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IDENTIFYING THE POTENTIAL OF TRANSCRIPTION FACTOR SOX9 GENE TRANSFER IN CHONDROCYTES DIFFERENTIATION AND ARTICULAR CARTILAGE FORMATION IN VITRO

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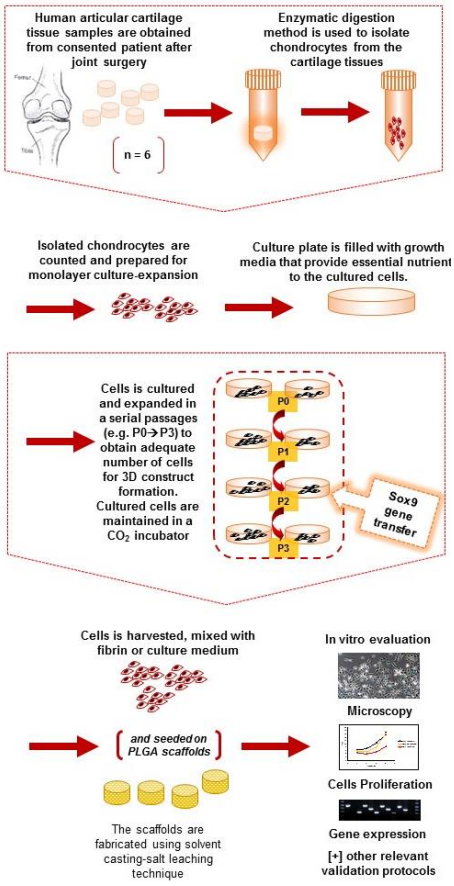
Graphical abstract



Abstract

Sox9 plays an important role as transcription factor for chondrogenesis; the formation of cartilage. This study aimed to identify the potential of the transiently overexpressed Sox9 gene in human chondrocytes differentiation and tissue engineered cartilage (TEC) formation in vitro. Articular cartilage samples were obtained from osteoarthritic patients who underwent joint replacement surgery. The isolated chondrocytes were cultured and transfected with pcDNA3-Sox9 using lipofection technique. The TEC constructs were formed by incorporating the transfected and the non-transfected cells onto poly(lactic-co-glycolic acid) (PLGA) scaffold with or without fibrin. This approach allows a comparison between four groups i.e. (1) transfected chondrocytes seeded on PLGA/fibrin [PFTC], (2) non-transfected chondrocytes on PLGA/fibrin [PFC], (3) transfected chondrocytes on PLGA [PTC] and (4) non-transfected chondrocytes on PLGA [PC]. All TEC constructs were cultured and evaluated at each time point of 1, 2 and 3 weeks in vitro. All TEC constructs were analysed for gross observation, histology, immunohistochemistry, cell proliferation activity, gene expression and sulphated glycosaminoglycan (sGAG) production assay. After 3 weeks, all PFTC and PFC showed higher cell viability, higher sGAG content, better histological features and distribution of extracellular matrix in concert with positive glycosaminoglycan (GAG) accumulation when compared to the PTC and PC. However, at week 3, the PFC and PC exhibited significantly higher sGAG production than PFTC and PTC. Chondrogenic properties of the constructs were evidenced by the expression of cartilage-specific markers; collagen II, collagen XI and aggrecan core protein. In this study, due to the nature of a new cartilage formation, the co-expression of collagen I in all constructs can be an indication of early cartilage development. Based on the outcomes, it is hoped that this study will provide a good ground for future tissue engineering application.

Keywords: Sox9 gene transfer, articular cartilage, transient transfection, chondrocytes, PLGA, fibrin

1.0 INTRODUCTION

Articular cartilage plays a crucial role in synovial joints as it has remarkable mechanical properties contributed primarily by its well-organized extracellular matrix produced by highly specialized cells, chondrocytes. The extracellular matrix consists of collagens predominantly, proteoglycans and

glycoproteins [1]. The extracellular matrix homeostasis is regulated and maintained to preserve the anatomical structure and mechanical strength of articular cartilage.

Unfortunately, articular cartilage is often subject to damage by trauma or diseases and it has limited capacity to repair itself due to the avascular nature of the cartilage [2]. Prevailing over this shortcoming has become an area of focus for tissue engineering and regenerative medicine (TERM).

The use of Sox9 gene transfer to facilitate chondrocytes differentiation and function is becoming more common in TERM. Sox9 is a member of Sox (Sry-type HMG box) family genes and has been revealed to be expressed mainly in mesenchymal condensations and cartilage [3].Sox9 plays a crucial role as transcription factor for chondrogenesis and cartilage formation [3, 4]. It up-regulates the cartilage specific markers namely collagen II, IX and IX and aggrecan [5, 6, 7, 8].

A successful gene transfer requires the use of method capable to achieve high transfection efficiency and low toxicity [9]. Although, viral method often results in high transduction efficiency, the technique does not meet the demands for a safe gene transfer [10]. In this study, a transient transfection of Sox9 using non-viral method i.e. lipofection was used considering the safety issues and ease of handling.

Thus, this study aimed to identify the potential of the transiently overexpressed Sox9 gene in chondrocytes differentiation and tissue engineered cartilage (TEC) formation in vitro.

2.0 EXPERIMENTAL

2.1 Cartilage Harvest and Chondrocytes Culture

With the ethical approval (IREC-18 and NMRR-12-1383-14531), articular cartilage samples were obtained from consented osteoarthritic patients (n= 6; age: 55-70 years old) after joint replacement surgery. Each sample was digested in 0.6% Collagenase A at 37°C for 6 hours.

Cells were cultured (passage 0; P0) with initial seeding of 5,000 cells/cm^2 in Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 (Ham) (1:1) DMEM/F12 (Gibco, Life Technologies, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco, Life Technologies, USA), 1% of antibiotics-antimycotic (Gibco, Life Technologies, USA), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer (Gibco, Life Technologies, USA), L-glutamine (Gibco, Life Technologies, USA) and L-ascorbic acid (Sigma®, Sigma-Aldrich Corporation, USA), as previously described [11].

Upon 80% confluency, the chondrocytes were subculture into passage 1 (P1) and 2 (P2), and grown in chondrogenic medium containing recombinant insulin, insulin-like growth factor - 1 (IGF-1), basic fibroblast growth factor (bFGF) and transforming growth factor- 2 (TGF-2) (Chondro-ENHANCE™, Tela Technologies, Malaysia) until passage two.

2.2 Sox9 Gene Transfer

Upon 80 % confluency, the cells in passage 2 (P2) were transfected with pcDNA3-sox9 plasmid using Lipofectamine 2000 according to manufacturer's protocol. The plasmid encoding green fluorescent protein (GFP) namely pmaxGFP™ was co-transfected with each reaction for transfection efficiency evaluation purpose using fluorescence inverted microscope (Nikon Eclipse *Ti*, Nikon, Japan).

2.3 In vitro 3-Dimensional Constructs Formation

The 3D microporous poly(Lactide-co-glycolic acid) or, PLGA (mole ratio 50:50, molecular weight 33,000 g/mole, pore size 180-250µm) scaffold was fabricated by solvent casting/salt leaching technique using dichloromethane solvent as previously described [11,12].

The TEC constructs were formed by incorporating the transfected and the non-transfected cells onto PLGA scaffold with or without fibrin, using the previously described procedure [11]. This approach allows a comparison between the following four groups (Table 1). Approx. 5x10^5 cells per scaffold were incorporated and suspended in (1) plasma-derived human fibrin, for PFTC and PFC group or (2) culture medium, for PTC and PC groups.

All constructs were cultured and evaluated at 1, 2 and 3 weeks *in vitro*. The evaluations involved gross morphology, histology, immunohistochemistry, cell proliferation activity, gene expression and sulphated glycosaminoglycan (sGAG) production.

Table 1 showing the four experimental groups and their relevant abbreviations assigned for the study

Scaffolds		Cells source	Abbreviation used
PLGA and Fibrin **(PF)**	seeded with	transfected chondrocytes **(TC)**	**PFTC**
		non-transfected chondrocytes **(C)**	**PFC**
PLGA only **(P);** **without fibrin*	seeded with	transfected chondrocytes **(TC)**	**PTC**
		non-transfected chondrocytes **(C)**	**PC**

2.4 Histological and Immunohistochemistry

For histological evaluation, Haematoxylin and Eosin (H&E) (Leica, Leica Biosystems, Germany), Alcian Blue (Merck, Merck KGaA, Germany) and Safranin O (Merck, Merck KGaA, Germany) to assess tissue histoarchitecture, GAG accumulation and presence of proteoglycans-rich matrix, respectively [11]. For immunohistochemistry evaluation, the staining was carried out according to protocol using monoclonal antibody (MAb) mouse anti-collagen I (1:4000) (Calbiochem EMD Biosciences

Inc., USA) and MAb mouse anti-collagen II (1:300) (Abcam®, Abcam, Inc., USA).

2.5 Cell Proliferation Assay

Cell proliferation assay was measured using thiazolyl blue tetrazolium bromide (MTT) (Merck, Merck KGaA, Germany) assay at day 7, 14 and 21.The absorbance intensity was measured using microplate reader at 570 nm and 660 nm as reference wavelength.

2.6 Gene Expression

Cartilaginous markers expression of each construct was evaluated using two-step reverse-transcription polymerase chain reaction (RT-PCR) at each time point of 1, 2 and 3 week in vitro [11]. Primers used were prepared by Integrated DNA Technologies, Singapore (Table 2). Housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as internal controls.

Table 2 Primers used in PCR

Gene	GenBank accession number	Sequence 5'– 3'	Product size (bp)
GAPDH	NM_002046.5	F:TCCCTGAGCTGAACGGGAAG R:GGAGGAGTGGGTGTCGCTGT	218
β-actin	NM_001101.3	F:AGTCCTGTGGCATCCACGAAA R:GTCATACTCCTGCTTGCTGA	281
Collagen type II	NM_001844.4	F:CTATCTGGACGAAGCAGCTGGCA R:ATGGGTGCAATGTCAATGATGG	210
Collagen type IX	NM_001853.3	F:GAAACCAGGGTGACAGAGGAG R:CCCTCCTAACACGGCTCCTT	236
Collagen type XI	NM_001854.3	F:GGATCAAATGATGAGGAGATGTCCTATG R:CTAAATGGTACCTGTATATGCAGCGTTG	345
Sox9	NM_000346.3	F:GCGGAGGAAGTCGGTGAAGA R:CCCTCTCGCTTCAGGTCAGC	237
Aggrecan core protein	NM_001135.3	F:CACTGTTACCGCCACTTCCC R:ACCAGCGGAAGTCCCCTTCG	184
Collagen type I	NM_000088.3	F:AGGGCTCCAACGAGATCGAGATCCG R:TACAGGAAGCAGACAGGGCCAACG	223
Collagen type X	NM_000493.3	F:CCCTTTTTGCTGCTAGTATCC R:CTGTTGTCCAGGTTTTCCTGGCAC	468

2.7 Sulphated Glycosaminoglycan (sGAG) Assay

In sGAG production assay, the wet weight and sGAG content (%) were measured to further evaluate the synthesis of cartilaginous extracellular matrix in the TEC constructs. All constructs were digested with papain digestion solution at 60°C for 16 hours. The sGAG content was analysed according to manufacturer's protocol. The absorbance of each sample was measured at 600-620 nm.

2.8 Statistical Analysis

Statistical analysis was performed using the non-parametric Kruskal-Wallis test followed by post-hoc Whitney *U* test with Bonferroni's correction level test. $P< 0.05$ was considered significant. Data were expressed as median and interquartile range (IQR) [13, 14].

3.0 RESULTS AND DISCUSSION

Sox9 transcription factor is important for normal cartilage development and function. Recent experiments revealed that Sox9 has the ability to bind more than 30 genes involved in the regulation of the chondrocyte phenotype. It regulates not only matrix proteins but also other transcription factors and cellular signalling proteins [15].

The efficiency was assessed directly based on the relative GFP expression level in the cells, which should have approximately 40% of transfected cells using lipofection (Figure 1). Although it is considered low, this method proved to give low toxicity effect, parallel with previous finding [16].

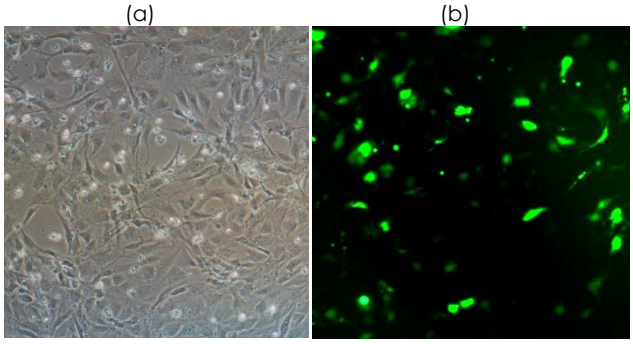


Figure 1 Human articular chondrocytes view under fluorescence inverted microscope after co-transfected with pcDNA3-sox9 and pmaxGFP plasmids. The magnification of (a) bright field image is 100X and (b) fluorescence images is 100X (Scale bar: 200m)

Macroscopically, there were no significant changes in shape and size of all constructs throughout 3 weeks in culture. At this stage, it was difficult to identify differences between the transfected and the non-transfected groups. However, it was quite clear that the constructs without fibrin i.e. PC and PTC became smaller by week 3 (Figure 2). The constructs with fibrin i.e. PFC

and PFTC outweigh the PC and PTC constructs by having whitish, cartilage-like appearance.

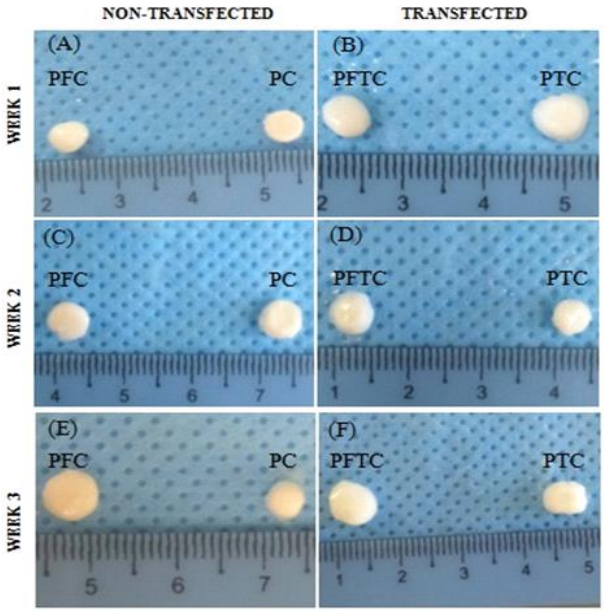


Figure 2 Macroscopic observation of all in vitro constructs at each time point of 1-, 2-, and 3-week

Microscopically, the H&E staining showed that chondrocytes were distributed mostly on the surface of scaffold in all groups at the first week of in vitro culture (Figure 3a). The presence of chondrocytes clusters with lacunae, embedded in basophilic ECM was evidenced in PFC and PFTC. Both PFC and PTFC exhibited better cartilaginous histoarchitecture in concert with more intense Alcian Blue staining confirming GAG accumulations (Figure 3b). By week 2 and 3, PFC and PFTC constructs exhibited cellular infiltration as the cells started to fill up void spaces in the scaffolds (a). Low accumulation of GAGs manifested by the weak Alcian Blue staining shown in PC and PTC constructs elucidated the correlation between cell density and GAG synthesis.

Lack positive Safranin O staining in all constructs suggestive lack proteoglycan production in the in vitro TEC constructs (*result not shown*). Instead, they were stained intensely with fast green solution, the counterstaining for collagen in the protocol. The result also contradicted with continuous expression of aggrecan gene (Figure 5e). It is well-known that proteoglycans consist of a core protein and one or more covalently attached glycosaminoglycan such as chondroitin sulphate (CS), keratan sulphate (KS) and dermatan sulphate (DS) [17].

The major proteoglycan presents in articular cartilage is aggrecan which consist of largely CS and KS chains attached to its core protein (18, 19). Therefore, in parallel with co-expression of collagen type I, we speculated that the engineered constructs might be at an early development stage with low amount of proteoglycans have been synthesized. With routine fixation and tissue processing techniques, the proteoglycans may be washed out during the processes [20, 21]. Furthermore, it has been reported that Safranin O is not a sensitive indicator of proteoglycan content in tissues containing a very low level of proteoglycans [22].

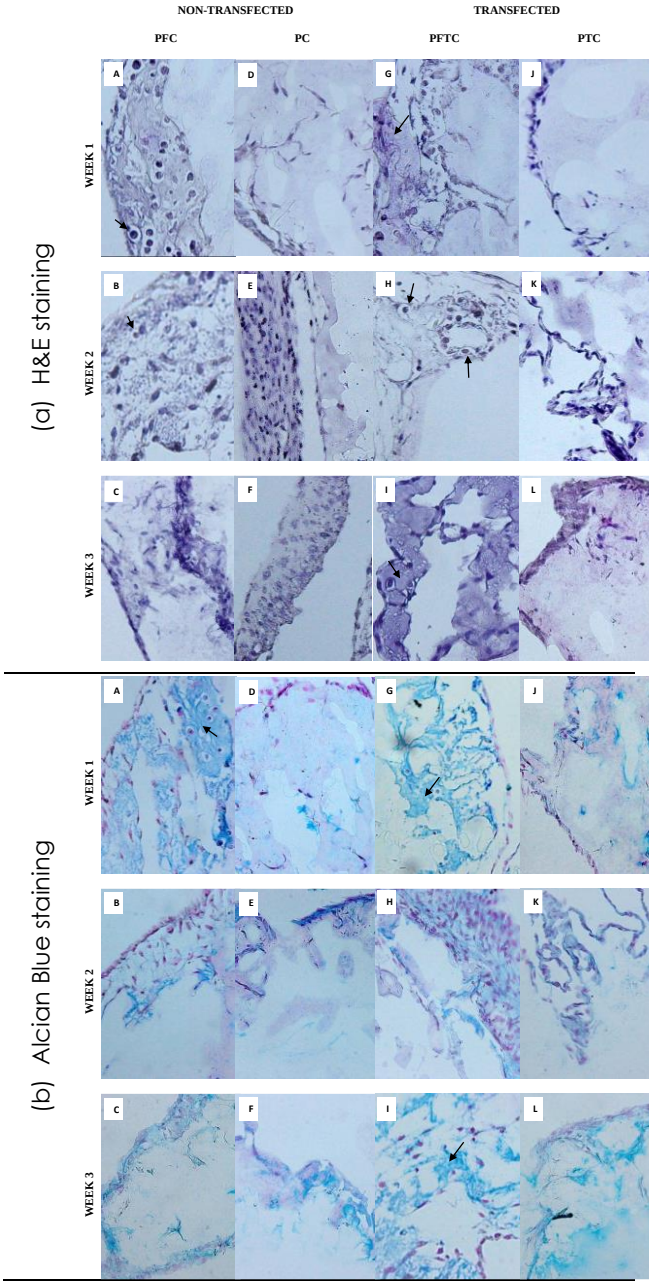


Figure 3 Histological evaluation of the in vitro constructs. (a) H&E and (b)Alcian Blue are shown (magnification of 200× and scale bar: 200 μm). Arrow demonstrated the cells and extracellular matrix

Based on immunohistochemistry evaluation (Figure 4), the peripheral surface area of the constructs that largely covered with cells embedded in matrix exhibited intense brownish precipitation indicative of collagen II (a) and I (b) immunopositivity more than the central regions of scaffold after the first two weeks of culture. At week 3, all constructs showed reduction in collagen II staining intensity. While, collagen I staining was steadily expressed.

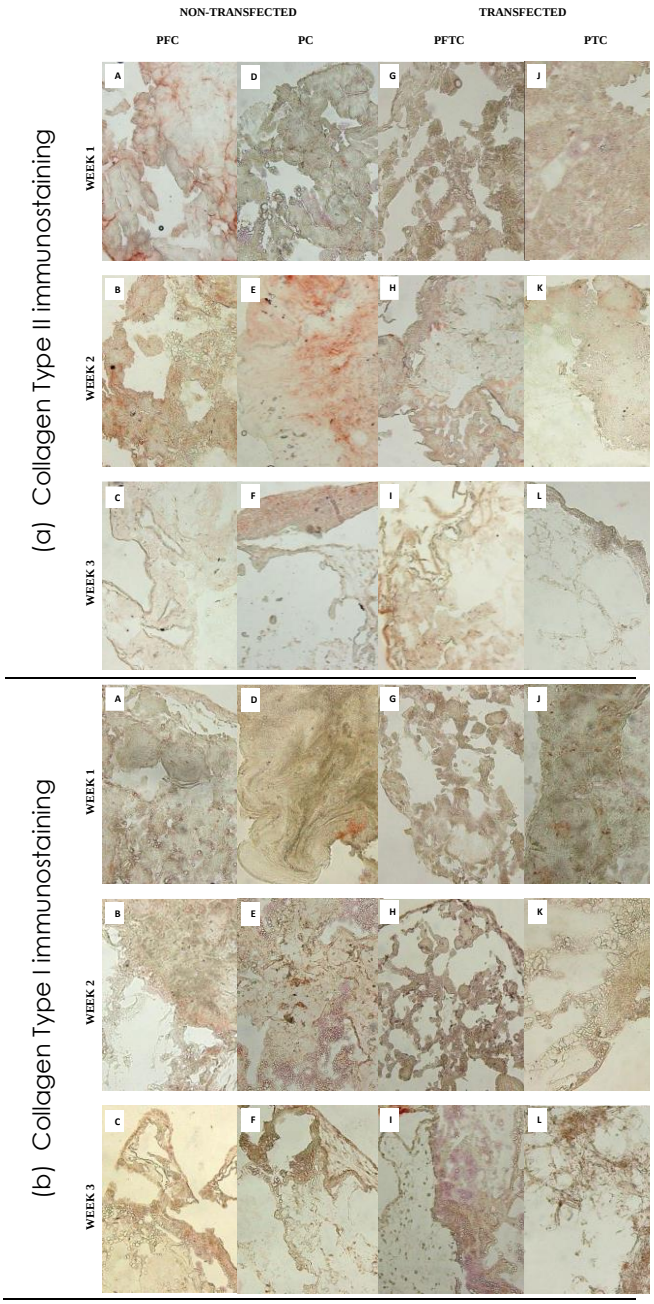


Figure 4 Immunohistochemistry observation of in vitro constructs stained with (a) collagen type II and (b) collagen type I staining. (Magnification of 200X, scale bar: 200 µm)

This present finding, later corroborated the qualitative gene expression analysis showing the down-regulation expression pattern of mature cartilage-specific marker e.g. collagen II and the stable expression pattern of mature cartilage dedifferentiation marker i.e. collagen I throughout the in vitro culture (Figure 5). The expression of other mature cartilaginous phenotypic markers Sox9 (c), aggrecan core protein (e) and collagen XI (g) was comparable in all constructs. A weak expression of collagen IX (f) was observed in the non-transfected and some transfected constructs at each time point. Hypertrophic property characterized by the expression of collagen X (i) was more consistent in the non-transfected group. Our overall observation in this study is that the TEC constructs indicated the early cartilage development based on the nature of a newly construct formation as mentioned in previous study [23].

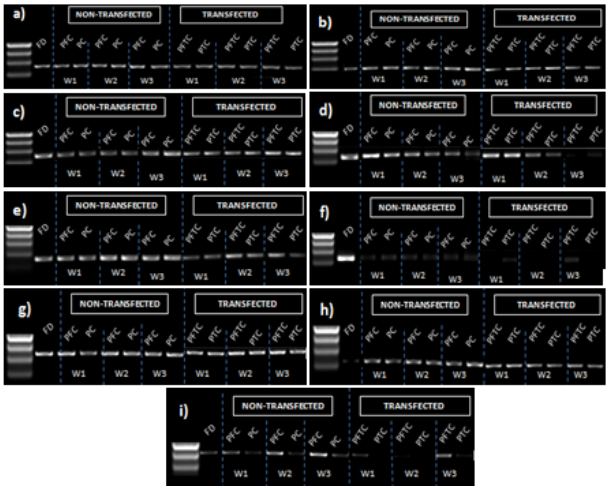


Figure 5 Two-step reverse transcriptase polymerase chain reaction of in vitro constructs. RT-PCR analysis of the mRNA expression of the housekeeping genes namely (a) β-actin and (b) GAPDH; the specific cartilaginous markers namely:(c) Sox9, (d) Collagen II, (e) Aggrecan core protein, (f), Collagen IX and (g) Collagen XI; dedifferentiation marker (h) Collagen I and hypertrophy marker (i) Collagen X (M: 100bp DNA ladder; FD: Freshly-digested cartilage as control)

Cell proliferation assay showed increment in cells growth activities at each time pint of day-7, -14 and -21 but, no statistical difference was found between each pair of compared groups (Table 3).

Table 3 Cell proliferation activity of in vitro constructs. Data was expressed as median (IQR)

		DAY-7	DAY-14	DAY-21
NON-TRANSFECTED	PFC	0.151 (0.109)	0.208 (0.228)	0.335 (0.187)
	PC	0.071 (0.061)	0.114 (0.116)	0.145 (0.158)
TRANSFECTED	PFTC	0.130 (0.177)	0.271 (0.145)	0.336 (0.287)
	PTC	0.105 (0.174)	0.105 (0.113)	0.128 (0.067)

From the data gathered, the wet weight of most constructs increased from week 1 to week 3, except for PC group (Table 4). Overall results indicated that the wet weight of the transfected groups was higher than the non-transfected groups.

Table 4 Wet weight (mg) of in vitro constructs. Data was expressed as median (IQR)

	NON-TRANSFECTED		**TRANSFECTED**	
	PFC	PC	PFTC	PTC
WEEK1	80.5 (31.50)	46.00 (37.25)	131.00 (38.50)	65.50 (46.75)
WEEK2	109.50 (43.50)	71.00 (47.25)	143.50 (34.75)	102.00 (30.50)
WEEK3	114.00 (73.75)	65.00 (16.25)	180.5 (65.75)	182.5 (90.00)

For sGAG production evaluation, the relative content of most constructs increased from week 1 to week 3 (Table 5). When normalized by the dried-weight of each construct, the sGAG content of the non-transfected constructs was relatively higher than the transfected groups.

Table 5 sGAG content (%) of in vitro constructs. Data was expressed as median (IQR)

	NON-TRANSFECTED		TRANSFECTED	
	PFC	PC	PFTC	PTC
WEEK1	0.307 (0.226)	0.260 (0.152)	0.193 (0.168)	0.158 (0.198)
WEEK2	0.375 (0.276)	0.376 (0.392)	0.278 (0.112)	0.207 (0.042)
WEEK3	0.556 (0.145)	0.514 (0.207)	0.352 (0.196)	0.323 (0.155)

Despite a few satisfactory findings, inconsistencies occurred between the four groups. Some transfected and non-transfected groups showed lack cartilaginous properties based on some aspects of examination. As reported by several prior studies [24, 25], this could be due to the nature of osteoarthritic or degeneratedcartilage samplesfrom elderly used in this study. Moreover, the transfection procedure was carried out on cultured cells at later passages, P2, where the initially cultured chondrocytes are known to adopt a fibroblastic trait at this stage [26, 27]. Perhaps, this is an indication that more signalling factors are needed for chondrogenic differentiation.

On the other hand, studies on the role of epigenetic regulation indicated that DNA methylation and histone modifications involvement in cells differentiation should be considered when studying and inducing cells towards a certain cell-specific lineage (28, 29). These factors perhaps could cause variation in chondrogenic differentiation efficiency.

However, in this present study we could not ascertain the mechanism of epigenetic modifications responsible for chondrogenic differentiation. It would be of great interest to explore the epigenetic regulation of chondrocytes, while determining the relevant components that facilitate chondrocytes redifferentiation in order to achieve efficient and reliable management of osteoarthritis in the near future.

4.0 CONCLUSION

Taken together, this study indicated that there may be more than transcription factor Sox9 gene transfer needed to maintain a prolonged chondrogenic phenotype in differentiated chondrocytes for better cartilage regeneration. Hence, it is hoped that this study provides a good ground for future cartilage TERM research and application.

Acknowledgement

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