



**THE POTENTIAL OF TRANSCRIPTION FACTOR SOX9
GENE TRANSFER IN OSTEOARTHRITIC
CHONDROCYTES DIFFERENTIATION AND
ARTICULAR CARTILAGE FORMATION**

BY

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**A thesis submitted in fulfillment of the requirement for the
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**Kulliyyah of Allied Health Sciences
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ABSTRACT

Articular cartilage has little capacity for repair, thus making it an area of focus for tissue engineering and regenerative medicine (TERM) research. The use of Sox9 gene transfer in facilitating chondrocytes differentiation is becoming more common since it plays an important role as transcription factor for chondrogenesis. This study aimed to identify the potential of the transiently overexpressed Sox9 gene in chondrocytes differentiation and in vitro and in vivo tissue engineered cartilage (TEC) formation. Articular cartilages were obtained from consented patients underwent joint surgery. Chondrocytes were isolated, cultured and transfected with pcDNA3-Sox9 using lipofection technique. The TEC constructs were formed by the incorporation of the transfected or the non-transfected chondrocytes onto poly (lactic-co-glycolic acid) (PLGA) scaffold with or without fibrin. Accordingly, comparisons between four experimental groups i.e. (1) transfected chondrocytes incorporated 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] were made. Transfection efficiency was optimized and assessed by the cartilaginous genes (Sox9, collagen type II and aggrecan) expression. All constructs were evaluated at 1, 2 and 3 weeks in vitro and, at 2 and 4 weeks in vivo (post-implantation). The evaluations involved gross morphology, histology, immunohistochemistry, cell proliferation activity, gene expression, sulphated glycosaminoglycan (sGAG) production and biomechanical property. From the results, optimum transfection efficiency was achieved using 2.0 µg and 4.0 µg DNA concentration. After 3 weeks in vitro, PFTC and PFC constructs showed better histoarchitecture and extracellular matrix (ECM) distribution in concert with positive glycosaminoglycan accumulation when compared to PTC and PC. Chondrogenic properties were evidenced by the expression of cartilaginous markers i.e. collagen type II, XI and aggrecan. The 2-week post-implanted constructs indicated better cartilaginous appearance, a more notable proteoglycans and glycosaminoglycan deposition than the 4-week constructs. The expression of collagen type II, XI and aggrecan was upregulated along with positive immunohistochemistry staining against collagen type I and II. The co-expression of collagen type I in the constructs indicated the early cartilage development based on the nature of a newly construct formation in this study. Despite a few satisfactory findings, inconsistencies occurred between the four groups. Some transfected and non-transfected groups showed lack of cartilaginous properties in some aspects of examination. Down-regulation of chondrogenic markers, particularly in PC and PTC constructs at 4-week post-implantation indicated that the constructs were unable to maintain specific cartilaginous phenotype. The sGAG content and compressive strain were comparable among all 4 groups. Taken together, this study indicated that there may be more than transcription factor Sox9 gene transfer needed for cartilage regeneration. There is always room for improvement in research. Hence, it is hoped that this study provides a good ground for future cartilage TERM research and application.

ملخص البحث

هدفت هذه الدراسة إلى التعرف على إمكانية جين (Sox9) المعبرة كلياً بشكل مؤقت في تمايز الخلايا الغضروفية. وتشكل الغضاريف المهندسة الأنسجة (TEC) مخبرياً خارج الجسم الحي وداخله. تم عزل واستزاع الخلايا الغضروفية وإدخال الحمض النووي (pcDNA3-Sox9) فيها باستخدام تقنية التعديـة بالجسيمات الشحمية. تم تشكيل بنيات الغضاريف المهندسة الأنسجة (TEC) عن طريق دمج الخلايا الغضروفية المعالجة وغير المعالجة علسقالة مكونة بـPLGA بـفبرين أو من دونه. وفقاً لذلك، فقد أجريت مقارنات بين أربع مجموعات تجريبية، وهي: (1) خلايا غضروفية معالجة مدمجة على PLGA/fibrin [PFTC] ، (2) خلايا غضروفية غير معالجة على PLGA/fibrin [PTC] ، (3) خلايا غضروفية معالجة مدمجة على PLGA [PTC] ، (4) خلايا غضروفية غير معالجة مدمجة على PLGA [PC]. تم تقييم جميع التراكيب في الأسبوع الأول والثاني والثالث مخبرياً خارج الجسم الحي مابعد الزرع. شملت عمليات التقييم التشكل الإجمالي، النسجيات، الكيمياء النسجية المناعية، نشاط تكاثر الخلايا، التعبير الجيني، إنتاج كبريتات غليكوزامينوغليكان، و الخصائص البيولوجية الميكانيكية. لوحظ من النتائج أن الكفاءة المثلى للمعالجة بالامكان تحقيقها باستخدام تركيز 2.0 مكغ و 4.0 مكغ للحمض النووي. بعد ثلاثة أسابيع في المختبر ، أظهرت التركيبات PFTC و PFC مقارنة بـ PTC و PC تصميم خلوي أفضل، وتوزيع المصفوفة خارج الخلية كان منسقاً معتراًم غليكوزامينوغليكان الإيجابي. اتضحت الخصائص المولدة للغضروف من تعبير المؤشرات الغضروفية : كولاجين نوعانين، واحد عشر، وأقريكان. أشارت بنيات الأسبوع الثاني ما بعد الزرع مظهراً أفضل وترسيباً أكثر تبيانياً للبروتيوغليكان وغليكوزامينوغليكان، مقارنة بنيات الأسبوع الرابع. التعبير عن الكولاجين نوعانين و احدى عشر قد ضبطاازديادياجنبنا إلى جنب مع التلويبالكيميائياتالنسجياالمناعي الإيجابي ضد كولاجين نوع و احد واثنين. وأشار التعبير المشترك لكولاجين نوع واحد في البنيات تطوراً غضروفياً مبكراً بالاعتماد على التشكل الجديد للبنيات في هذه الدراسة. فقد أظهرت بعض المجموعات المعالجة وغير المعالجة نقصاً في الخصائص الغضروفية من بعض جوانب الفحص. الضبط التخفيضي للمؤشرات المولدة للغضروف ولا سيما في بنيات PC و PTC في الأسبوع الرابع بعد الزرع أشار إلى أن البنيات لم تتمكن من الحفاظ على نمط ظاهري غضروفي معين. محتوى sGAG والتوتر الضاغط كانا قابلين للمقارنة بين جميع المجموعات الأربع. أشارت هذه الدراسة على أنه قد يكون هناك أكثر من عامل واحد غير نقل جين (Sox9) لتجديد الغضاريف.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences (Biomedical Science).

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DECLARATION

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*This thesis is dedicated to my future self.
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In the name of Allah, The Most Gracious, The Most Merciful.

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LIST OF ARTICLES / MANUSCRIPTS / PROCEEDINGS / ABSTRACTS RELATED TO THIS THESIS

Main Publications.

1. Norhamiza Mohamad Sukri, Muhammad Aa'zamuddin Ahmad Radzi, Rozlin Abdul Rahman, Ahmad Hafiz Zulkifly, Abdurezak Abdulahi Hashi, Munirah Sha'ban. (2015). Identifying the Potential of Transcription Factor Sox9 Gene Transfer in Chondrocytes Differentiation and Articular Cartilage Formation In Vitro. [POSTER PRESENTATION].
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2. Rozlin Abdul Rahman, Noradibah Abdul Majid, Nor Athirah Azeman, Norhamiza Mohamad Sukri, Noorhidayah Md. Nazir Muhammad Aa'zamuddin Ahmad Radzi, Ahmad Hafiz Zulkifly, Aminudin Che Ahmad, Suzanah Abdul Rahman, Munirah Sha'ban. Cell Proliferation Assay and Sulphated Glycosaminoglycan Production in Poly (lactic-co-glycolic acid)-based Scaffolds Seeded with Bone Marrow Mesenchymal Stem Cells for Cartilage Tissue Engineering. *Regenerative Research* 3 (2) 2014.161-162.
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LIST OF ABBREVIATIONS

ACI	Autologous chondrocyte implantation
BMSC	Bone marrow mesenchymal stem cells
cDNA	Complementary deoxyribonucleic acid
cmd	Cartilage matrix deficiency
COPCORD	Community-oriented programme for control of rheumatic diseases
CS	Chondroitin sulphate
DS	Dermatan sulphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FD	Nutrient Mixture F-12 (Ham)/Dulbecco's Modified Eagle Medium (1:1) medium
FGF	Fibroblast growth factor
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia-inducible factor
IGF	Insulin-like growth factor
IHH	Indian Hedgehog
IQR	Interquartile range
KS	Keratan sulphate
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide

NaHCO ₃	Sodium hydrogen bicarbonate
NSAIDS	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OATS	Osteochondral autologous transplantation system
pcDNA	Partial complementary deoxyribonucleic acid
PLGA	Poly (lactic co-glycolic) acid
RT-PCR	Reverse transcriptase polymerase chain reaction
sGAG	Sulphated glycosaminoglycans
S.O.C	Super optimal with catabolite repression medium
Sox9	Sry (sex determining region Y)-box 9
TE	Tris-EDTA
TEC	Tissue engineered cartilage
TERM	Tissue Engineering and Regenerative Medicine
TGF-β	Transforming growth factor-β family
TKA	Total knee arthroplasty

LIST OF SYMBOLS/EQUATIONS

cm	Centimeter
et al.,	(<i>et alia</i>): and others
g	Gram
kg	Kilogram
L	Litre
M	Molarity
mg	Milligram
ml	Milliliter
MPa	Megapascal
N	Normality
®	Registered patent
rpm	Revolutions per minute
™	Trademark
v/v	volume/volume
w/v	weight/volume
μl	Microlitre
μm	Micrometer

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Articular cartilage plays a crucial role as a load-bearer in the synovial joints. It has remarkable mechanical properties contributed primarily by its well-organized extracellular matrix (ECM) produced by the highly specialized cartilage cells; chondrocytes. The ECM consists of predominantly collagens, large aggregated proteoglycans and glycoproteins (Buckwalter, Mankin and Grodzinsky, 2005). The matrix homeostasis is regulated and maintained mainly by chondrocytes to preserve the anatomical structure and mechanical strength of the cartilage. Unfortunately, articular cartilage is often subject to damage by trauma or diseases. If left nontreated, this avascular connective tissue is prone to degenerate as it has limited capacity to repair itself (Chiang and Jiang, 2009; Berta, Duska, Tóth and Hangody, 2015). The progressive degeneration of the cartilage ultimately leads to joint pain and dysfunction that characterize osteoarthritis (OA).

Osteoarthritis is a leading cause of chronic disability among the middle aged and elder people, although younger individuals may be affected due to injury or overuse (Tanna, 2004; Amoako and Pujalte, 2014). The disease is capable of causing gradual loss of cartilage primarily in weight bearing joints such as hips, knees and spine. Knee OA is the most common type of OA compared to others, which affected approximately 13% of women and 10% of men aged 60 years and older (Michael, Schluter-Brust and Eysel, 2010; Heidari, 2011). Older people in Asia have high risk to get knee OA and with rapid ageing and increasing obesity in most countries, more concern should be given to reduce the prevalence in Asia (Fransen, Bridgett, March,

Hoy, Penserga and Brooks, 2011; Chopra, 2013). A community-oriented programme for control of rheumatic diseases (COPCORD) studies on several developing countries in Asia showed different prevalence rate of knee OA, ranging from 2.3 to 29% of the rural and/or urban populations (Chopra, 2013). In addition, Asian people tend to use the kneeling and squatting postures in their daily activities which have appeared as one of the risk factors for knee OA (Kasahara, Majima, Kimura, Nishiike and Uchida, 2013).

This disease gradually worsens with time and to date, finding the most effective treatment that not only can relieve pain, but capable to completely regenerate and restore the hyaline cartilage functionally for long-term period using less invasive procedures remains challenging to researchers (Musumeci, Castrogiovanni, Mazzone, Szychlinska, Castorina and Loreto, 2014). Available therapeutic drugs for OA such as analgesics, anti-inflammatory and anti-cytokine drugs are limited largely to symptomatic relief of pain and the effectiveness varies between patients. They do not provide cartilage regeneration and only do little to block disease progression. Moreover, the frequent use of oral drugs has been associated with adverse effects on gastrointestinal tract and cardiovascular system (Sudano et al., 2010; O'Neil, Hanlon and Marcum, 2012; Scarpignato, Lanas, Blandizzi, Lems, Hermann and Hunt, 2015). The use of intra-articular injections for drug administration are somehow safer, but the short-term therapeutic effect requires the patients to get repeated injection which may predispose them to the risk of infection (Gerwin, Hops and Lucke, 2006; Iannitti, Lodi and Palmieri, 2011; Ishijima et al., 2014).

The accepted surgical treatment options for OA till date include microfracture (Salzmann, Sah, Sudkamp and Niemeyer, 2013), osteochondral autologous transplantation system (OATS) mosaicplasty (Ulstein, Årøen, Røtterud, Løken,

Engebretsen and Heir, 2014), autologous chondrocyte implantation (ACI) (Bentley, Biant, Vijayan, Macmull, Skinner and Carrington, 2012) and total knee arthroplasty (TKA) (Rönn, Reischl, Gautier and Jacobi, 2011). Certainly, encouraging outcomes of using these procedures such as pain relief and improvement of joint function until a certain time point have been reported by many clinical studies (Basad, Ishaque, Bachmann, Stürz and Steinmeyer, 2010; Solheim, Hegna, Oyen, Austgulen, Harlem and Strand, 2010; Crawford, DeBerardino and Williams, 2012; Ulstein et al., 2014). However, to date, none have succeeded in a complete hyaline cartilage restoration and long-lasting improvement of joint function (Kessler, Ackerman, Dines and Grande, 2008; Gobbi, Karnatzikos and Kumar, 2014; Filardo, Kon, Perdisa, Tetta, Di Martino and Marcacci, 2015). There are insufficient clinical data to support significant superiority of any of the techniques over one another (Ely Zarina and Tunku Kamarul, 2015). The poor resulting repair tissue and its deterioration with time, the limited availability of donor site, graft failure, arthrofibrosis and periosteal hypertrophy, all represents the drawbacks of such operative procedures (Evans, Miniaci and Hurtig, 2004; Concaro, 2010; Gobbi et al., 2014; Filardo et al., 2015).

For severe form of OA, total knee arthroplasty (TKA) is often the ultimate procedure when drug therapy and other surgical procedures failed to restore the joint function. While this procedure may improve the quality of life in a few months post-surgery, the functional limitations of prosthesis are insufficient for a young active or working individual. Post-operative complications such as infection, pulmonary embolism or deep venous thrombosis and mechanical loosening have also been reported (Werner, Evans, Carothers and Browne, 2015; Houdek, Watts, Shannon, Wagner, Sems and Sierra, 2015). Moreover, this procedure is inaccessible primarily