MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Sukri *et al*. | Malaysian Journal of Fundamental and Applied Sciences, Vol. 18 (2022) 304-322

RESEARCH ARTICLE

The Feasibility of Using Human Primary Chondrocytes Derived from Osteoarthritic Patients Overexpressed with *SOX9* Seeded on PLGA-Fibrin Hybrid Scaffolds for Cartilage Engineering

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Abstract This study aimed to find an optimal formulation to form 3D hyaline-like cartilage substitutes using the tissue engineering triads. The primary cells taken from osteoarthritic patients were overexpressed with transcriptional factor SRY (Sex Determining Region Y)-Box 9 (SOX9) using Lipofectamine 2000™ through a non-viral transfection method. The transfected and non-transfected cells were seeded on poly(lactic-co-glycolic acid) (PLGA) based scaffolds with and without fibrin. The arrangement resulted in four experimental groups. The 3D 'cells-scaffolds' tissue constructs were cultured for three weeks and implanted ectopically in nude mice for four weeks. The evaluations include macroscopic and microscopic study, gene expression analyses, and sulfated glycosaminoglycan (sGAG) assay, focusing on the cartilage properties. A biomechanical evaluation was performed only on post-implanted constructs. All in vitro, two- and four-week post-implanted constructs exhibited firm and smooth hyaline-like cartilage appearance. In vitro constructs showed sparse cells distribution with minimal cartilaginous tissue formation. However, a high density, lacunae-encapsulated chondrocytes embedded within the basophilic ground substances was observed in all post-implanted constructs. It is supported by positive-brownish precipitation immunolocalisation against collagen type II. Besides, molecular analysis showed that COL2A1 and other cartilaginous markers were also expressed. Increased sGAG content and compressive strain could be observed in vitro and in vivo. Although quantitatively, no significant statistical differences were found between the four groups, the qualitative results indicated that SOX9-overexpressed cells, PLGA, and fibrin combination guides hyaline-like cartilage formation better than other groups. Hence, the combination may be studied in a big animal model to develop its potential for future clinical application.

Keywords: Articular cartilage; chondrocytes; osteoarthritis; SOX9; Overexpression; gene transfer; PLGA; fibrin; ectopic implantation model.

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Received: 19 July 2021
Accepted: 25 June 2022

Introduction

After several decades of research and development, knowledge and understanding of articular cartilage tissue engineering have substantially evolved [1,2]. Cartilage is a resilient, highly specialised tissue bearing human body weight. It reduces friction impacts by cushioning and protecting the bones of the joints from gliding on each other. Histologically, cartilage represents a simple tissue structure with only one cell type, i.e., chondrocyte, sparsely distributed and embedded in an abundant, homogeneous extracellular matrix (ECM) [3]. Cartilage is also an avascular tissue [4]. From a wound healing perspective, without blood vessels, it is difficult to trigger inflammatory reactions and supply regenerating cells at the injury site [5]. Chondrocyte's low mitotic activity further reduces cartilage intrinsic healing capacities when responding to injury. Consequently, if left untreated, the condition may lead to gradual loss of articular cartilage and predispose an individual to bone and joint inflammation, i.e., osteoarthritis (OA) [6,7]. To our knowledge, there is no known cure for OA. The available non-invasive pharmacological and non-pharmacological treatments showed encouraging outcomes and relieve the associated pain to some degree [8]. Nevertheless, surgery may be prescribed to the patients as the last option if the medical conditions worsen. The surgical procedures in practice include microfracture [9], mosaicplasty [10], and autologous chondrocyte implantation (ACI) [11,12]. Their clinical outcomes seem satisfactory. However, post-surgery complications and restored cartilage quality are still arguable. This condition is a primary concern in the orthopaedic [5]. Despite numerous medical and health advancements, there is still a need to fill this gap in the long run.

Tissue engineering [13,14] and gene transfer [15] approaches have been trying to address clinical needs to improve therapeutic interventions. A simple online literature search using "gene transfer" and "tissue engineering" keywords showed an increasing trend and growing interest in the areas over the past few years. Transfecting transcriptional factors or growth factors genes could be an excellent option to help the cells differentiate and develop into tissues accordingly. The feasibility of using a tissue engineering approach for cartilaginous tissue formation has been studied in vitro [16–18] and in vivo [19–21]. Good cells sources, relevant signalling factors, and compatible biomaterial scaffolds are the essential elements in tissue engineering [13,14,18,22,23]. These three elements, known as the tissue engineering triads, can be used individually or in combination [24,25]. Obtaining quality cells as starting materials to form cartilage tissues remains challenging and requires thorough research using proper cells and tissue models. Many studies have incorporated gene transfer techniques early in cartilage tissue engineering research to enhance cell properties before using them for tissue formation [17,26,27]. Gene transfer helps regulate the expression of therapeutic gene products over extended periods and maintains the regenerated hyaline articular cartilage [7,28–30].

As a master regulator, transcription factor SOX9 has been extensively studied and used in gene transfer techniques for cartilage regeneration [7,28,31–35]. It promotes chondrogenic differentiation and cartilaginous ECM synthesis. It is also used to delay hypertrophy and degenerative changes in various cell types, including articular chondrocytes [26,32,34,35], bone marrow mesenchymal stem cells (BMSCs) [7,28,36], adipose stem cells [37–40], and embryonic stem cells [41]. The genetically modified cells provide exogenous repair stimulus through SOX9 overexpression, the signalling factor for cartilage development [7,31,34,41,42]. Unlike epithelial tissues, connective tissues like cartilage have a high ECM to cell ratio, indicating ECM's substantial role in maintaining the structural integrity of cartilage [3,4]. Although cells source is the functional living unit of the human body and central for cartilage engineering, a recent review suggested that biocompatible scaffolds are still needed for in vitro cartilage formation [1]. Scaffolds made from degradable biomaterials act as a temporary artificial ECM or three-dimensional (3D) platform for cell adhesion before the cells secrete biological ECM and start forming the tissue naturally. Ideally, the scaffolds would degrade, leaving only the cellular component behind [43].

The feasibility of using hybrid scaffolds based on natural and synthetic materials in cartilage tissue engineering has been demonstrated in ectopic nude mice models and autologous in vivo systems by some studies [14,17,44,45]. Plasma-derived fibrin and PLGA have shown potential as 3D cell delivery vehicles [16,19,20,39]. The natural plasma-derived fibrin can be easily prepared using the freshly donated whole blood [18,25] or purchased from commercially available resources. Several PLGA-based

systems have been approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) and are widely used clinically to treat and diagnose diseases [46]. Fibrin and PLGA hybrid scaffolds promote cellular proliferation and cartilaginous tissue formation of rabbit's articular chondrocytes after in vivo implantation in nude mice [19]. In other studies, fibrin and PLGA hybrid scaffolds were used as a 3D platform to evaluate the gene transfer effect in cartilage repair [20,47]. The full-thickness cartilage defects of rabbit knee joints were successfully restored by a composite construct comprising BMSCs, plasmid DNA encoding TGF-ß1 and PLGA/fibrin gel hybrids [47]. The tissue construct formed smooth, well-integrated neocartilage, resembling typical articular cartilage histological characteristics [47].

Our previous preliminary study used a monolayer cell culture model to identify the potential of SOX9 to restore typical chondrocyte properties of cells source derived from the knee joint of osteoarthritic patients [33]. Mediated by the lipofection gene transfer technique, the serially passaged SOX9-overexpressed cells preserve their chondrogenic properties [48]. However, the outcomes varied in the latter attempt to form 3D cartilaginous tissue constructs in vitro. It was unsatisfying since the same monolayer cultured cell source was used and seeded on PLGA with and without fibrin as a cells' carrier. The in-vitro 3D cell culture was expected to yield results better than the 2D monolayer cell culture. However, since the in vitro static culture can never mimic the actual environment, this present study aimed to explore further and evaluate the 3D tissue constructs after ectopic implantation using the nude mice model. The in vitro constructs were placed in subcutaneous pockets of the animal for up to four weeks. It was short-term implantation, but the duration was adequate to address the biocompatibility aspect if need be [43].

The present study aims to look into the feasibility of using the primary chondrocytes derived from human osteoarthiritic patients overexpressed with SOX9 seeded on PLGA-fibrin hybrid scaffolds for cartilage engineering. This study chose a small animal model because it gives a simple yet practically conducive, dynamic microenvironment for tissue differentiation and maturation. Although the implantation is ectopic and not performed orthotopically (at the actual lesion site), the model is minimally invasive and still helpful for cartilage tissue engineering research, especially in understanding the basic science of tissue regeneration in vivo [49]. This paper presents both qualitative and quantitative results for more scientific rigour. The outcomes from this study may benefit and contribute to the progress of connective tissue research, especially the articular cartilage.

Materials and methods

Human cartilage harvest and primary cell culture procedure

Following the principles of the Declaration of Helsinki, IIUM Research Ethical Committee (IREC18) and National Medical Research Register (NMRR-12-1383-14531) approved this study. After having informed consent, human articular cartilage samples were obtained from osteoarthritic patients (n= 6; female, age range from 55 to 70 years old) who underwent total knee replacement (TKR). These irreparable osteoarthritic tissues were no more beneficial for patients and usually discarded after surgery. The healthy portions of cartilage tissues were identified grossly and dissected from the TKR samples. All samples were processed within 24 hours post-surgery. Each sample was washed with phosphate-buffered saline (PBS) (Gibco, Life Technologies, USA) containing 1% antibiotic-antimycotic (100X, Gibco). Each sample was minced and digested in 0.6% Collagenase A (Roche, Roche Diagnostics, Switzerland) at 37°C for 6 hours in an orbital shaking incubator (Stuart Scientific, Redhill, UK). After the enzymatic digestion, chondrocytes suspension was centrifuged (Hettich Zentrifugen, Andreas Hettich GmbH & Co. KG, Germany) at 6000 rpm for 5 minutes. The resulting cell pellet was washed with PBS, centrifuged, and suspended in PBS for total cell count using a haemacytometer (Silverlite, Rohem Instruments PVT., India).

Cells were plated in monolayer culture using a 6-well plate (Nunc™, Thermo Scientific, USA) with an initial seeding of 50,000 cells per well (or, 5000 cells per cm^2) in cells culture medium. The cells were then incubated in a 5% CO_2 incubator (Barnstead Lab-Line, Thermo Scientific, USA) at 37°C, and the

medium was changed every two days. Once the cells reached 80% to 90% confluency, the primary cultured cells were detached from the culture plate through trypsinisation with 0.25% Trypsin-EDTA (Gibco, Life Technologies, USA). The harvested cells were subcultured at a similar cell density in a commercially available chondrogenic medium (ChondroENHANCE™, TELA Technologies, Malaysia) and grown until passage two (P2) [33].

Porous 3D PLGA scaffolds formation using solvent casting and salt leaching technique

A stepwise procedure using solvent casting and salt leaching technique was used to construct a 3D macroporous PLGA (mole ratio 50:50, molecular weight 33,000 g/mole Resomer RG 305 H, Evonik, Boehringer Ingelheim, Germany) scaffold [18,25]. In this method, sieved sodium chloride (NaCl) particles (size: 180-250 µm) (Fisher chemicals, Fisher Scientific, UK) were mixed in a 9:5 ratio with a polymer/solvent solution (20% w/v concentration of PLGA in dichloromethane (DCM, Merck, Merck KGaA, Germany) and then cast to make a cylindrical disk scaffold using silicone moulds (7 mm in diameter and 3 mm in thickness) [16,19,50]. The salt-leaching step made a porous scaffold. The scaffolds were soaked in ultrapure water continuously for 48 hours, wherein the used water was replaced every 6 hours. The scaffolds were then freeze-dried for 24 hours in a freeze-dryer (EyeLa FDU-1200, Tokyo Rikakikai, Japan). The scaffolds were sterilised with 70% ethanol (Merck, Merck KGaA, Germany) and washed with PBS three times before 3D tissue construct formation.

Fresh plasma-derived fibrin collection and preparation

Whole blood was collected in 10 ml buffered sodium citrate tubes (BD Vacutainer, BD, USA). It was carefully transferred into a conical tube (Nunc™, Thermo Scientific, USA) and centrifuged at 4800 rpm for 6 min at 4◦C. The upper plasma layer was gently separated from the red blood cells. The resulting plasma was filtered using a sterile 0.22 µm filter membrane (Minisart, Sartorius Stedim Biotech, Taiwan) into a new tube (Nunc™) [22] and kept in a freezer at -20°C (Samsung, Korea) until further usage.

SOX9 overexpression using lipofection gene transfer method

The passaged two (P2) cells were transfected with the pcDNA3-SOX9 plasmid vectors using Lipofectamine 2000® (Invitrogen, Life Technologies, USA) upon 90% confluency, according to the manufacturer's protocol. The transfection complex containing 4.0 µg of pcDNA3-SOX9 vector and 10.0 µl of Lipofectamine 2000® in Opti-MEM Serum-free Medium (Gibco, Life Technologies, USA) was added in each culture well and mixed by rocking the plates gently back and forth. The transfected cells were incubated in a 5% CO_2 incubator at 37°C for 48 to 72 hours [33]. They were then incorporated onto 3D PLGA scaffolds with or without fibrin as cells carrier. The transfection media were changed after 4 hours of incubation. The plasmid encoding green fluorescent protein (GFP), pmaxGFP (Amaxa, Amaxa Biosystem, Germany), was co-transfected with each reaction for transfection efficiency evaluation. The transfection efficiency was evaluated qualitatively using blue excitation B-2A filter of an inverted fluorescence microscope (Nikon Eclipse Ti, Nikon, Japan). Approximately 40% transfection efficiency can be achieved via this method [33].

3D 'cells-scaffolds' construct formation

The 3D 'cells-scaffolds' constructs were formed using the transfected and the non-transfected cells seeded onto the PLGA scaffolds with and without fibrin [24,25,33]. When the monolayer cultured cells reached 80% to 90% confluency, they were detached from the culture plate by trypsinisation and counted for total cell and viability. An approximately 5 x 10^5 cells per scaffold were incorporated and suspended in plasma-derived fibrin or the culture medium. This approach allows a comparison between the following four groups (Table 1). For in vivo part, each group had four samples for gross and histology, gene expression study, sGAG analysis, and compression test. This number is essential to determine the total number of animals needed for implantation.

Table 1. Various combinations of tissue engineering triads resulted in four experimental groups in the study. The abbreviations will be used throughout this document.

Scaffolds		Cells ± signalling factor	Groups & abbreviations
1. PLGA and plasma-derived fibrin (PF)	seeded with	1. *SOX9*-transfected chondrocytes (TC)	**1. PFTC**
			2. PFC
2. PLGA only (P) **without plasma-derived fibrin*		2. non-transfected chondrocytes (C)	**3. PTC**
			4. PC

For PFTC and PFC groups, PLGA scaffolds were soaked in cells suspended in plasma-derived fibrin. This preparation resulted in the fibrin-filled group. The admixture was solidified into a gel-like substance with the addition of 50 to 100 µl of 1M calcium chloride ($CaCl_2$) (Fisher chemicals, Fisher Scientific, UK). For PTC and PC constructs, the PLGA scaffolds were seeded with cells suspended in a culture medium. This preparation made the non-fibrin-filled group. All constructs were cultured for three weeks in vitro before implantation in the nude mice [33].

Short-term ectopic implantation model

Following the Animal Welfare Act 2015, the IIUM Animal Care and Use Committee (IACUC) [IIUM/IACUCApproval/2015/(5)(22)] approved the procedure. The animal number used was 16 and determined based on the 3D 'cells-scaffolds' constructs (the subjects) that need to be implanted (see Appendix I). The Balb/c nude mice (BALB/c Nude-IVV, Prima Nexus, Malaysia) were acclimatised for one week before the implantation. Each animal was housed in an individually ventilated cage (IVC, Tecniplast, West Chester, PA) with corn cob bedding (Biocob, Biosys Corporation, Singapore) and free access to sterile water and food pellets (Altromin #1324 Rodent Maintenance diet, Altromin, Germany). The IVC maintains a microbiologically controlled environment. They were cleaned and sterilised twice a week. After three weeks of in vitro culture, the constructs from each PFTC, PFC, PTC, and PC group were implanted subcutaneously at the dorsum of 6-week-old male nude mice [25,51]. The general anaesthesia was a combination of ketamine (50 mg/ml per kg), xylazine (50 mg/ml per kg) (Ilium, Troy Laboratories Pty Limited, Australia), and zoletil (25 mg/ml per kg) (Virbac, France) at a ratio of 2:2:1. The drugs were given intramuscularly, following the manufacturer's protocol. Under an aseptic condition, the mice's skin was cleaned and applied with povidone-iodine. A subcutaneous pocket was created on the nude mice dorsum through a small non-invasive horizontal incision (<1.0 cm) of the skin. The constructs from each group were carefully inserted into the subcutaneous pocket [25,51]. The incision was closed and sutured with non-absorbable monofilament suture 5.0 (Brilon, Vigilenz, Malaysia). A non-absorbable monofilament suture showed slightly less foreign body reaction [52]. The mice were returned and kept in a new IVC and monitored daily to ensure they had no post-operative complications. The implanted constructs were harvested at two- and four-week post-implantation. The euthanasia procedure was carried out using an overdose of anaesthetic drugs. The dead mice and their cage contents were transferred to well-sealed biohazard plastic bags and disposed of following the standard operating procedure set by the IA-CUC. The resulting post-implanted constructs were observed grossly at room temperature immediately after the dissection. They were then analysed for histology and immunohistochemistry (IHC), sGAG production assay, and biomechanical testing.

Gross observation, histology, and immunohistochemistry evaluation

The shape, size, and appearance of in vitro and two- and four-week post-implanted 3D 'cells-scaffolds' constructs were observed macroscopically. They were manually palpated using forceps briefly to assess their mechanical rigidity or stiffness. The constructs were fixed in 10% neutral phosphate-buffered formalin (Leica, Leica Biosystems, Germany) and were then processed, embedded, and sectioned to prepare six µm-thick sections for histological analysis. The sections were stained with hematoxylin and eosin (H&E, Leica, Leica Biosystems, Germany), alcian blue (MERCK, Merck KGaA, Germany), and safranin O (MERCK, Merck KGaA, Germany). Each staining facilitates the assessments of overall tissue histoarchitecture involving cell and ECM distribution and organisation, GAG accumulation, and cartilage-specific proteoglycans-rich matrix, respectively [16]. Alcian Blue and Safranin O/Fast Green staining intensity is proportional to total GAG and proteoglycans deposition [53,54]. For IHC evaluation, immunolocalisation was carried out against type I and type II collagen. This procedure used Ultratech HRP Streptavidin Biotin Universal Detection System and UltratechAEC Chromogen kits protocols

(Beckman Coulter, Beckman Coulter, Inc., USA) [19,55].

Total RNA isolation, cDNA synthesis, and gene expression analysis

Cartilaginous markers expression of each 3D 'cells-scaffolds' construct was evaluated using qualitative, two-step reverse-transcription polymerase chain reaction (RT-PCR) at each time point of two- and four-week after implantation. Total RNA was extracted from each construct using 1.0 ml of Trizol reagent according to the manufacturer's protocol (Invitrogen, Life Technologies, USA). The yield and purity of RNA were determined using NanoDrop™ 1000 Spectrophotometer (NanoDrop 1000, Thermo Scientific, USA). Reverse transcription was performed using Superscript™ II Reverse Transcriptase (Invitrogen, Life Technologies, USA), according to manufacturer's protocol under the following conditions: 65°C for 5 minutes, 42°C for 2 minutes, 42°Cfor 50 minutes and lastly, 70°C for 15 minutes (TC-512, Techne, Inc., USA) [33,55]. Reverse transcription is the synthesis of single-stranded DNA, also known as complementary DNA (cDNA), using single-stranded RNA as a template, mediated by reverse transcriptase (RT). The resulted cDNA was used as a template for amplification by polymerase chain reaction (PCR). The PCR was carried out using a gradient thermal cycler (TC-512, Techne, Inc., USA). The details of primers (Integrated DNA Technologies, 1st BASE, Singapore) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), homo sapiens actin beta (*ACTB*), collagen type II (*COL2A1*), collagen type IX (*COL9A3*), collagen type XI (*COL11A2*), *SOX9*, aggrecan-core protein (*ACAN*), collagen type I (*COL1A1*), and collagen type X (*COL10A1*) genes used in this study are described in Table 2. Two housekeeping genes (*GAPDH* and *ACTB*) were used as the internal controls. The PCR products were separated by 1.5% agarose gel electrophoresis (EC 300 XL, Thermo Scientific, USA) and stained with ethidium bromide (EtBr) (Merck, Merck KGaA, Germany). The PCR product (band) was visualised by UV transillumination using a gel documentation system (Alpha Imager HP System, Alpha Innotech, USA) [33,48].

Table 2. Primers used in PCR. F – Forward, R – Reverse.

Genes	GenBank accession number	Sequence 5'– 3'	Product size (bp)
GAPDH	NM_002046.5	F:TCCCTGAGCTGAACGGGAAG R:GGAGGAGTGGGTGTCGCTGT	218
ACTB	NM_001101.3	F:AGTCCTGTGGCATCCACGAAA R:GTCATACTCCTGCTTGCTGA	281
COL2A1	NM_001844.4	F:CTATCTGGACGAAGCAGCTGGCA R:ATGGGTGCAATGTCAATGATGG	210
COL9A3	NM_001853.3	F:GAAACCAGGGTGACAGAGGAG R:CCCTCCTAACACGGCTCCTT	236
COL11A2	NM_001854.3	F:GGATCAAATGATGAGGAGATGTCCTATG R:CTAAATGGTACCTGTATATGCAGCGTTG	345
SOX9	NM_000346.3	F:GCGGAGGAAGTCGGTGAAGA R:CCCTCTCGCTTCAGGTCAGC	237
ACAN	NM_001135.3	F:CACTGTTACCGCCACTTCCC R:ACCAGCGGAAGTCCCCTTCG	184
COL1A1	NM_000088.3	F:AGGGCTCCAACGAGATCGAGATCCG R:TACAGGAAGCAGACAGGGCCAACG	223
COL10A1	NM_000493.3	F:CCCTTTTTGCTGCTAGTATCC R:CTGTTGTCCAGGTTTTCCTGGCAC	468

Sulfated glycosaminoglycan (sGAG) production assay

All 3D 'cells-scaffolds' constructs were digested with papain digestion solution (125 µg/ml of papain, 5 mM L-cysteine, 100 mM Na_2HPO_4, 5 mM EDTA, pH 6.8) at 60°C for 16 hours. According to the manufacturer's protocol, relative sGAG contents were quantitatively analysed using the sGAG Assay Kit (K-Assay®, Kamiya Biomedical Company, USA) [33]. The assay principle is based on the specific interaction between sGAG and the cationic dye Alcian Blue. Before the assay began, 150 ml of Alcian Blue working solution was prepared by mixing 50 ml of SAT solution, 90 ml of distilled water, and 10 ml of Alcian Blue stock solution [25]. After digestion of samples with the papain enzyme digestion solution, the insoluble materials were removed by centrifugation at 12,000 rpm for 15 minutes. The supernatant from each sample was transferred into a new vial. Then, 50 µl of 8M GuHCl was added to each vial,

mixed, and incubated at room temperature for 15 minutes. Next, 50 µl of SAT solution was added to each vial, mixed, and incubated again at room temperature for 15 minutes. Then, 750 µl of Alcian Blue working solution was added, mixed, and incubated for another 15 minutes. The samples were then centrifuged for 15 minutes at 12,000 rpm [24,25,33]. The supernatant was carefully removed by suction with a syringe and discarded. Then, the pellet was suspended and mixed thoroughly in 500 µl of dimethyl sulfoxide (DMSO) solution for 15 minutes on a shaker at room temperature. Again, the mixture was centrifuged at 12,000 rpm for 15 minutes. After discarded the supernatant, the pellet was dissolved in 500 µl of Gu-Prop solution and mixed for 15 minutes. For each sample, 240 ul/well was dispensed into a flat bottom 96-well microplate. Finally, the absorbance of each sample was measured at 600-620 nm using a microplate reader [25,33].

Biomechanical analysis

The biomechanical properties of harvested in vivo constructs were determined using compressive stress (MPa). A static compression mechanical test using a biomechanical analyser (ElectroPuls™ E3000, Instron, Singapore) determined the compressive strain of two and four weeks post-implanted constructs (approx.. 3 mm in diameter and 1 mm in thickness). The test was performed at a compressive strain rate of 0.1 mm/min until a break, and the maximal compressive load was reached. Each construct's average compressive stress (MPa) was calculated and statistically analysed at each time point [25]. No compression test was done on in vitro constructs because they have not had proper mechanical rigidity in this study.

Statistical analysis

Statistical analyses of quantitative data were performed using the SPSS Software 16.0. Based on the initial calculation [56–58], it was expected that the data would be normally distributed so that parametric statistical tests could be used to analyse them. However, based on the SPSS Kolmogorov-Smirnov test for normality, the data turned out to be not normally distributed; thus, the assumptions were not met. Hence, differences among four independent PFTC, PFC, PTC, and PC groups were determined using the non-parametric Kruskal-Wallis test [59] with $p<0.05$ was considered significant. A post hoc test was carried out to compare relevant significant results using the Mann-Whitney *U* test with Bonferroni's correction α-level. The results were expressed as median (interquartile range, IQR).

Results

Animal conditions

All mice remained alive and active throughout the study. No post-surgery complications were observed. As described earlier in this paper, it should be appreciated that the mice in this study are the host for the implanted 3D 'cells-scaffolds' constructs. They are not the subjects. Hence, no analysis on the animal part throughout the implantation. The subcutaneous nude mice implantation model helps simulate and represent a living bioreactor model for the tissue to grow and develop in vivo. This animal lacks a thymus and is unable to produce T-cells. Therefore it is immunodeficient and will not reject the tissue constructs. The animals were used to achieve the objective that focuses on the human hyaline-like cartilage formation in vivo.

3D constructs exhibited hyaline-like cartilage macroscopically

All in vitro 3D 'cells-scaffolds' constructs exhibited smooth, white, and glossy hyaline-like cartilage appearance (Figure 1). The two- and four-week post-implanted constructs were firmed and easily dissected from the surrounding subcutaneous tissue. A thin vascularised reddish capsule layer was observed in all constructs. However, based on a brief macroscopic examination, the constructs had no sign of vascular invasion or inflammation. Regardless of *SOX9* overexpression, the fibrin-filled PFTC and PFC constructs exhibited firmer, smoother, and better hyaline-like cartilage appearance at two weeks post-implantation than the non-fibrin-filled PTC and PC constructs (see the four experimental groups descriptions in Table 1). The former preserved their initial shape and size better than the latter throughout the experiment. All week-2 post-implanted constructs maintained their initial rounded shape, with a slight size reduction compared to the in vitro constructs. The transfected groups, i.e., PFTC and

PTC constructs, exhibited a hyaline-like cartilage appearance better than the non-transfected groups. After four weeks of implantation, all constructs exhibited 30 – 50% reduction in their size. The macroscopic appearance and changes of the in vitro and in vivo constructs are shown in Figure 1.

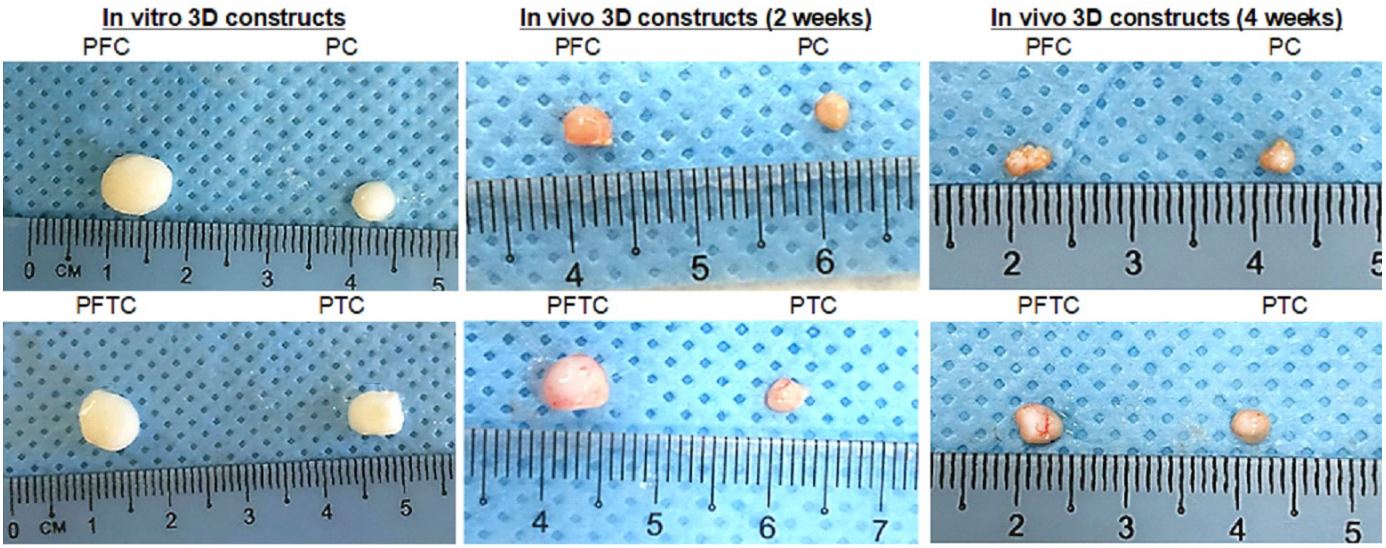


Figure 1. Macroscopic observations of the four groups of in vitro constructs (before implantation) and in vivo constructs (after two and four weeks of implantation).

3D constructs exhibited hyaline-like cartilage microscopically

The H&E stain depicts overall cell morphology, cartilaginous ECM distribution, and organisation of in vitro and in vivo constructs. Based on the microscopic observation, all transfected (PFTC and PTC) and no-transfected (PFC and PC) groups exhibited minimal hyaline-like cartilage formation. However, regardless of transfection, constructs comprised of fibrin (PTFC and PFC) exhibited a closely packed cellular distribution and ECM organisation more than the two groups without fibrin (PTC and PC). At 2-week post-implantation, both fibrin-filled PFTC and PFC constructs once again exhibited a more compact, homogenous organisation of hyaline-like cartilage histoarchitecture than the PTC and PC constructs. The inner region of the constructs showed a distribution of well-isolated chondrocytes with lacunae structure embedded in the basophilic ground substance. More lacunae-encapsulated chondrocytes were observed in PFTC, PFC, PTC, and PC after four weeks of implantation. The overall ECM deposition was comparable in all four groups (Figure 2, row 1). Intense Alcian Blue stain was seen in all constructs at two- and four weeks post-implantation, indicating the presence of accumulated GAG (Figure 2, row 2). Based on the intensity, once again, regardless of transfection, the fibrin-filled PFTC and PFC exhibited better GAG accumulation than the non-fibrin-filled PTC and PC constructs. Cartilage-specific proteoglycan-rich matrix was shown by the orange-red to red-violet region stained by Safranin O/Fast Green (Figure 2, row 3). Again, regardless of transfection, the fibrin-filled PFTC and PFC exhibited better proteoglycan-rich matrix deposition than the non-fibrin-filled PTC and PC constructs. After four weeks of implantation, the non-fibrin-filled PTC and PC constructs consistently showed a lighter Alcian Blue and Safranin O stain intensity than the fibrin-filled PFTC and PFC constructs.

3D constructs exhibited immunopositivity against collagen type II

Collagen type II and collagen type I immunolocalization were performed in all in vitro and in vivo constructs. Indicated by brownish precipitation, Collagen type II was detected in all constructs. After two weeks of implantation, brownish precipitation could be seen surrounding the pericellular matrix of the outer and inner regions tissue sections. However, regardless of SOX9 overexpression, the fibrin-filled PFTC and PFC groups exhibited colour intensity more or better than the non-fibrin-filled PC and PTC groups. After four weeks, all constructs continue expressing collagen type II (Figure 3, row 1). On the same account, collagen type I (Figure 3, row 2) was co-expressed with collagen type II and showed constant immunopositivity in all constructs at both time points

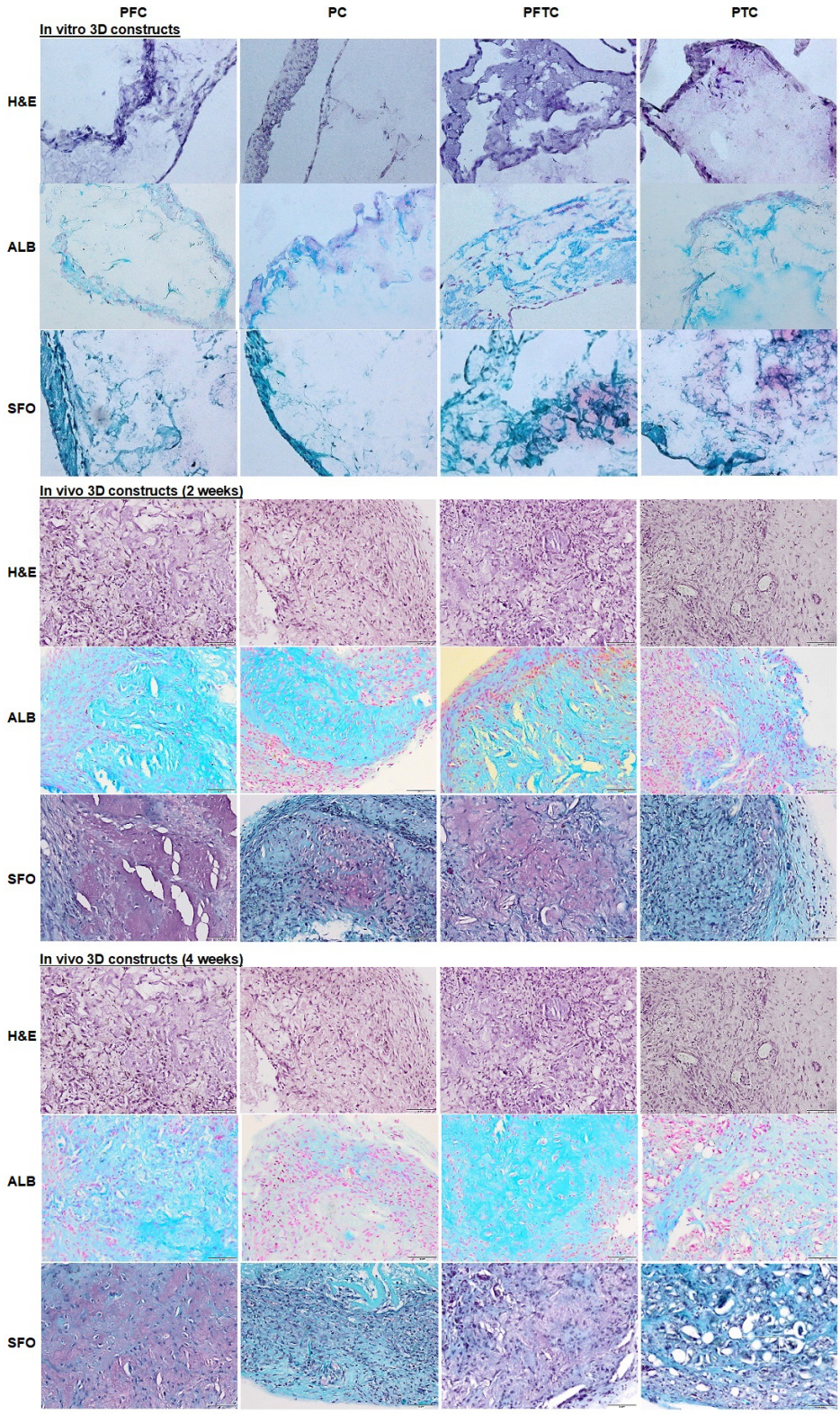


Figure 2. H&E (row 1), Alcian blue (ALB, row 2) and Safranin O/Fast Green (SFO, row 3) staining progress of the four groups of in vitro constructs (before implantation, after three weeks in culture) and in vivo constructs (after two and four weeks of implantation).

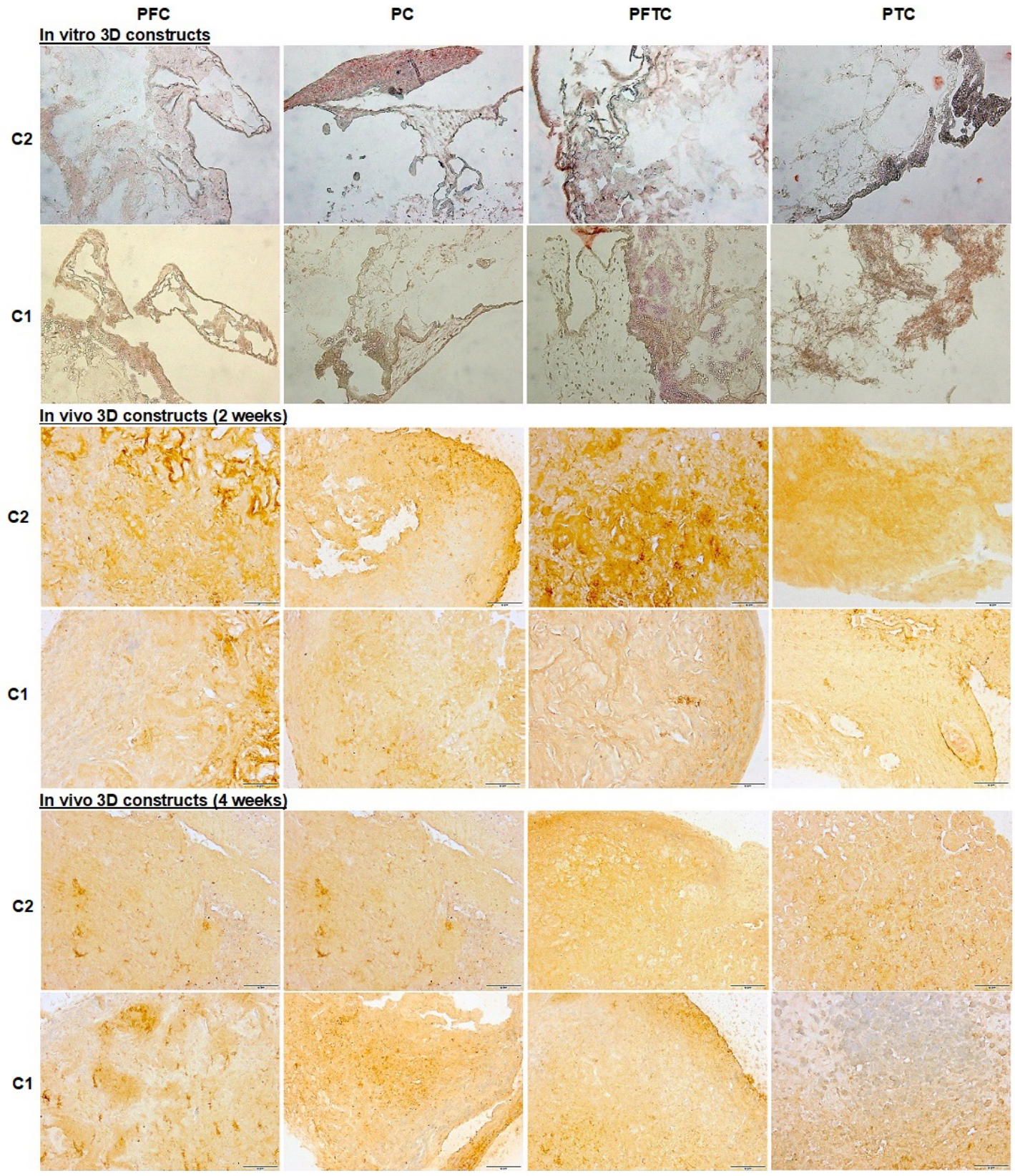


Figure 3. Collagen type II (C2) and type I (C1) IHC of the four groups of in vitro constructs (before implantation, after three weeks in culture) and in vivo constructs (after two and four weeks of implantation).

Cartilaginous gene expression in 3D constructs

When comparing the qualitative mRNA expression of in vitro and in vivo constructs, after visualising the PCR products, both transfected and non-transfected groups showed mixed results yet depicted comparable retentive potential for specific cartilage phenotypes expression (Figure 4). Chondrocytes derived from freshly digested (FD) cartilage samples served as standard (or normal control). The FD cells expressed the necessary cartilage-specific markers——*COL2A1, SOX9, ACAN, COL9A3,* and *COL11A2*. Meanwhile, *COL1A1* and *COL10A1* appear as thin-dim bands for FD cells. Although *COL2A1* exhibited a down-regulation pattern after three weeks of in vitro culture, the marker was re-expressed two weeks post-implantation. All constructs could not sustain *COL2A1* expression until week four. A similar pattern was shown in the *COL11A2* expression. Unlike *COL2A1* and *COL11A2, SOX9* and *COL1A1* were steadily expressed in all in vitro and in vivo constructs. The *ACAN* expression was detected at each time point, but it was down-regulated in vivo compared to the in vitro constructs. The hypertrophy marker *COL10A1* was detected only after two weeks of implantation, primarily in PFC and PTC. No expression of *COL9A3* was detected in all in vivo constructs (result not shown).

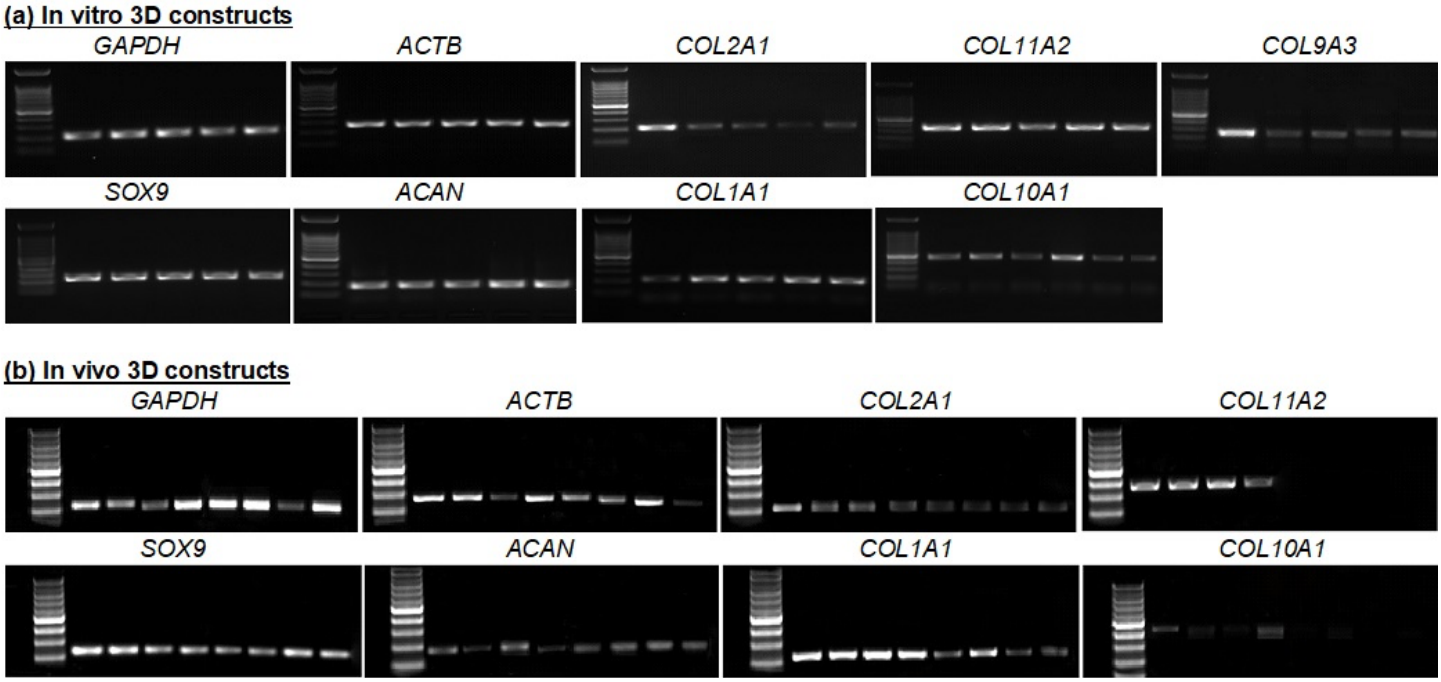


Figure 4. Gene expression results: (a) In vitro constructs (before implantation), from left to right, lane 1=100bp DNA ladder, lane 2=fresh-digested chondrocytes; lane 3=PFC, lane 4=PC, lane 5=PFTC, lane 6=PTC. (b) In vivo constructs (after two and four weeks of implantation), from left to right, lane 1=100bp DNA ladder*, Week 2=lane 2 to 5 (PFC, PC, PFTC and PC), and Week 4=lane 6 to 9 (PFC, PC, PFTC and PC), respectively. Cells from freshly digested (FD) cartilage samples served as standard (or normal control) because the cells had not yet undergone ex vivo manipulations, e.g., cells culture and subcultures as well as transfection. The two housekeeping genes (GAPDH and ACTB) presence indicated reliable analysis.

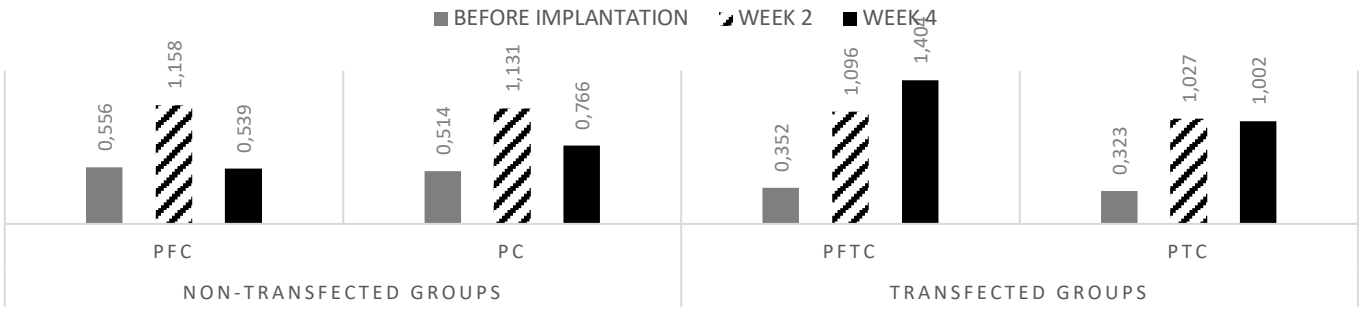
Sulphated glycosaminoglycan (sGAG) content in 3D constructs

The relative sGAG content (%) was computed and normalised by dried-weight (n=4, data not shown) of in vitro 'cells-scaffolds' constructs before implantation and in vivo constructs after two- and four-week implantation. Since the data were not normally distributed, they were expressed as median (IQR) (Table 3). From the results, before implantation the PFC accumulated 0.556 (0.145) % sGAG content, 1.158 (0.852) % at week two post-implantation, and 0.539 (0.738) % at week four post-implantation. The PC group sGAG contents were 0.514 (0.207) % before implantation, 1.131 (0.267) % at week two post-

implantation and 0.766 (0.578) % at week four post-implantation. The PFTC group exhibited increment from in vitro 0.352 (0.196) % to in vivo 1.096 (0.519) % (week 2) and 1.404 (0.726) % (week 4), respectively. The PTC group's sGAG accumulation exhibited a similar pattern as PFC and PC groups from in vitro 0.323 (0.155) % to in vivo 1.027 (0.495) % (week 2) and 1.002 (0.299) % (week 4). They had a higher sGAG content in week two post-implanted constructs than the before implantation and week four post-implanted constructs (Table 3). However, no significant differences were detected between all groups despite some promising trends in PFTC over other groups indicated in the supplementary picture below Table 3.

Table 3. Relative sGAG content (%) normalised by dried-weight of in vitro 'cells-scaffolds' constructs before implantation and in vivo constructs after 2- and 4-weeks implantation. The data were not normally distributed. Hence, they were expressed as median (IQR). Although quantitatively, no significant statistical differences between all groups, some trends can be seen when the median values are visualised using a bar chart below the table.

	PFC (%)	**PC** (%)	**PFTC** (%)	**PTC** (%)
Before implantation	0.556 (0.145)	0.514 (0.207)	0.352 (0.196)	0.323 (0.155)
Week 2	1.158 (0.852)	1.131 (0.267)	1.096 (0.519)	1.027 (0.495)
Week 4	0.539 (0.738)	0.766 (0.578)	1.404 (0.726)	1.002 (0.299)



A compressive strain of in vivo 3D constructs

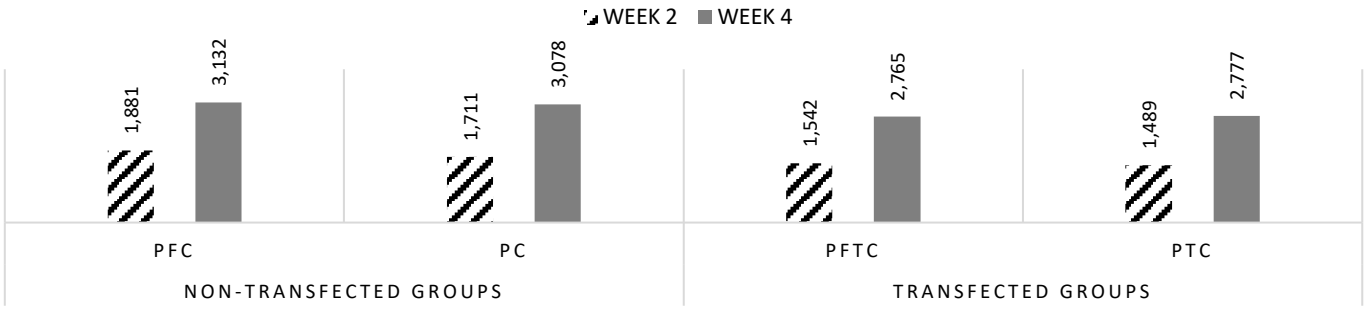
The compressive strain (MPa) data of two- and four-week post-implanted constructs (n=4) were not normally distributed when statistically examined. Expressed as median (IQR), the results for all groups can be appreciated as follows: PFC = 1.881 (1.522) MPa and 3.132 (0.675) MPa, PC = 1.711 (1.142) MPa and 3.078 (0.740) MPa, PFTC = 1.542 (1.103) MPa and 2.765 (1.289) MPa, and PTC = 1.489 (0.466) MPa and 2.777 (1.232) MPa, at two- and four-week post-implantation, respectively. The compressive strain readings in the four-week post-implanted constructs showed an increment by 1.251 MPa in PFC, 1.367 in PC, 1.223 in PFTC, and 1.288 in PTC compared to the two-week implanted constructs. However, there was no statistically significant difference in compressive strain between the transfected and non-transfected groups. The results and trends visualisation were shown in Table 4.

Discussion

As indicated earlier in this paper, this study continues our research efforts in finding an optimal solution or formulation of hyaline-like cartilage formation following the previous in vitro studies based on the monolayer cell culture model [48] and the 3D cell or tissue culture model [33]. This present study still utilised the SOX9 overexpression in human articular chondrocytes seeded on PLGA-based scaffolds with and without fibrin. The samples were obtained from female patients with an age range from 55 to 70 years. This present study moved one step further using short-term in vivo subcutaneous implantation or the ectopic implantation model using nude mice. This small animal model has long been the 'gold standard' to evaluate the construct maturation before the orthotopic study [25] or before proceeding to a large-animal model [60–62]. The dynamic microenvironment offered by the nude mice model was expected to help promote cartilage tissue formation [25,51].

Table 4. Biomechanical strength was evaluated using compressive strain (MPa) of the 3D 'cells-scaffolds' constructs 'after 2- and 4-weeks implantation only. The data were not normally distributed. They were expressed as median (IQR). Although quantitatively, no significant statistical differences between all groups, increasing trends can be seen when the median values are visualised using a bar chart below the table.

	PFC (MPa)	**PC** (MPa)	**PFTC** (MPa)	**PTC** (MPa)
Week 2	1.881 (1.522)	1.711 (1.142)	1.542 (1.103)	1.489 (0.466)
Week 4	3.132 (0.675)	3.078 (0.740)	2.765 (1.289)	2.777 (1.232)



This study shows that the 'cells-scaffolds' constructs made from SOX9-overexpressed or -transfected chondrocytes incorporated with PLGA and fibrin developed and formed a minimal cartilage tissue in vitro into a firm hyaline-like cartilage tissue after implantation. It is also noteworthy that the fibrin-filled 'cells-scaffolds' (PFTC and PFC) constructs showed a better gross appearance than the non-fibrin-filled (PTC and PC) constructs. Since PLGA's acidic by-products could trigger inflammatory reactions in the host body [63], plasma-derived fibrin has been used to reduce inflammatory reactions other than promoting cartilage formation [19,25]. This study indicated no signs of inflammation or vascular invasion surrounding the in vivo constructs. This aspect is crucial since previous studies have shown that a biomaterial can affect the cells' survival if it does not allow homogenous cell distribution, exchange of necessary molecules, or degradation by-products that are toxic [64].

With or without transfection, all constructs exhibited size reduction after implantation. These findings were contrary to previous studies in which the resulting four weeks constructs were still able to maintain the macroscopic and microscopic cartilage-like phenotypes [19,45]. As the scaffolds degrade over time, the study anticipated the articular cartilage cells would produce their ECM, leading to neocartilage formation and retain tissue size. However, that is not the case in this study. The cells in this study were taken from the knee joints of osteoarthritic patients who underwent TKR. It could be appreciated that the subcutaneous pocket is not similar to synovial joints. The subcutaneous pocket may provide a dynamic microenvironment for tissue formation, but it is still not the articular cartilage's actual environment, i.e., the joints.

Multiple studies using nasal septum [65–67] and ear [68,69] cartilages showed that the constructs formed by these two cells sources survived subcutaneously even after six weeks [69] and six months [65] of implantation in nude mice. Unlike nasal septum and ear cartilages, articular cartilage lines the epiphyses of joint ends of the bone. Its free surfaces faced the synovial fluid-filled cavity [3,4]. It could be appreciated that the structural and functional arrangements for articular, nasal septum and ear cartilages are different [70]. Hence it is not surprising that articular cartilage could not survive subcutaneously. However, it is worth noting that for clinical applications, it is essential to retain the construct's size and shape during the regeneration or healing process [21]. Size reductions in non-fibrin-filled PTC and PC constructs may indicate that they disintegrate faster than the fibrin-filled PFTC and PFC constructs. Inversely, the results showed that the fibrin-filled constructs could survive longer subcutaneously than the non-fibrin-filled constructs. Therefore, this ectopic implantation model is still helpful for articular cartilage studies to a certain extent.

Other than appealing gross appearances, the fibrin-filled 'cells-scaffolds' constructs (PFTC and PFC) also exhibited histoarchitectures better than the non-fibrin-filled constructs (PTC and PC). It is evident by high-density lacuna-encapsulated chondrocytes distributed and embedded within ECM-rich collagen and proteoglycans in both in vitro and in vivo post-implanted constructs. The results are comparable to previous cartilage tissue engineering studies [21,23,25]. The fibrin-filled 'cells-scaffolds' constructs (PFTC and PFC) had an abundant, homogenous ECM production. Hence, they exhibited a more uniform alcian blue (GAG) and safranin O (proteoglycan-rich matrix) colour shade/intensity than the non-fibrin-filled (PTC and PC) constructs, especially the PC group. The PC non-transfected, non-fibrin-filled constructs showed colour diminution after four weeks of implantation. These findings supported fibrin's numerous advantages and essential roles in tissue engineering applications [16,19,20,37,39,71,72] other than facilitating typical wound healing cascades in the human body.

Unlike the satisfactory gross and histological findings, the qualitative gene expression study of cartilage-specific markers exhibited mixed results. Interestingly, the freshly digested chondrocytes (the native control samples) showed typical cartilage gene expression despite the 55 to 70 years samples' age range, comparable to the previous study [22]. Both cartilage dedifferentiation and hypertrophic markers were barely detected in the freshly digested chondrocytes. This result suggests that a healthy cartilage portion is still available in the TKR samples. The in vitro non-transfected PFC and PC constructs exhibited better cartilaginous gene expression than the transfected PFTC and PTC groups, as previously reported [33]. All in vivo constructs expressed all the necessary cartilage-specific genes at two weeks post-implantation. However, *COL2A1* and *COL11A2* exhibited a down-regulation pattern after four weeks of implantation. The transcription factor *SOX9* and aggrecan core protein were steadily expressed throughout the experiment, consistent with *SOX9* roles in driving aggrecan expression [73]. *COL1A1* and *COL10A1* were frequently reported to co-express with the cartilage-specific markers in the in vivo construct [19,25,50]. However, these respective two dedifferentiation and hypertrophic markers were down-regulated in this present study. The *COL2A1* and *COL1A1* molecular analysis oppose the positive IHC staining against collagen type II and I. The scenario only affirmed differences between mRNA and protein expression levels. Inconsistencies are no stranger in cartilage study because it has been discussed comprehensively that many of the up-regulated genes during dedifferentiation also play roles in cartilage development [2]. These authors also argued that some up-regulated genes in cultured chondrocytes are highly expressed in an embryonic stage, neonatal epiphyseal, and articular cartilage. Their expression decreases after birth, suggesting a primary role in cartilage development but a lesser role in maintaining phenotype [2].

Only the PFTC group exhibited increment in the relative sGAG content from in vitro to in vivo. The other three groups had approximately 50% increment in the relative sGAG content after two weeks of implantation compared to in vitro. However, their relative sGAG content had then reduced between 35 – 50% after four weeks of implantation, especially in the non-transfected groups, PFC and PC. There was no significant statistical difference between *SOX9* transfected and non-transfected constructs regarding sGAG content and compressive strain.

Chemical and mechanical environments are essential to regulate the synthesis and maintenance of functionally intact ECM to develop an implant [45]. It is because the biomechanical properties of tissue depend primarily on its ECM structural composition. Even small changes in the matrix composition may lead to significant alterations in its biomechanical properties [74]. In this study, the in vivo constructs were implanted for up to four weeks subcutaneously. Hence, the compressive strain of the constructs could not achieve the level of adult articular cartilage [75]. The biomechanical properties and other biological characteristics of engineered cartilage under the skin do not precisely resemble the native human articular cartilage since the knee joint environment is different from the subcutaneous environment [21,45]. Articular cartilage undergoes complex biomechanical changes associated with the structural remodelling during its maturation [76,77]. This process requires cross-linking of the interconnected collagen and proteoglycans network, contributing to the tissue's functional properties [3,4]. It was also reported that newly formed cartilage made of mostly collagen type I does not integrate with the adjacent surrounding matrix and cannot endure mechanical stress over time [28]. The current

four weeks implantation was too short for a complete 3D 'cells-scaffolds' constructs maturation. Prolonging in vivo implantation period would be helpful to confirm this phenomenon.

Ectopic implantation models have been long used as a host for cartilage tissue grafts since their first application in the 1980s [78]. The T cell deficiency in nude mice allows tissue grafts to mature under the skin for a specific time point. This model provides a minimum yet dynamic in vivo microenvironment that mimics the physiology milieu for tissue growth [78]. A few studies using nude mice models include evaluating in vivo chondrogenic differentiation of articular chondrocytes or mesenchymal stem cells and the feasibility of the regenerated cartilage [21,36,79]. It was also used to compare how TGF-ß1 transfection affects human chondrocytes [23] and assess the hypoxic environment's effects and overexpression of hypoxia-inducible factor-1α in BMSCs human articular chondrocytes to produce cartilage-like tissue in vivo [80]. It is noted that animal use in research continually probes many questions and ethical concerns worldwide. Using a small animal model in research is a practical effort to support the expansion of replacement, reduction, and refinement (3R) principles to 5R, including 'robustness' and 'reproducibility' [81] before seriously considering embarking on a study using a pre-clinical large-animal model [60–62,77]. A large-animal model ethically requires highly skilled personnel, high-end facilities for animal care and use (many go for accredited labs), and literally, more budget.

In vivo cartilage formation using transient transfection of *SOX9* in the present study could be improved further [82]. Since this study used OA-derived chondrocytes, restoring a native phenotype in such cells may require more robust systems [2,25,36,40,41,73,83] to revive them. Previous studies demonstrated that other than *SOX9, L-SOX5* and *SOX6* also decrease with the advancement of OA in human articular cartilage [42,83]. Furthermore, *SOX* trio (*L-SOX5, SOX6*, and *SOX9*) are essential to promote chondrogenesis in chondroprogenitor cells and chondrocytes [36,37,39–41,83]. Co-transfection of the *SOX* trio genes in human ASCs was more efficient in enhancing chondrogenesis in vitro than *SOX9* transfection alone [40]. Proteoglycan deposition and reduced calcification were also demonstrated in the *SOX* trio transfected explants after three-week implantation in mice [40]. Therefore, future works involving the *SOX* trio gene transfer approach would be advantageous to restore the chondrogenic phenotypes of OA-derived chondrocytes in tissue-engineered cartilage.

Future studies should expand on the quantitative mRNA expression to verify further the effect of *SOX9* overexpression on in vivo 3D constructs. The formulation of the scaffold should be improved to avoid the size reduction of the constructs. The sample size should also be increased. Autologous implantation of an engineered cartilage construct in larger animal models is an exciting approach for future works [55]. The microenvironment in bigger animal models provides a better platform to mimic the physiological milieu of the normal knee joint [62]. Improvement to the culture system, such as dynamic culture system, may enhance the transient transfection effect of a therapeutic gene on matrix component deposition and distribution, thus form a better cartilage structure with enhanced biomechanical properties in vitro and in vivo [31,84].

Conclusions

Quantitatively, no significant statistical differences were found between the four 'cells-scaffolds' construct groups. However, it could be seen that the qualitative results indicated that *SOX9*-overexpressed cells, PLGA, and fibrin (PFTC) provides optimal and feasible combination which guides hyaline-like cartilage formation better than the other three groups (PFC, PTC, and PC). Hence, this 'cells-scaffolds' construct combination may be studied further in a pre-clinical, large animal model to develop its potential for future clinical applications while observing the 5R's principles [81].

Funding statement

Malaysia Research Assessment Instrument (MyRA) Research Grant Scheme [MIRGS13-01-002-0003], Ministry of Higher Education (MOHE) Malaysia, and International Islamic University Malaysia.

Acknowledgements

The authors thank Malaysia Research Assessment Instrument (MyRA) Research Grant Scheme (MIRGS13-01-002-0003), Ministry of Higher Education (MOHE) Malaysia, and International Islamic University Malaysia (IIUM) for research funding and facilities, IIUM Tissue Engineering and Regenerative Medicine Research Team, and Assoc. Professor Dr Tong-Chuan He from the University of Chicago Medical Center, USA, for the pcDNA3-SOX9 plasmid gift.

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