ORIGINAL ARTICLE

Matrix Production in Chondrocytes Transfected with Sex Determining Region Y-Box 9 and Telomerase Reverse Transcriptase Genes: An *In Vitro* Evaluation from Monolayer Culture to Three-Dimensional Culture

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Abstract

BACKGROUND: This study aimed to observe the cartilaginous matrix production in SRY (sex determining region Y)-box 9 (*SOX9*)- and/or telomerase reverse transcriptase (*TERT*)-transfected chondrocytes from monolayer to three-dimensional (3D) culture.

METHODS: The genes were transferred into chondrocytes at passage-1 (P1) via lipofection. The post-transfected chondrocytes (*SOX9*-, *TERT*- and *SOX9/TERT*) were analysed at P1, P2 and P3. The non-transfected group was used as control. The 3D culture was established using the chondrocytes seeded in a disc-shaped PLGA/fibrin and PLGA scaffolds. The resulting 3D “cells-scaffolds” constructs were analysed at week-1, -2 and -3. The histoarchitecture was evaluated using haematoxylin and eosin, alcian blue and safranin o stains. The quantitative sulphated glycosaminoglycan (sGAG) content was measured using biochemical assay. The cartilage-specific markers expression were analysed via real-time polymerase chain reaction.

RESULTS: All monolayer cultured chondrocytes showed flattened, fibroblast-like appearance throughout passages. Proteoglycan and sGAG were not detected at the pericellular matrix region of the chondrocytes. The sGAG content assay indicated the matrix production depletion in the culture. The cartilage-specific markers, *COL2A1* and *ACAN*, were down-regulated. However, the dedifferentiation marker, *COL1A1* was upregulated. In 3D “cells-scaffolds” constructs, regardless of transfection groups, chondrocytes seeded in PLGA/fibrin showed a more uniform distribution and produced denser matrix than the PLGA group especially at week-3. Both sGAG and proteoglycan were clearly visualised in the constructs, supported by the increment of sGAG content, quantitatively. Both *COL2A1* and *ACAN* were upregulated in *SOX9/TERT*-PLGA and *SOX9/TERT*-PLGA/fibrin respectively. While, *COL1A1* was downregulated in *SOX9/TERT*-PLGA.

CONCLUSION: These findings indicated that the *SOX9/TERT*-transfected chondrocytes incorporation into 3D scaffolds facilitates the cartilage regeneration which is viable structurally and functionally.

Keywords Cartilage · Chondrocytes · Glycosaminoglycan · Proteoglycan · Tissue engineering

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1 Introduction

Tissue engineering basic elements consist of cell source, signalling molecule and biomaterial scaffold has the potential to facilitate tissue regeneration [1]. These elements assist each other during tissue development whereby signalling molecule supplies sufficient nutrient and biomaterial scaffold provides a temporary frame to stimulate cell growth [2]. In the clinical setting, it is well-known that limited healthy cell source can be retrieved from the patient. Hence, the other elements are important to support tissue maturation even if started with small cell source amount.

Chemical signalling molecule is essential in supplementing necessary nutrients to the cultured cells and stimulating the cells' behaviour. The therapeutic materials (DNA) delivery via gene transfer is one of the techniques to introduce signalling molecule into the cells. A number of therapeutic genes including *SOX9* [3], transforming growth factor beta (*TGF-β*) [4] and insulin-like growth factor-I (*IGF-I*) [5] are usually used in cartilage regeneration experimentation. The gene is either delivered through non-viral and viral approaches [6] into the targeted cell so that the cell could benefit from the expressed protein. In this study, it is hypothesised that *SOX9* and *TERT* genes promote cartilage tissue formation. *SOX9* is essential for cartilage development as it is known as the master regulator for chondrogenesis [7]. While, *TERT* regulates telomere length elongation during DNA replication which is crucial for sustaining cell's lifespan [8]. It is expected that both genes synergistically improve chondrocytes quality. A previous finding showed a combination of *SOX9* and *TERT*-transfected chondrocytes has comparable cell proliferation activity with the non-transfected chondrocytes in 3D culture [9], indicated that the transfection procedure is non-toxic to the cultured cell.

3D biomaterial scaffold provides a conducive environment for cells attachment, proliferation and differentiation [10–12]. The scaffold structure also allows extracellular matrix distributed through its interconnected pores [13] and helps the cells regain its phenotype [14]. A synthetic biomaterial, poly(lactic-co-glycolic) acid (PLGA) is widely used as scaffold because of its biodegradability and compatibility properties [15]. With the use of PLGA scaffold, encouraging outcomes on cartilaginous tissue formation have been noted through the help of natural biomaterial, fibrin (PLGA/fibrin) [2, 16, 17]. It is undeniable that natural biomaterials help to enhance tissue development whereby the biologically active components assist cell adhesion and proliferation [18]. However, it has limited mechanical strength which may not be suitable for certain load-bearing tissue application [19]. Both types of biomaterials complement their ability as a scaffold by providing adequate structural strength and biological components for cell growth.

Based on the literature, we aimed to observe matrix production in *SOX9*- and/or *TERT*-transfected chondrocytes from *in vitro* monolayer to 3D culture. The chondrocytes and “cell-scaffold” constructs were evaluated using selected histological stains, quantitative sGAG content assay and cartilage-specific markers expression using RT-PCR.

2 Materials and methods

2.1 Chondrocytes isolation

Animal ethical approval was granted by the Institutional Animal Care and Use Committee of International Islamic University Malaysia (IACUC-IIUM) (IIUM/IACUC/Approval 2015/[5]/[24]). The 6-months old New Zealand White rabbits ($n = 6$) were obtained from a commercially available source in Kuantan, Pahang. Articular cartilage was aseptically dissected from the knee joint and washed with phosphate buffered saline (PBS, pH 7.4) (Gibco, Grand Island, NY, USA) containing 1% antibiotic-antimycotic (AA) (Gibco, Grand Island, NY, USA) solution. The sample was enzymatically digested with 0.6% collagenase A (Roche Applied Science, Mannheim, Germany) in an incubator shaker (Stuart Scientific, Redhill, UK) at 37 °C for 4 h. The chondrocytes suspension was centrifuged at 2500 rpm, 5 min (Mikro 220R, Hettich Zentifugen, Germany). The resulting cell pellet was washed twice with PBS to remove the excess enzyme. After final centrifugation, the cell pellet was suspended in PBS for cell viability assessment using Trypan Blue dye (Gibco, Grand Island, NY, USA) exclusion test. The chondrocytes were plated with an initial seeding of 50,000 cell/cm² in 6-well plates (Thermo Scientific, Nunclon Delta Surface, Denmark) supplemented with 1:1 of Ham's F12 nutrient mixture (F12) and Dulbecco's Modified Eagles Medium (DMEM) culture medium supplemented with 1% (v/v) AA (Gibco, Grand Island, NY, USA), 10% (v/v) fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 1% (v/v) GlutaMAXTM (Gibco, Grand Island, NY, USA), 1% (v/v) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Gibco, Grand Island, NY, USA) and 1% (v/v) 50 µg/ml of ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA). The cultures were maintained in a 5% CO₂ incubator (Thermo Scientific, Barnstead lab Line, Asheville, NC, USA) at 37 °C. The culture medium was changed every other day. The cells were sub-cultured to passage-1 (P1), P2 and P3 when reaching 70–90% confluency.

2.2 *SOX9* and *TERT* genes transfection and chondrocytes expansion

Upon 70–90% confluency, the P1 chondrocytes were transfected with pcDNA3-*SOX9* and/or pBABE-neo-*hTERT*

plasmids via lipofection (LipofectamineTM 3000, Invitrogen, Carlsbad, CA, USA) by following the manufacturer's protocol. The green fluorescent protein (*GFP*) plasmid, pMaxGFPTM (AMAXATM 4D-NucleofectorTM, Lonza, Cologne, Germany) was co-transfected for transfection efficiency observation [20]. The transfected chondrocytes were observed using CyTeLLTM Cell Imaging System (GE Healthcare Life Sciences, Marlborough, MA, USA) [20]. The chondrocytes were divided into the following groups: (a) non-transfected chondrocytes (control), (b) *SOX9*-transfected chondrocytes, (c) *TERT*-transfected chondrocytes and (d) *SOX9/TERT*-transfected chondrocytes. The chondrocytes groups were detached from the culture plate with Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Grand Island, NY, USA) and sub-cultured to P2 and P3 for evaluation. The remaining cells from each group were cultured with initial seeding of 5000 cell/cm² into 8-well chambered slides (Lab Tek II Chambered Slide with Cover RS Glass Slide Sterile (Thermo Fisher Scientific, Rochester, NY, USA) until it reaches 30–50% confluency.

2.3 3D “cell-scaffold” construct formation

Disc-shaped of 20% (w/v) PLGA (mole ratio: 65:35, Resomer RG653H, Sigma-Aldrich, St. Louis, MO, USA) scaffold with 85.2% porosity was fabricated using solvent casting/salt leaching method based on our reported method [15]. The sieved sodium chloride (NaCl) (Merck KGaA, Darmstadt, Germany) particles with the size of 180–250 µm were added to the dissolved PLGA and cast into 3 mm height × 7 mm diameter silicon mould. 1 × 10⁵ chondrocytes groups at P1 were seeded in sterilised PLGA scaffold with or without rabbit's fibrin (Table 1). The chondrocytes seeded in PLGA with 250 µl fibrin (PLGA/fibrin) was coagulated by adding 50 µl calcium chloride (CaCl₂) (Sigma-Aldrich, St. Louis, MO, USA). The formed “cell-scaffold” constructs were cultured and harvested at week-1, -2, -3.

2.4 Histological staining

The chondrocytes in chambered slides were fixed with 4% formaldehyde. Whereas the “cell-scaffold” constructs were fixed in 10% neutral buffered formalin (NBF) (Surgipath[®], Leica Microsystem, Buffalo Grove, IL, USA), processed and sectioned to 5 µm. Haematoxylin and eosin (H&E) (Haematoxylin 560MX[®], Leica Biosystem Richmond Inc, Canada), alcian blue, pH 2.5 (Merck KGaA, Darmstadt, Germany) and 0.1% safranin o (Merck KGaA, Darmstadt, Germany) were conducted to evaluate the structural and functional aspects.

2.5 sGAG assay

The samples were digested with 125 µg/ml papain enzyme (Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 60 °C. The sGAG detection was carried out by using Alcian Blue assay as described in Karlsson and Björnsson [21]. The absorbance was measured using VersaMax microplate reader (Molecular Devices, San Jose, CA, USA) at 620 nm. The data were normalised with the sample's dried weight. The data were expressed as mean ± standard error of the mean (SEM). The data was tested with Shapiro–Wilk normality test. One-way ANOVA was used with $p < 0.05$ was considered statistically significant.

2.6 Genes expression analysis

Aggrecan (*ACAN*) and collagen type-II (*COL2A1*), collagen type-I (*COL1A1*) were evaluated using RT-PCR. The primers used are as listed in the primer sequences table (Table 2). The reaction was conducted by using CFX96 TouchTM Real-Time PCR Detection System (BIO RAD, Hercules, CA, USA). The PCR profile for the respective markers are as follows; initial denaturation, 95 °C for 5 min, 39 cycles of 95 °C for 5 s and 60.3 °C for 30 s. The data were expressed as mean ± standard error of the mean (SEM). The data was tested with

Table 1 The experimental groups and their relevant abbreviations

Scaffolds	Cells source	Abbreviation used
PLGA with fibrin		
Seeded with	SOX9 and TERT-transfected chondrocytes	SOX9/TERT-PLGA/fibrin
	SOX9-transfected chondrocytes	SOX9-PLGA/fibrin
	TERT-transfected chondrocytes	TERT-PLGA/fibrin
	Non-transfected chondrocytes (control)	Control-PLGA/fibrin
PLGA without fibrin		
Seeded with	SOX9 and TERT-transfected chondrocytes	SOX9/TERT-PLGA
	SOX9-transfected chondrocytes	SOX9-PLGA
	TERT-transfected chondrocytes	TERT-PLGA
	Non-transfected chondrocytes (control)	Control-PLGA

Table 2 The rabbit's cartilaginous-specific markers primer sequences

Primer's name	Sequences	Accession numbers	PCR product (bp)
<i>HPRT1</i>	F:5'-GCA GAC CTT GCT TTC CTT GGT-3' R:5'-GCA GGC TTG CGA CCT TGA C-3'	NM_001105671.1	62
<i>COL1A1</i>	F:5'-GGT GGT TAC GAC TTT GGT TA-3' R:5'-GCA TCG ACT TCA TAG TCC TT-3'	NM_001195668.1	95
<i>COL2A1</i>	F:5'-TGC AGG AGG GGA AGA GGT AT-3' R:5'-GGC AGT CCT TGG TGT CTT CA-3'	NM_001195671.1	123
<i>ACAN</i>	F:5'-ACA CTG GCG AGT ACT GTG AC-3' R:5'-TGT GAA AGA GTC GCT GGC AT-3'	XM_008251722.2	95

Shapiro–Wilk normality test. ONE-WAY ANOVA used with $p < 0.05$ was considered statistically significant.

3 Results

3.1 Monolayer culture

3.1.1 Cartilaginous matrix components in the cultured chondrocytes

All chondrocytes groups have flattened, fibroblast-like appearance throughout passages (Fig. 1). The chondrocyte's diameter became larger as it progresses to P2 and P3. The blue stain of alcian blue indicated sGAG accumulated in the pericellular matrix of all chondrocyte groups at P1 and P2 (Fig. 2). However, the blue stain was absent in all group at P3. For safranin o (Fig. 3), the orange-red stain that serves as positive indicator of proteoglycan was absent in all groups until P3.

3.1.2 sGAG content in the cultured chondrocytes

For sGAG content, there was no significant differences between the groups, $F(3,20) = 0.082$, $p = 0.969$ and no significant difference across the passages, $F(2,40) = 0.891$, $p = 0.418$ (Fig. 2A). There was no interaction between passages and chondrocytes groups, $F(6,40) = 0.113$, $p = 0.994$. At P1, sGAG amount started at 1.03 ± 0.48 $\mu\text{g}/\text{mg}$, 1.35 ± 0.40 $\mu\text{g}/\text{mg}$, 1.14 ± 0.69 $\mu\text{g}/\text{mg}$, 1.25 ± 0.51 $\mu\text{g}/\text{mg}$ for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively. At P3, sGAG amount was at 0.73 ± 0.37 $\mu\text{g}/\text{mg}$, 0.86 ± 0.27 $\mu\text{g}/\text{mg}$, 0.84 ± 0.34 $\mu\text{g}/\text{mg}$, 0.39 ± 0.58 $\mu\text{g}/\text{mg}$ for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively.

3.1.3 Aggrecan, collagen type-II and collagen type-I expressions in the cultured chondrocytes

For *ACAN* expression in cultured chondrocytes (Fig. 3A), there was no difference between the groups, F

$(3,20) = 2.809$, $p = 0.066$ and no significant difference across the passage, $F(1.001,20.023) = 3.381$, $p = 0.81$. There was no interaction between passages and cultured chondrocytes groups, $F(3.004, 20.023) = 3.060$, $p = 0.052$. The expression started at 1.00 ± 0.50 -fold, 1.00 ± 0.55 -fold, 1.00 ± 0.52 -fold and 1.00 ± 0.22 -fold for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively. At P2, the expression was 1.04 ± 1.02 -folds, 0.74 ± 0.90 -fold, 5.97 ± 0.77 -fold and 0.99 ± 1.18 -fold for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively. While, *ACAN* at P3 was 0.69 ± 0.06 -fold, 0.84 ± 0.069 -fold, 0.31 ± 0.35 -fold and 0.94 ± 0.32 -fold for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively. For *COL2A1* expression (Fig. 4), there was no difference between groups, $F(3,20) = 3.048$, $p = 0.52$ and there is significant difference across the passages, $F(1.004, 20.081) = 18.848$, $p = 0.000$. There was a significant interaction between passages and chondrocytes groups, $F(3.012, 20.081) = 3.359$, $p = 0.039$. The expression at P2 was 1.67 ± 0.64 -folds, 3.15 ± 0.64 -fold, 7.02 ± 0.43 -fold and 0.84 ± 0.41 -fold for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively. While, at P3, 0.11 ± 0.29 -folds, 0.41 ± 0.72 -fold, 0.05 ± 0.44 -fold and 0.04 ± 0.41 -fold for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively. While, there was a significant difference across the passages, $F(1.242, 24.845) = 118.883$, $p = 0.000$ and there was a difference between groups, $F(3,20) = 14.196$, $p = 0.000$ for *COL1A1* expression (Fig. 4). There was also a significant difference in interaction between passages and chondrocytes groups, $F(3.727, 24.845) = 14.572$, $p = 0.000$. At P2, *COL1A1* expression were 0.11 ± 0.58 -folds, 3.90 ± 0.88 -fold, 0.28 ± 0.46 -fold and 17.51 ± 1.21 -fold for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively. At P3, *COL1A1* expression were 29.24 ± 0.08 -folds, 11.55 ± 0.76 -fold, 11.71 ± 0.42 -fold and 21.56 ± 0.35 -fold for *SOX9/TERT*-, *SOX9*-, *TERT*-, non-transfected chondrocytes respectively.

Fig. 1 **A** The representative images of fibroblast-like shaped chondrocytes at passage-1 (P1), P2 and P3 in all monolayer cultured chondrocytes group. **B** The representative images of "cell-scaffold"

construct morphology at week-2 and week-3 in all group. Scale bar = 100 μ m (yellow coloured). (Color figure online)

Fig. 2 A The reduction of sulphated glycosaminoglycan (sGAG) content in monolayer cultured chondrocytes from P1 to P3. $*p < 0.05$ that considered as significant. **B** The representative images of sGAG

in monolayer cultured chondrocytes indicated as blue stain of alcian blue staining. Scale bar = 100 μm (yellow coloured). (Color figure online)

Fig. 3 A Aggrecan (ACAN) expression in monolayer cultured chondrocytes. Significant upregulation in *TERT*-transfected chondrocytes at P2 and downregulation at P3 while the expression level maintained constant in other groups throughout the passages.

* $p < 0.05$ that considered as significant. **B** The representative images showed non-detectable proteoglycan in monolayer cultured chondrocytes groups through the absent of orange-red stain of safranin O. Scale bar = 100 μm (yellow coloured). (Color figure online)

Fig. 4 A The significant downregulation of collagen type-II (*COL2A1*) expression in monolayer cultured chondrocytes groups at P3. **B** The collagen type-I (*COL1A1*) expression level was significantly upregulated in monolayer cultured chondrocytes groups at P3. **C** *COL2A1* expression was upregulated in *SOX9/TERT*-PLGA throughout the culture period. The expression was significantly upregulated at week-2 in *SOX9/TERT*-PLGA/fibrin and downregulated at the following week. **D** *COL1A1* expression showed no significant difference in *SOX9/TERT*-PLGA/fibrin and *SOX9/TERT*-PLGA. Both “cell-scaffold” construct groups maintained the expression level throughout the culture period. The other “cell-scaffold” constructs significantly expressed *COL1A1* within the culture period. * $p < 0.05$ that considered as significant

3.2 3D culture

3.2.1 Cartilaginous matrix components in the “cell-scaffold” constructs

In “cell-scaffold” constructs, the chondrocytes morphology, density and extracellular matrix were visualised through H&E staining at week-2 and week-3 (Fig. 1B). The chondrocytes enclosed in lacunae surrounded by extracellular matrix were visualised at both time point. The chondrocytes and extracellular matrix concentrated at the peripheral part of the scaffolds and became denser at week-3. It was noted that the “cell-scaffold” constructs from chondrocytes seeded in PLGA/fibrin have a denser extracellular matrix as compared to the chondrocytes seeded in PLGA. The extracellular matrix component, sGAG was visualised as the blue stain of alcian blue in all “cell-scaffold” construct groups (Fig. 5). While, proteoglycan was also noted at both week-2 and week-3 with the orange-red stain of safranin o (Fig. 6).

3.2.2 sGAG content in the “cell-scaffold” constructs

There was no significant difference between the groups, $F(7,40) = 1.032$, $p = 0.425$ and significant difference was observed across the weeks, $F(1.707, 68.290) = 104.980$, $p = 0.000$ (Fig. 5A) in term of sGAG content. There was no interaction between weeks and “cell-scaffold” construct groups, $F(11.951, 68.290) = 1.452$, $p = 0.165$. At week-1, sGAG content started at *SOX9/TERT*-PLGA/fibrin (1.82 ± 0.41 µg/mg), *SOX9/TERT*-PLGA (1.21 ± 0.37 µg/mg), *SOX9*-PLGA/fibrin (1.82 ± 0.40 µg/mg), *SOX9*-PLGA (1.17 ± 0.67 µg/mg), *TERT*-PLGA/fibrin (1.75 ± 0.66 µg/mg), *TERT*-PLGA (2.40 ± 0.53 µg/mg), control-PLGA/fibrin (1.76 ± 0.66 µg/mg) and control-PLGA (1.22 ± 0.22 µg/mg). Whereas, at week-3, *SOX9/TERT*-PLGA/fibrin (4.71 ± 1.07 µg/mg), *SOX9/TERT*-PLGA (3.61 ± 1.22 µg/mg), *SOX9*-PLGA/fibrin (5.95 ± 1.83 µg/mg), *SOX9*-PLGA (3.89 ± 2.06 µg/mg), *TERT*-PLGA/fibrin (4.83 ± 2.42 µg/mg), *TERT*-PLGA ($4.49 \pm$

1.77 µg/mg), control-PLGA/fibrin (4.97 ± 1.58 µg/mg) and control-PLGA (2.81 ± 1.41 µg/mg).

3.2.3 Aggrecan, collagen type-II and collagen type-I expressions in the “cell-scaffold” construct

In “cell-scaffold” constructs, the ACAN expression in “cell-scaffold” constructs at week-2 was *SOX9/TERT*-PLGA/fibrin (215.27 ± 0.23 -fold), *SOX9/TERT*-PLGA (77.80 ± 0.29 -fold), *SOX9*-PLGA/fibrin (2.08 ± 0.19 -fold), *SOX9*-PLGA (0.11 ± 0.11 -fold), *TERT*-PLGA/fibrin (1.83 ± 0.25 -fold), *TERT*-PLGA (46.62 ± 0.56 -fold), control-PLGA/fibrin (1.20 ± 0.60 -fold), control-PLGA (1.72 ± 0.44 -fold) (Fig. 6A). While, at week-3, *SOX9/TERT*-PLGA/fibrin (404.50 ± 0.79 -fold), *SOX9/TERT*-PLGA (6.67 ± 0.57 -fold), *SOX9*-PLGA/fibrin (1.70 ± 0.57 -fold), *SOX9*-PLGA (0.10 ± 0.10 -fold), *TERT*-PLGA/fibrin (1.47 ± 0.25 -fold), *TERT*-PLGA (11.97 ± 0.48 -fold), control-PLGA/fibrin (1.31 ± 0.38 -fold), control-PLGA (1.26 ± 0.34 -fold). There was no significant difference across the weeks, $F(1.022, 40.892) = 3.354$, $p = 0.074$. There was a difference between groups, $F(7,40) = 7.797$, $p = 0.000$. There was a difference in interaction between weeks and “cell-scaffold” construct groups, $F(7.156, 40.892) = 3.460$, $p = 0.005$. The *COL2A1* expression at week-2 was *SOX9/TERT*-PLGA/fibrin (179.56 ± 0.40 -fold), *SOX9/TERT*-PLGA (7.77 ± 0.41 -fold), *SOX9*-PLGA/fibrin (22.19 ± 0.20 -fold), *SOX9*-PLGA (0.03 ± 0.25 -fold), *TERT*-PLGA/fibrin (2.92 ± 0.23 -fold), *TERT*-PLGA (11.12 ± 0.55 -fold), control-PLGA/fibrin (3.59 ± 0.43 -fold), control-PLGA (1.07 ± 0.47 -fold) (Fig. 4C). Whereas, at week-3 was *SOX9/TERT*-PLGA/fibrin (150.64 ± 0.93 -fold), *SOX9/TERT*-PLGA (14.79 ± 0.66 -fold), *SOX9*-PLGA/fibrin (5.50 ± 0.47 -fold), *SOX9*-PLGA (0.02 ± 0.32 -fold), *TERT*-PLGA/fibrin (2.34 ± 0.28 -fold), *TERT*-PLGA (6.98 ± 0.60 -fold), control-PLGA/fibrin (1.29 ± 0.38 -fold), control-PLGA (0.63 ± 0.24 -fold). There was no significant difference across the weeks, $F(1.041, 41.654) = 2.065$, $p = 0.158$. There was a difference between groups, $F(7,40) = 7.180$, $p = 0.000$ for *COL2A1* expression. There was no difference in interaction between weeks and “cell-scaffold” construct groups, $F(7.289, 41.654) = 1.627$, $p = 0.152$. The *COL1A1* expression at week-2 was *SOX9/TERT*-PLGA/fibrin (0.66 ± 0.46 -fold), *SOX9/TERT*-PLGA (0.30 ± 0.29 -fold), *SOX9*-PLGA/fibrin (4.82 ± 0.13 -fold), *SOX9*-PLGA (7.56 ± 0.12 -fold), *TERT*-PLGA/fibrin (3.44 ± 0.24 -fold), *TERT*-PLGA (0.52 ± 0.56 -fold), control-PLGA/fibrin (4.44 ± 0.52 -fold), control-PLGA (14.18 ± 0.44 -fold) (Fig. 4D). At week-3, the expression was *SOX9/TERT*-PLGA/fibrin (1.78 ± 0.82 -fold), *SOX9/TERT*-PLGA (0.23 ± 0.54 -fold), *SOX9*-PLGA/fibrin (0.91 ± 0.35 -fold), *SOX9*-PLGA (7.45 ± 0.11 -fold), *TERT*-PLGA/fibrin

Fig. 5 **A** The increment of sGAG content in all “cell-scaffold” construct from week-1 to week-3. * $p < 0.05$ that considered as significant. **B** The representative images of sGAG present in all “cell-

scaffold” construct extracellular matrix stained with alcian blue at week-2 and week-3. Scale bar = 100 μm (yellow coloured). (Color figure online)

(0.00 ± 0.33 -fold), *TERT*-PLGA (4.77 ± 0.30 -fold), control-PLGA/fibrin (5.97 ± 0.37 -fold), control-PLGA (6.22 ± 0.21 -fold). There was a significant difference

across the weeks, $F(1.489, 59.546) = 97.155$, $p = 0.000$. There was a difference between groups, $F(7, 40) = 37.025$, $p = 0.000$. There was a difference in interaction between

Fig. 6 **A** ACAN expression in engineered cartilages. Significant upregulation in *SOX9/TERT-PLGA/fibrin* from week-1 until week-3. While *SOX9/TERT-PLGA* and *TERT-PLGA* were significantly upregulated at week-3 and downregulated at week-3. $*p < 0.05$ that considered as significant. **B** The representative images showed the

present of proteoglycan in all “cell-scaffold” construct through the orange-red stain of safranin o. Positive proteoglycan stain was intense at week-3 as compared to week-2. Scale bar = 100 μ m (yellow coloured). (Color figure online)

weeks and “cell-scaffold” construct groups, $F(10.421, 59.546) = 26.094$, $p = 0.000$.

4 Discussion

The study observed the cartilaginous matrix formation in chondrocytes transfected with *SOX9*- and/or *TERT* genes in monolayer culture and 3D culture. Previously, we have reported the potential of PLGA-based scaffold for cartilage regeneration [2, 15–17, 37]. PLGA is an FDA-approved, biocompatible and biodegradable polymer. PLGA scaffold alone could facilitate *in vitro* cartilage formation. Our previous findings suggested that the incorporation of PLGA with fibrin showed homogenous chondrocytes distribution with better histoarchitecture than PLGA only group in both *in vitro* and *in vivo* studies [2, 15–17]. Another basic tissue engineering component, signalling molecules in the form of human's encoded genes, pCDNA3-*SOX9* and pBABE-neo-*hTERT* were used instead of rabbit due to the limited genetic databases of the species. Studies on transfection of human's gene in animal cells have shown that the gene was able to regulate its function in animal cells. For example, *hTERT* activity has been verified in human and domestic animal chondrocytes as it inhibits the cells ageing from both species [22–29]. The similar gene, *hTERT* also showed its compatibility with rabbit telomerase template RNA (rTR) as demonstrated via TRAP assay and immunology test [30]. Other studies noted the ability of human *SOX9* to induce chondrogenic properties in mouse embryonic stem cell [31] and maintain the chondrocytic phenotype of rabbit's intervertebral disc cells [32].

Around 22.23% transfection efficiency was obtained from the used transfection reagent [20]. The efficiency percentage is higher than the one reported on normal human articular cartilage, 20.7% and osteoarthritic articular chondrocytes, 7.8% [33]. The transfection efficiencies of both normal and osteoarthritic cartilage are said to be sufficient enough for transfection-based molecular assessments [33]. Moreover, fibroblast growth factor-1 or platelet-derived growth factor B gene activity was able to provide *in vivo* biological effect with an average of 0.1–1% transfection efficiency on the arterial wall [34–36]. For this study, because of limited cell number retrieved during cell harvesting, the cells were transfected once at P1 to minimise cell damage that potentially induced by the transfection procedure [38]. The transient gene transfer initiates gene to regulate its activity in the cell and provides signals for cell to maintain the phenotype. Based on our unpublished data, the upregulation of *SOX9* and *TERT* was observed after transfection at P1 in the respective transfected cell groups compared to control, showed the effectiveness of the transfection (unpublished data). This study

further evaluated the establish 3D tissue from the cell groups seeded on PLGA and PLGA/fibrin.

Regardless of transfection groups, the cultured chondrocytes changed from polygonal-shaped to flattened, fibroblast-like appearance throughout passages (Fig. 1). This phenomenon suggested the occurrence of chondrocytes dedifferentiation as the cells could not maintain their polygonal shape in the culture [37]. The dedifferentiation event or the loss of chondrocytic phenotype in monolayer culture setting was similarly reported in several studies [37–39]. In term of the cell count, the cumulative cell number of transfected chondrocyte groups was comparable to control. *SOX9/TERT*-transfected chondrocytes and *TERT*-transfected chondrocytes have higher cumulative number among the groups (unpublished data). The transfected cells survival indicated the used transfection reagent have low cytotoxicity. An important cartilage matrix component, sGAG, controls cartilage homeostasis by attracting water molecules into the tissue. However, faint alcian blue visualisation in the chondrocytes' pericellular matrix region and sGAG quantity reduction, supported the loss of chondrocytic phenotype in all group (Fig. 2). The non-detectable proteoglycan was anticipated because it is formed by sGAG chains attached to the core protein (Fig. 3B). Although it was not detected in protein level, *ACAN*, a major proteoglycan marker was expressed in the cultured chondrocytes. The level of protein expression is known to be determined by the transcript amount of respective gene [40, 41]. However, the gene's protein level may deviate in a certain condition such as during cellular differentiation or stress [40]. Therefore, it is believed that the cells are in the proliferating state instead of producing protein. In term of collagens expression, *COL2A1* and *COL1A1* were co-expressed in all group. *COL2A1* was downregulated from P2 to P3 by 15.18 times, 7.68 times, 140.4 times, 21 times in *SOX9/TERT*-transfected chondrocytes, *SOX9*-transfected chondrocytes, *TERT*-transfected chondrocytes and control, respectively. Whereas, *COL1A1* was upregulated from P2 to P3 by 265.82 times, 2.96 times, 41.82 times, 1.23 times in *SOX9/TERT*-transfected chondrocytes, *SOX9*-transfected chondrocytes, *TERT*-transfected chondrocytes and control, respectively. These collagen expression patterns once again highlight the loss of chondrocytic phenotype that may lead to the unwanted fibrocartilage development instead of hyaline cartilage [37, 42, 43].

The cell seeding density of 1×10^5 cells/scaffold was standardised for the “cell-scaffold” construct formation. At day-3 of cell proliferation assay, the cell number of all “cell-scaffold” construct was more than 1×10^5 cells, showed the efficiency of cell seeding technique (data not shown). In our previous report, cartilage formation was accomplished by seeding 1×10^6 cells/scaffold [15]. In

this present study, the used cell density is sufficient to regenerate cartilage as visualised through histological stains. The result suggested that, functional cartilage histoarchitecture can be achieved with the minimal cell seeding density. This is because, high cell density culture has the tendency to promote chondrocytes hypertrophy in the culture [45]. The occurrence of cartilage hypertrophy contributes to extracellular matrix mineralisation which commonly associated with fibrocartilage and bone formation. The “cell-scaffold” constructs regardless of transfection groups, formed using chondrocytes seeded in PLGA/fibrin have developed and denser extracellular matrix at week-3 than chondrocytes seeded in PLGA. It is suggested that the absence of external help limits the attachment efficiency to anchor the chondrocytes into the PLGA. While, the fibrin in PLGA/fibrin scaffold glued the chondrocytes to PLGA. In addition, fibrin accommodates chondrocytes adaptation in the culture medium, because of the acidic PLGA by-product affects the culture medium pH [44, 45]. From week-2 to week-3, the chondrocytes migrated towards the centre of the PLGA/fibrin and PLGA scaffold. The tissue developed steadily as the secreted matrix helped to maintain the “cell-scaffold” construct structure during the tissue processing procedure at both time points. Whereas, the week-1 “cell-scaffold” construct structure collapsed during the process possibly because of the extracellular matrix was not well developed, thus the result could not be tabulated.

The quantitative sGAG content was accumulated in all “cell-scaffold” construct throughout the culture period, supported by the visualisation of sGAG at the extracellular matrix (Fig. 5). The proteoglycan was also visualised in all “cell-scaffold” construct at both week-2 and week-3. The orange-red stain was prominent at week-3 as compared to week-2 indicated cartilaginous maturation of the formed “cell-scaffold” construct (Fig. 6B). The positive expression of sGAG or proteoglycan in “cell-scaffold” construct is crucial as the molecules itself provide hydration and sturdiness to the tissue. In the molecular level, *ACAN* was upregulated from week-1 to week-2 by 215.27 times and 1.88 times from week-2 to week-3 in *SOX9/TERT*-PLGA/fibrin. The gene was downregulated in other groups from week-2 to week-3. Regardless of expression level dissimilarity, proteoglycan protein was expressed in all “cell-scaffold” construct visualised through histology. This unexplainable result showed the dynamic of cells behaviour as explained by Liu et al. [42] and Vogel and Marcotte [43]. For collagens expression, *COL2A1* was upregulated from week-1 to week-2 by 7.77 times and 1.90 times from week-2 to week-3 in *SOX9/TERT*-PLGA. Whereas, it was downregulated in other “cell-scaffold” constructs at week-3. *COL1A1* which supposedly well-expressed in fibrocartilage or degenerated cartilage also

expressed in all “cell-scaffold” construct. Nonetheless, only *SOX9/TERT*-PLGA showed *COL1A1* downregulation from week-1 until week-3 by 4.91 times. In normal condition, mature cartilage specifically expressed collagen type-II. However, co-expression of collagen type-II and collagen type-I cannot be avoided during newly cartilage development. As reported by Sasano et al. [46], collagen type-I was expressed during rat tibial articular cartilage development via immunohistochemistry and in situ hybridisation. Thus, co-expression of both collagens is expected for this experiment. Based on the overall gene expression profile, the “cell-scaffold” construct formed using *SOX9/TERT*-transfected chondrocytes has better cartilaginous markers expression than the “cell-scaffold” construct formed from other chondrocyte groups.

Both PLGA/fibrin and PLGA provide a suitable environment for cartilaginous tissue formation indicated by extracellular matrix accumulation in all “cell-scaffold” construct particularly PLGA/fibrin scaffold. Fibrin is essential to prevent fluid leakage by forming spiderweb-like structure clot during injury. Fibrin was incorporated with PLGA because of the signalling components in the fibrin promote cell attachment and proliferation during tissue regeneration [16]. It is also known to have minimal inflammatory and foreign substance response during the wound healing process [15]. The described characteristics make fibrin as one of the good options to be used as scaffolding material [47, 48].

The study demonstrated “cell-scaffold” construct formation through TE triad application. Monolayer culture is essential to initiate cell proliferation and provide sufficient cell number for the construct formation. With minimal cell seeding density, PLGA/fibrin and PLGA scaffolds improved the cells ability in maintaining its phenotypic characteristics as the “cell-scaffold” construct formed by all chondrocyte groups could express cartilaginous property as shown by the presented result. “Cell-scaffold” construct from chondrocytes seeded in PLGA/fibrin has better cartilage histoarchitecture at week-3 than PLGA group. In term of cell groups, “cell-scaffold” construct formed using *SOX9/TERT*-transfected chondrocytes has better cartilaginous markers profile compared to the other groups. It seemed that the co-transfected genes assist chondrocytes in expressing and maintaining the phenotype during 3D culture. Currently, the formed “cell-scaffold” construct’s potential is being validated through an ongoing *in vivo* experimentation for further understanding.

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Compliance with ethical standards

Conflict of interest The authors have declared that there is no conflict of interest.

Ethical statement Animal ethical approval was granted by the Institutional Animal Care and Use Committee of International Islamic University Malaysia (IACUC-IIUM) (Reference No. IIUM/IACUC/Approval 2015/[5]/[24]).

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