all alginate cultures. By qPCR analysis, type II collagen and aggrecan were significantly upregulated by PPS relative to the control at 5 (P=0.002), 15 (P=0.03) and 40 µg/mL (P=0.036) and 5 (P=0.012) and 15 µg/mL (P=0.019), respectively (Figure 2D). Type I and X collagen were both significantly down-regulated (P<0.05) by PPS at all concentrations in a dose-dependent pattern relative to the control (Figure 2D). Runx2 mRNA was only significantly down-regulated at 1 and 5 µg/mL of PPS relative to the control (Figure 2D).

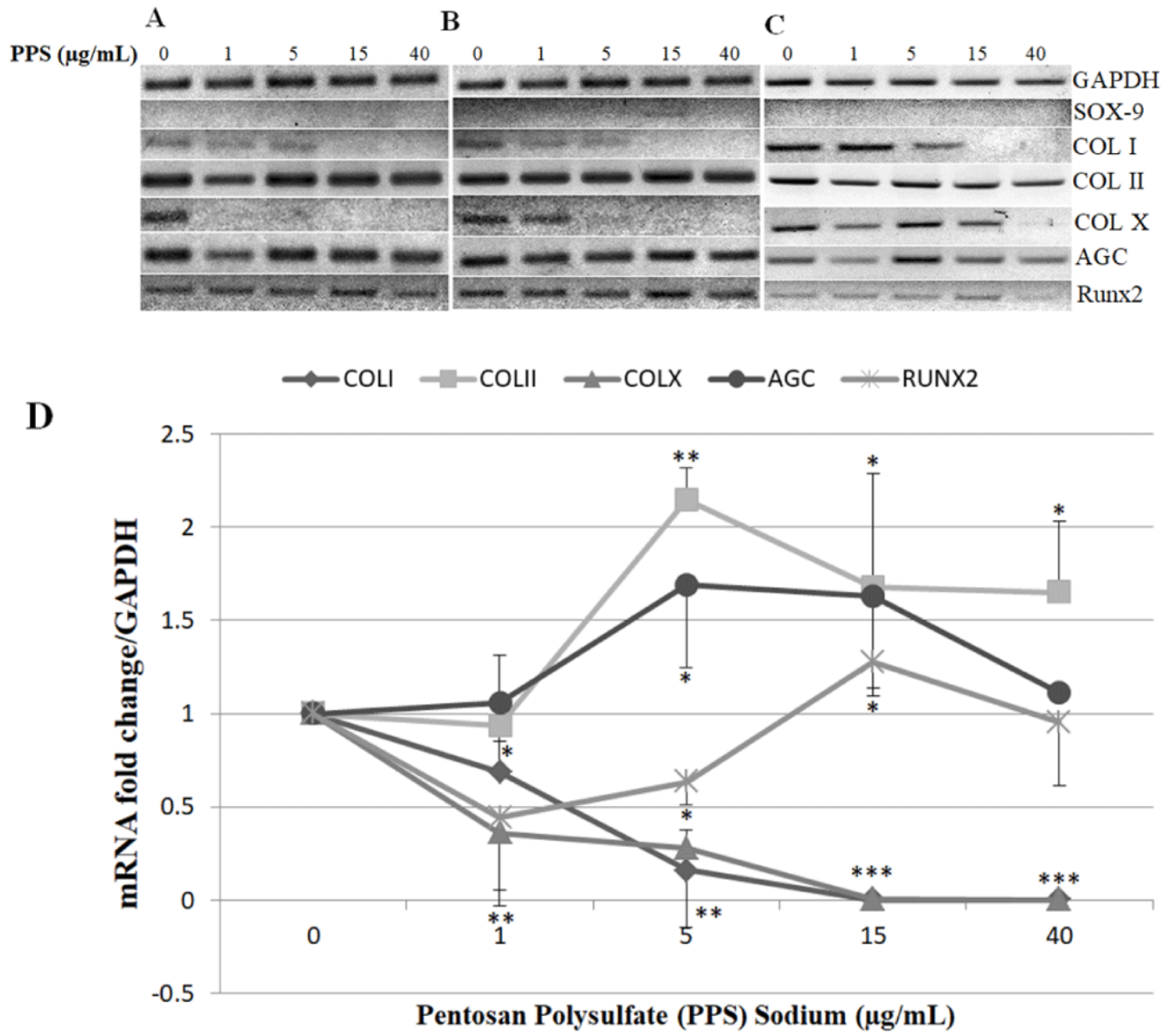

Figure 2: Effects of pentosan polysulfate (PPS) sodium on Sox-9, type I, II and X collagen, aggrecan and Runx2 mRNA expression in chondrocytes cultured in alginate beads. First passage canine articular chondrocytes isolated from: (a) femoral head cartilages of 10 month old, (b) left and (c) right humeral head cartilages of 3 year old dog were encapsulated in alginate beads and cultured for 18 days in 0 (control), 1, 5, 15 and 40 µg/mL of PPS (d) shows concentration effects of PPS on the target genes as quantified by real-time PCR (qPCR). PPS enhanced the expression of cartilage-specific genes, type II collagen (COL II) and aggrecan (AGC). Type I (COL I) and X collagen (COL X) were inhibited in a concentration-dependent manner. Significant difference was defined as *P<0.05, **P<0.01, ***P<0.001. Quantitative results represent the mean (± 95% confidence interval) of four (4) independent experiments. GAPDH; internal control gene.

Sox-9 protein expression is reduced to low 'physiological' levels in alginate beads cultured CACs

To verify whether the suppression of Sox-9 at mRNA level by alginate encapsulation of CACs correlated with the protein level, Western Blot analysis using 20 µg of cellular protein lysates loaded per lane was performed as previously described with Rabbit polyclonal Anti-Sox-9 antibody (Abcam, Tokyo, Japan, Cat. #:ab26414) at 1/500 dilution. CAC were cultured as previously described [27] as MMC for 18 days in 20% FBS DMEM supplemented with PPS concentrations of 0, 5 and 40 µg/mL as positive control for Sox-9 protein expression. Surprisingly, while Sox-9 protein was detected in all the MMC, it could not be detected in all alginate beads cultured CAC with or without PPS (Figure 3A). To verify if the non-detection of Sox-9 protein was due to actual suppression of the transcription factor in alginate encapsulated CAC, 80 µg of cellular protein lysates from each alginate cultured CAC

was precipitated by acetone method and Western blot was repeated as previously described. Interestingly, Sox-9 protein expression was detected at 80 µg protein lysates load per lane in all alginate beads culture although the expression levels were very low compared, highlighting that Sox-9 protein although downregulated in alginate cultures is still involved at very low 'physiological' levels in maintaining the chondrocyte phenotype (Figure 3B).

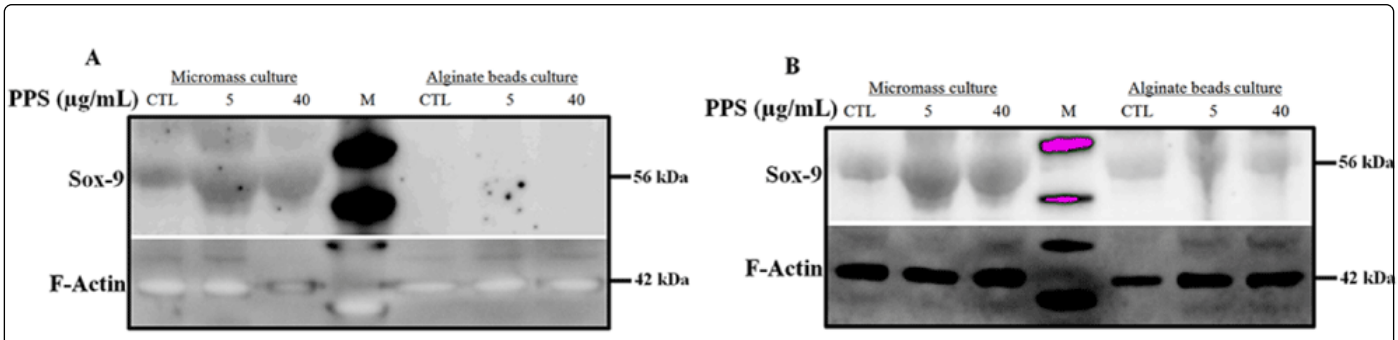

Figure 3: Sox-9 protein expression in canine articular chondrocytes cultured as micromass and alginate beads. (A) Shows that compared to MMC, Sox-9 protein was undetected in alginate cultured CAC from cellular protein lysates loaded at 20 µg per lane for each culture model. (B) However, when Western blot was repeated with 80 µg cellular protein lysates load per lane for alginate cultured CAC, Sox-9 protein was detected although at very low levels. M: Protein standards marker for blots. Predicted molecular weight for Sox-9: 56 kDa and F-Actin: 42 kDa.

PPS promotes proteoglycan (PG) deposition in alginate beads culture

Alginate encapsulated chondrocytes in the presence of PPS were characterized by an enhanced Alcian blue staining indicating increased deposition of PG (Figures 4A and 4B). Semi-quantitative analysis of PG depositions, demonstrated a peak PG deposition at 5 µg/mL of PPS which was characterized by enhanced and homogenously stained matrix with almost all cells showing PG synthesis activity (Figures 4A and 4B). PPS significantly increased ($P<0.01$) PG synthesis at concentrations of 1-15 µg/mL compared to the control (0 µg/mL of PPS). However, while PG deposition tended to be increased at 40 µg/mL of PPS compared to the control, there was no significant difference ($P>0.05$) between treatments (Figure 4B).

Alginate beads culture leads to HIFs protein stabilization; PPS has no effect on Runx2 and HIF-2α protein expression but significantly stabilizes HIF-1α expression

Normally, under normoxic culture condition HIF-α isoforms proteins are rapidly degraded in the cell as there is sufficient oxygen for the HIF-targeting prolyl hydroxylases (PHD) to target them for von Hippel-Lindau-mediated proteosomal (pVHL) degradation, therefore neither HIF-α isoform is normally detectable [28-31]. Nonetheless, HIF-1α has been shown to be expressed in suspension cultures of human normal and OA articular chondrocytes under normoxic conditions [32]. However, little is known about the expression at protein level of HIF-α isoforms in dedifferentiated monolayer articular chondrocytes encapsulated in alginate beads under normoxic culture condition and in response to PPS. As expected, by RT-PCR, HIF-1α and HIF-2α bands were expressed and readily detectable at mRNA level (Figure 5A). Interestingly, both HIF-1α and HIF-2α protein were detectable under normoxic culture condition in alginate beads culture, an indication of stabilization of the HIF-α isoforms (Figure 5A). HIF-2α protein levels were not significantly different (ANOVA, $P=0.74$) between the treatments (Figure 5B). In contrast, HIF-1α protein was prominently expressed and significantly stabilized (ANOVA, $P<0.0001$) at all PPS concentrations compared to the control (Figure 5B). The identity of the extra band at ~94 kDa level is unknown although a previous study which also observed two spaced bands under normoxia condition that became consolidated into a single band under hypoxia condition proposed this to be due to an increase in HIF-1α protein degradation [32]. There was no significant difference (ANOVA, $P=0.059$) in Runx2 protein expression between the treatments (Figures 5A and 5B). The two bands of Runx2 that were detected have been attributed to represent the full-length (upper band) and truncated form of Runx2 (lower band) [33].

Runx2 colocalizes with PPS

As expected, control chondrocytes without TRITC-PPS predominantly showed nuclear localization of Runx2 protein (Figure 6A). Notably, chondrocytes incubated with TRITC-PPS predominantly demonstrated cytoplasmic and perinuclear colocalization of Runx2 (green) with TRITC-PPS (red) with evidently reduced Runx2 nuclear localization in some cells (Figure 6B). The merged image with white arrows (yellow/orange areas) clearly demonstrates that Runx2 protein colocalizes with TRITC-PPS and possibly interacting (Figure 6B). To quantitatively the colocalization, colocalization analysis was performed on a pixel by pixel basis of Runx2 and TRITC-PPS intensity from 40 random fields. The mean (± SD) co-localization coefficient for Runx2 was 0.88 (± 0.06) (88%) and that of TRITC-PPS was 0.44 (± 0.12) (44%) with a mean overlap coefficient of 0.91 (91%). This indicates that 88% of Runx2 protein co-localized with 44% of TRITC-PPS (Figure 6C).

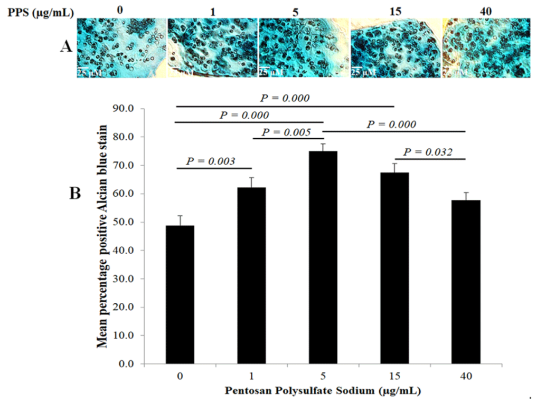

Figure 4: Effects of pentosan polysulfate (PPS) sodium on chondrocytes proteoglycan (PG) deposition in alginate beads culture. (A) Photomicrographs and (B) PG deposition semi-quantified using a semi-automatic grading technique of first passage (P1) articular chondrocytes cultured in 0 (control), 1, 5, 15 and 40 µg/mL of PPS for 18 days. Alginate beads were fixed with 4% formalin, sectioned and stained with 1% Alcian blue solution for PG deposition. PPS concentrations of 1-15 µg/mL significantly enhanced PG deposition compared to control with peak effect observed at 5 µg/mL. Five (5) random selected fields (n=20) for each treatment image were captured and quantified. Quantitative results represent the mean (± 95% confidence interval) of 20 quantified random fields. Scale Bars: 25 µm.

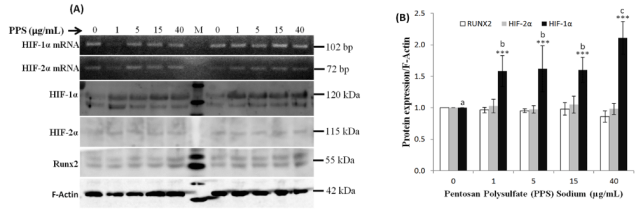

Figure 5: Effects of pentosan polysulfate (PPS) sodium on Runx2, HIF-1α and HIF-2α protein expression in chondrocytes cultured in alginate beads. First passage (P1) articular chondrocytes were encapsulated in alginate beads and cultured in 0 (control), 1, 5, 15 and 40 µg/mL of PPS for 18-days. (a) Expression of HIF-α isoforms mRNA as detected by RT-PCR, and detection of HIF-1α and HIF-2α proteins and Runx2 by Western blot. (b) Bar graphs showing the concentration effects of PPS on HIF-1α, HIF-2α and Runx2 proteins as quantified by densitometry. Quantitative data represents the mean ± 95% confidence interval of four (4) independent experiments. Significant difference was defined as ***, P<0.001, **, P<0.01, *, P<0.05. Different letters represent significantly different treatments. M: Protein standards marker for blots and 100 bp DNA ladder for gels. F-Actin (internal control): 42 kDa, Runx2: 55 kDa, HIF-1α: 120 kDa and HIF-2α: 115 kDa.

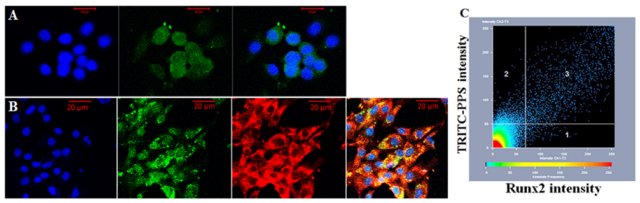

Figure 6: Co-localization analysis of Runx2 and pentosan polysulfate in immunofluorescence (IF) stained methanol fixed articular chondrocytes. First passage (P1) articular chondrocytes were incubated overnight 0 (control) and 20 µg/mL tetramethyl rhodamine (TRITC) labeled pentosan polysulfate (TRITC-PPS) in serum free (SF) DMEM. (a) Control cells showing predominantly nuclear localization of Runx2 protein (Green), while (b) Chondrocytes treated with 20 µg/mL of TRITC-PPS showing perinuclear and nuclear co-localization of TRITC-PPS (Red) and Runx2 protein. The merged section indicated by white arrows (Yellow areas) demonstrates Runx2 colocalization with TRITC-PPS. (c) Scatterplot representing Runx2 (green intensity) on the x-axis and TRITC-PPS (red intensity) on the y-axis. The three quadrants designated by crosshairs represent; quadrant 1-pixel that have high Runx2 (green) intensities and low TRITC-PPS (red) intensities; quadrant 2-pixels that have high TRITC-PPS (red) intensities and low Runx2 intensities (green), and quadrant 3-pixels with high intensity levels in both Runx2 (green) and TRITC-PPS (red) considered to be colocalized. The mean (± SD) colocalization coefficient for Runx2 was 0.88 (± 0.06) and that for TRITC-PPS was 0.44 (± 0.12) with a mean overlap coefficient of 0.91 of 40 random fields. Scale Bars: 20 µM.

Discussion

Redifferentiation after *in vitro* expansion has long been proposed as the best hope for returning chondrocytes to their native articular cartilage mode of expression [3,5]. To the best of our knowledge, this is the first study to demonstrate that the phenotype of dedifferentiated monolayer CAC is completely restored by combining alginate encapsulation with culture in standard medium supplemented with only PPS without the addition of established chondrocytic growth factors or maintaining the cells in culture for ≥ 4 weeks. Our results demonstrate selective upregulation of type II collagen, aggrecan and PG deposition with complete suppression of type I and X collagen in the presence of PPS within 18 days of culture. The observed positive anabolic effect of PPS are in agreement with previous studies [18-20,34] and inhibitory effects of PPS on type I and X collagen mRNA expression have also been previously reported in chondrogenic differentiated MPC in MMC [18]. Surprisingly, alginate encapsulation of CAC resulted in complete suppression of Sox-9 mRNA expression and significantly downregulated levels of Sox-9 protein expression which was only detectable at high cellular protein lysates load of 80 µg. While Runx2 mRNA was significantly downregulated at PPS concentrations of 1 and 5 µg/mL, by immunoblotting, there was no significant difference in Runx2 protein expression between the treatments. Nonetheless, the inhibitory effects of PPS on Runx2 mRNA expression have also been previously reported in MPC [18]. Intriguingly, in spite of using normoxic culture conditions, both HIF-α isoforms were detectable by immunoblotting, an indication of HIFs

stabilization in 3D alginate beads culture. The stabilization of HIF-1α protein expression under normoxia culture condition has been previously reported in human normal and OA articular chondrocytes in suspension pellet culture [32]. However, the detection of the HIF-α isoforms at protein level in alginate culture is being reported for the first time. In agreement with previous studies, the present study results also supports partial phenotype restoration of dedifferentiated monolayer chondrocytes cultured at high density in alginate beads. However, the addition of PPS was essential for the complete suppression of type I and X collagen gene expression. The outcome of this study confirms our working hypothesis and further confirms that PPS is a suitable alternative novel chondroinductive factor to established growth factors like TGF-β to induce not only chondrogenic differentiation of MPC as previously demonstrated by Ghosh et al. [18], but also redifferentiation of dedifferentiated monolayer articular chondrocytes as established by this study.

The transcription factor Sox-9, is a key positive regulator of articular cartilage differentiation, chondrocyte proliferation, and transition to a non-mitotic hypertrophic state [35,36] that stimulates the transcription of type II collagen and aggrecan [37]. Alginate encapsulated P3 human articular chondrocytes (HAC) after 4 weeks in 5% oxygen have been shown to fully regain type II collagen expression with aggrecan and Sox-9 levels exceeding encapsulated primary chondrocytes levels cultured in 20% oxygen [17]. The induction of Sox-9 has also been demonstrated in dedifferentiated P5 HAC encapsulated in alginate beads in 7 days cultures [12] and in adult P4 porcine chondrocytes cultured in alginate beads up to 4 weeks [38]. However, earlier studies that investigated the use of alginate encapsulation to redifferentiate rabbit articular chondrocytes [8,11] including recent studies [14,15] did not evaluate Sox-9 expression and therefore it is not clear whether the suppressive effects of alginate beads on Sox-9 observed in our study also occurs in these chondrocytes. The observed induction of type II collagen in the absence of detectable Sox-9 mRNA in our study strongly suggests that it may not be the key regulator of type II collagen promoter activity in CAC although in agreement with observations made by others [39,40], it could still be involved at very low physiological levels in maintaining the chondrocyte phenotype. In support of the observed suppressive effects of alginate encapsulation of CAC on Sox-9 mRNA expression, Western blotting with 20 μg per lane of cellular protein lysates from alginate beads cultured CAC also showed no detectable Sox-9 protein compared to MMC chondrocytes that showed induced Sox-9 protein expression. Interestingly, Sox-9 protein was detected although at very low expression levels when the cellular protein lysates from alginate beads cultured CAC were loaded at 80 μg per lane, thus indicating that the transcriptional factor is still involved at very low 'physiological' levels in maintaining the chondrocyte phenotype. We have also previously reported Sox-9 mRNA induction in monolayer cultured canine bone marrow-derived mesenchymal stem cells (cBMSC) but it is suppressed in alginate culture. Nonetheless, cBMSC demonstrate a chondrocyte phenotype in both culture systems expressing type II collagen and aggrecan culture [27]. While the exact molecular mechanism leading to the suppression of Sox-9 in alginate beads cannot be fully elucidated, it could be speculated that this could be due to the reported inhibitory effects of alginate on the chondroinductive factor, TGF-β and its ability to inhibit cell-to-cell interactions, a well-known chondroinductive factor that induces Sox-9 protein expression in MMC and pellet cultures as confirmed in this study. TGF-β1 has been reported to bind to alginate when incubated in low pH environment by replacing Ca^{2+}, resulting in inactivation of the protein but this TGF-β1-alginate bond is reversible when the beads are exposed to neutral conditions (pH 7.4), leading to the release of the active form of TGF-β1 [41]. This results in an altered cellular biochemical microenvironment in which cells are constantly exposed to varying levels of activated TGF-β1 [41,42]. Therefore, additional studies will be required to fully elucidate the impact of alginate on cartilage tissue regeneration since the outcome of this study has serious implications on its use as a bioscaffold material for cartilage tissue engineering.

The transcription factor Runx2 drives the expression of type X collagen and stimulation of hypertrophy [43,44]. Therefore, the regulation of Runx2 expression is a potential target for preventing chondrocyte hypertrophy and promoting cartilage tissue formation. Although the mechanism by which PPS completely suppressed type X collagen mRNA was not fully elucidated in this study, immunocytochemistry and co-localization analysis strongly suggest that PPS may suppress the promoter activity of Runx2 by direct interaction. This has been previously suggested as the mechanism by which PPS [18,21,24,45] and other active glycosaminoglycans (GaGs) [46,47] exert their action through interaction with transcription factors. We also evaluated PTHrP mRNA expression to verify whether the observed inhibition of type X collagen was due to PPS effects and not due to overexpression of PTHrP. PTHrP has been shown as one of the strong negative regulator that participates in a negative feedback loop with Indian Hedgehog (Ihh) to regulate chondrocyte hypertrophy by suppressing Ihh, a stimulatory factor of hypertrophy and chondrocyte proliferation that is regulated by Runx2 [43,48]. While PTHrP mRNA was distinctly expressed in monolayer cultured P1 CAC, it was undetected in all alginate cultures suggesting that the observed inhibition of type X collagen was partly due to the effects of PPS. The non-detection of PTHrP could be due to limited cell-to-cell interactions in alginate culture since PTHrP mRNA has been shown to be continuously expressed in HAC pellet cultures [48] but low and undetectable in low cell density monolayer cultures [49]. These results taken together with findings of a previous study [49] indicate that endogenous PTHrP is not involved in the regulation of chondrocyte hypertrophy.

In agreement with previous findings [28,50], we demonstrate that HIF-α isoforms are normally expressed at mRNA level in cultured CAC. However, the most provocative finding of our study was the detectable HIF-α isoforms at the protein level under normoxia culture conditions, an indication of the HIFs stabilization. The detection of HIFs study could be strongly associated with reduced oxygen tension due to enhanced matrix proteins synthesis especially in the PPS treated beads. Chondrocytes in suspension cultures compared to monolayer cultures are assumed to experience differently low oxygen tension levels depending on the quantity of matrix deposited around them [12,16,32]. While there was no significant difference in HIF-2α protein levels between the treatments, HIF-1α protein was significantly stabilized and expressed at all PPS concentrations compared to the control. Previous studies have shown HIF-2α as an anabolic factor that promotes a chondrocyte phenotype [50-53]. HIF-2α has been shown to be essential for hypoxic induction of the HAC phenotype at both the gene and protein level [52] by acting as a promoter of both Sox-9-dependent and independent factors important for key cartilage matrix synthesis [31,53]. In fact, hypoxia has been shown to promote cartilage function by two complementary mechanisms involving the HIF-α isoforms in which HIF-2α increases matrix tissue production and HIF-1α inhibits cartilage destruction [29]. Our results demonstrate for the first time that both HIF-α isoforms could be stabilized to detectable

levels in CAC cultured in alginate beads under normoxic culture conditions and may therefore consequently accumulate in these cells translocating to the nucleus to activate the transcription of their target genes required for the survival and phenotype maintenance of CAC in what appears to be Sox-9 independent.

While the result of this study have a limited sample size of only four cartilages and the protocol was only evaluated in P1 chondrocytes, it is important to point out that this strategy was initially evaluated on previously cryopreserved CAC expanded up to P2 that were alginate encapsulated from a solution of 2.0×10^6 cells/mL and cultured in 10% FBS DMEM supplemented with similar PPS concentrations. Similar suppressive effects of PPS on type I and X collagen gene expression were observed with the cells maintaining a chondrocyte phenotype. This suggests that the protocol is reproducible at least up to P2, hence more studies are required to evaluate it in higher passages (P3-P5) since ACT requires isolating chondrocytes from a small cartilage biopsy and passaging them several times to obtain a high cell population prior to implantation. This protocol could be further optimized to produce a phenotypically stable chondrocytes population by combining the observed chondroinductive effects of PPS with already established chondroinductive factors such as low physiological oxygen tension culture conditions. Furthermore, *in vivo* long-term animal model experiments will be necessary to evaluate the ability of these constructs to maintain a chondrocyte phenotype and repair experimentally-induced articular cartilage defects with hyaline cartilage matrix.

Conclusion

In conclusion, this is the first study to demonstrate that the phenotype of dedifferentiated monolayer CAC is completely restored by combining alginate beads with culture in standard medium supplemented with only PPS. While further studies are required to fully elucidate the mechanism by which PPS restores the phenotype of chondrocytes, our results confirm that PPS is a suitable alternative novel chondroinductive factor that may offer a solution to the inherent challenges faced in cartilage tissue engineering and ACT.

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