THE POTENTIAL OF SRY (SEX DETERMINING REGION Y)-BOX 9 AND TELOMERASE REVERSE TRANSCRIPTASE GENES TRANSFECTION FOR ARTICULAR CARTILAGE TISSUE ENGINEERING

BY

NOORHIDAYAH BINTI MD NAZIR

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy in Health Sciences

Kulliyyah of Allied Health Sciences
International Islamic University Malaysia

MAY 2020

ABSTRACT

This study incorporates gene transfer with tissue engineering to evaluate the feasibility of cartilaginous tissue formed using SRY(Sex Determining Region Y)-Box 9 (*SOX9*) and Telomerase Reverse Transcriptase (*TERT*) genes transfected chondrocytes. The aim of this research is to improve on the current cartilage treatment strategies with undue limitations and to work toward an alternative treatment for cartilage damage. The experimental settings involve monolayer cell culture, *in vitro* three-dimensional (3D) culture and *in vivo* ectopic implantation. The cells were isolated from six rabbit's articular cartilage and cultured until passage-1 (P1). The P1 cells were transfected with *SOX9*/*TERT*-, *SOX9*-, and *TERT*-gene. The non-transfected chondrocytes serve as the control group. For monolayer study, the cells were sub-cultured until P3 and evaluated in each serial passage. The *in vitro* 3D construct was formed by seeding the P1 cells in poly(lactic-*co*-glycolic acid) (PLGA) and PLGA/fibrin hybrid scaffolds with cells density of 1×10^5 per scaffold. The resulted cell-scaffold constructs were evaluated at week-1, -2 and -3 of culture. For *in vivo* study, the week-3 *in vitro* constructs formed by *SOX9*/*TERT*-transfected chondrocytes were subcutaneously implanted at the dorsum of the athymic mice. The constructs were evaluated at week-2 and -4 post-implantation. The analyses include growth kinetics profile, cell proliferation analysis, compression-stress analysis, macroscopic, microscopic visualisation, histological stains, quantitative sulphated glycosaminoglycan (sGAG) content analysis and gene expression study using real-time polymerase chain reaction (RT-PCR) of cartilaginous markers (*SOX9*, *COL2A1*, *ACAN*), *COL1A2*, *TERT* and collagenolytic marker (*MMP13*). A total of 60.4% transfection efficiency can be achieved using Lipofectamine® 3000 reagent. The upregulation of the transferred genes was noted in the cell groups indicating the effectiveness of the procedure. The monolayer cultured cells were unable to retain their cartilaginous phenotype. However, the *in vitro* 3D culture successfully exhibited the cartilaginous tissue formation. The cells and extracellular matrix (ECM) were densely distributed in the constructs at week-3. The cell number was increased in the constructs. The ECM components (sGAG, proteoglycan and collagen type-II) were visualised in the constructs. The cartilaginous genes expression was upregulated in the *SOX9*/*TERT*-transfected chondrocytes constructs group. Hence, this group was selected for the *in vivo* study. The *in vivo* constructs have the appearance which resembles cartilage. In terms of the construct's rigidity, there are no changes in the groups from week-2 to week-4 post-implantation. The cells and ECM distribution were homogenous in the *in vivo* constructs, which is better than the one observed in the *in vitro* constructs. The presence of ECM components was noted in the constructs indicates the cartilaginous tissue development. The cartilaginous genes expression was particularly upregulated in *SOX9*/*TERT*-PLGA/fibrin construct. The *SOX9*/*TERT*-PLGA/fibrin construct has the potential to be developed into a functional cartilaginous tissue and translated into clinical application. Since the end goal of this present study is to benefit the humankind, proper research guidelines to ensure safety and efficacy of the engineered tissue must be followed with good intention and values. The approach is in-line with the teaching of Islam – there should be neither harming nor reciprocating harm.

خلاصة البحث

استخدمت هذه الدراسة نقل الجينات المتضمن لطرق هندسة الأنسجة لتقييم إمكانية تكوين الأنسجة الغضروفية باستخدام خلايا غضروفية تم تعداؤها بجينات SRY (المنطقة المحددة للجنس Y)-بوكس 9 (SOX9) وجين التيلوميراز المنتسخ العكسي (TERT). هدف هذا البحث إلى تحسين الاستراتيجيات الحالية المحدودة لمعالجة الغضاريف والعمل على علاج بديل للغضاريف المتضررة. تضمنت الإعدادات التجريبية كلا من المستنبتات الخلوية أحادية الطبقة، والمستنبتات المختبرية الثلاثية الأبعاد، والزرع المنتبذ داخل الجسم الحي. تم عزل الخلايا من الغضاريف المفصلية لستة أرانب واستنباتها حتى الطور 1 (P1). تم تعداء خلايا P1 بجينات SOX9/ TERT-، و SOX9-، و TERT-. وضعت الخلايا الغضروفية التي لم يتم تعداؤها في المجموعة الضابطة. لدراسة الطبقة الأحادية، تم استنبات الخلايا ثانويا حتى الطور 3 (P3) وتقييمها في كل طور تسلسلي. تم تكوين البنية المختبرية الثلاثية الأبعاد عن طريق زرع خلايا P1 في بولي(حمض اللاكتيك-كو- حمض الجليكول) (PLGA) والسقالات الهجينة بـ PLGA/فيبرين بكثافة خلوية قدرها 1×10^2 لكل سقالة. تم تقييم "السقالات الخلوية" التي تم انتاجها في الأسبوع الأول والثاني والثالث من الاستنبات. أما بالنسبة للدراسة داخل الجسم الحي، تم زرع التركيبات المختبرية من الأسبوع الثالث المكونة بالخلايا الغضروفية التي تم تعداؤها بجينات SOX9/TERT تحت الجلد على ظهر فئران عديمة الغدد الزعترية. تم تقييم التركيبات في الأسبوع الثاني والرابع بعد الزرع. شمل التقييم على بروفايل النمو الحركي، وتحليل تكاثر الخلايا، وتحليل الضغط والإجهاد، والتصور العياني والمجهري، والبقع الهيستولوجية، وتحليل محتوى الجليكوزامينوجليكان الكمي (sGAG)، ودراسة التعبير الجيني باستخدام تفاعل البوليميراز المتسلسل اللحظي (RT-PCR) للمعلمات الغضروفية (SOX9، COL2A1، ACAN)، COL1A2، TERT والمعلمات الكولاجينية (MMP13). كان بالامكان تحقيق نسبة 60.40٪ من كفاءة التعداء باستخدام كاشف Lipofectamine® 3000. تم ملاحظة التنظيم الرفعي للجينات المنقولة في مجموعات الخلايا مما يشير إلى فعالية العملية. لم تكن الخلايا الأحادية الطبقة المستنبتة قادرة على الحفاظ على النمط الظاهري للغضروف. ومع ذلك فقد أظهرت المستنبتة المختبرية الثلاثية الأبعاد بنجاح تكون الأنسجة الغضروفية. كانت الخلايا والمصفوفة خارج الخلية (ECM) موزعة بشكل كثيف في التركيبات في الأسبوع الثالث. وارتفع أيضا عدد الخلايا في التركيبات. وتم تصوير مكونات المصفوفة خارج الخلية (sGAG، وبروتيوجليكان، وكولاجين نوع 2) في التركيبات. تم تنظيم الجينات الغضروفية بشكل رفعي في مجموعة تركيبات الخلايا الغضروفية التي تم تعداؤها بجينات SOX9/TERT، وبالتالي فقد تم اختيار هذه المجموعة لدراستها في الجسم الحي، حيث يوجد في التركيبات داخل الجسم الحي مظهرا مشابها للغضروف. أما بالنسبة لصلابة التركيبة، فلم يكن هنالك أي تغير في المجموعات من الأسبوع الثاني إلى الأسبوع الرابع بعد الزرع. كانت الخلايا وتوزيع المصفوفة خارج الخلية متجانسة في التركيبات داخل الجسم الحي، وذلك بالطبع أفضل من تلك الملحوظة في التركيبات خارج الجسم الحي. كان وجود مكونات المصفوفة خارج الخلية ملحوظا في التركيبات ويدل ذلك على تطور الغشاء الغضروفي. كان لدى مجموعة SOX9/TERT-PLGA/fibrin القدرة على التطور إلى نسيج غضروفي وظيفي وتحويله إلى التطبيقات الإكلينيكية. بما أن الهدف النهائي لهذه الدراسة الحالية هو نفع البشرية فإنه من الواجب اتباع الإرشادات البحثية المناسبة لضمان سلامة وفعالية الأنسجة المهندسة بنية وقيم حسنة. توافقت طرق البحث مع التعاليم الإسلامية التي تستوجب عدم وجود الضرر والضرار.

APPROVAL PAGE

The thesis of Noorhidayah binti Md Nazir has been approved by the following:

_____
Munirah binti Sha'ban
Supervisor

_____
Ahmad Hafiz bin Zulkifly
Chairman of Supervisory Committee

_____
Kamarul Ariffin bin Khalid
Co-Supervisor

_____
Ismail bin Zainol
Co-Supervisor

_____
Zaitunnatakhin binti Zamli
Co-Supervisor

_____
Azran Azhim bin Noor Azmi
Internal Examiner

_____
Norzana binti Abd Ghafar
External Examiner 1

_____

Bakiah binti Shaharuddin
External Examiner 2

_____

Ahmed Jalal Khan Chowdhury
Chairman

DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Noorhidayah binti Md Nazir

Signature ..... Date .....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

THE POTENTIAL OF NON-VIRAL SRY (SEX DETERMINING REGION Y)-BOX 9 AND TELOMERASE REVERSE TRANSCRIPTASE GENES TRANSFER FOR ARTICULAR CARTILAGE TISSUE ENGINEERING

I declare that the copyright holders of this dissertation are jointly owned by the student and IIUM.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below

1. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.

2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.

3. The IIUM library will have the right to make, store in a retrieved system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Noorhidayah binti Md Nazir

……..…….. ……..
Signature Date

ACKNOWLEDGEMENTS

Firstly, it is my utmost gratitude above all due to *Allah SWT* (ﷻ) for His mercy and blessing, I have been able to complete this thesis.

I would like to express my special thanks to my respected supervisor, Assist. Prof. Dr. Munirah Sha'ban for her guidance, encouragement, advise and patience thru the years I am in her research team. I also would like to thank my co-supervisors, Prof. Dr. Ahmad Hafiz Zulkifly, Assoc. Prof. Dr. Kamarul Ariffin Khalid, Prof. Dr. Ismail Zainol and Assist. Prof. Dr. Zaitunnatakhin Zamli for their support and assistance throughout completing this work. My gratitude goes to Kulliyyah of Allied Health Sciences, International Islamic University Malaysia for providing me the facilities and opportunity to study. I acknowledge the Ministry of Energy, Science, Technology, Environment and Climate Change (MESTECC) or formerly known as Ministry of Science, Technology and Innovation (MOSTI) and MyBrain15 for granting the financial assistance to complete this study.

I wish to express my appreciation and thanks to the research team members and friends for their friendship, support and encouragement during my study years. Although I cannot list their names here, they will always hold a special place in my heart. I will never forget this meaningful journey in my life especially with the research team members and Dr. Munirah.

My deepest gratitude to my beloved parents, Md Nazir Md Taib and Kamisah Jaafar@Harun for always being there for me from the very beginning. I will be forever grateful. Finally, my appreciation goes to my dearest siblings, Muhammad Asyraf, Muhammad Syahmi, Muhammad Ismat Hanif, Muhammad Amirul Hanis, Muhammad Khairul Hamizan and Aisyah Fathonah for their continuous support, encouragement and motivation. Thank you very much for being part of my life.

TABLE OF CONTENTS

Abstract ii
Abstract in Arabic iii
Approval Page iv
Declaration vi
Copyright vii
Acknowledgements viii
Table of Contents ix
List of Tables xiv
List of Figures xv
List of Abbreviations xix

CHAPTER ONE: INTRODUCTION 1
1.1 Background of the Study 1
1.2 Problem Statement 3
1.3 Research Objectives 4
1.3.1 General Objectives 4
1.3.2 Specific Objectives 4
1.4 Research Hypotheses 5

CHAPTER TWO: LITERATURE REVIEW 6
2.1 Articular Cartilage Structure and Function 6
2.2 Articular Cartilage Degenerative Diseases and Current Treatment Modalities 7
2.3 Tissue Engineering and Gene Transfer 8
2.3.1 Tissue Engineering Principle 8
2.3.2 Cartilage Tissue Engineering 12
2.3.3 Gene Transfer 16
2.3.4 The Application of Gene Transfer in Cartilage Tissue Engineering 18
2.4 Safety and Efficacy Issues of Gene Transfer Application in TERM from the Islamic Perspective 20

CHAPTER THREE: METHODOLOGY 22
3.1 Research and Ethical Approval 22
3.2 Preparation of Solutions and Reagents 22
3.2.1 Preparation of Ampicillin Stock 22
3.2.2 Preparation of Kanamycin Stock 22
3.2.3 Preparation of LB Agar And Broth 23
3.2.4 Preparation of 50x TAE Buffer 23
3.2.5 Preparation of 1x TAE Buffer 24
3.2.6 Preparation of 1x Phosphate-Buffered Saline (Pbs), pH 7.2 24
3.2.7 Preparation of 0.1% (W/V) L-Ascorbic Acid Solution 24
3.2.8 Preparation of FD Stock and Complete Medium 24
3.2.9 Preparation of 0.6% (W/V) Collagenase A Solution 25

3.2.10 Preparation of 0.5% (W/V) 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) Solution 25
3.2.11 Preparation of Papain Enzyme Digestion Solution 25
3.2.12 Preparation of SAT Reagent 26
3.2.13 Preparation of Alcian Blue Reagent 26
3.2.14 Preparation of Dimethyl Sulfoxide (DMSO) Washing Solution 27
3.2.15 Preparation of Gu-Prop-H_2O Solution 27
3.2.16 Preparation of 0.3% (V/V) Acid Alcohol 27
3.2.17 Preparation of Weigert's Iron Haematoxylin Solution 27
3.2.18 Preparation of 1% (V/V) Acetic Acid 28
3.2.19 Preparation of 0.1% (W/V) Safranin O Solution 28
3.2.20 Preparation of 1% (W/V) Alcian Blue Solution, Ph 2.5 28
3.2.21 Preparation of 1% (W/V) Toluidine Blue Solution 28
3.2.22 Preparation of 0.1% (W/V) Proteinase K Solution 28
3.2.23 Preparation of Primary Antibody, Monoclonal Mouse Anti-Rabbit Collagen Type II (1:2000) 29
3.2.24 Preparation of Primary Antibody, Monoclonal Mouse Anti-Rabbit Collagen Type I (1:300) 29
3.3 Preparation of Plasmid Vector Containing *SOX9* and *TERT* Genes 29
3.3.1 Transformation and Confirmation 29
3.3.2 Amplification 33
3.4 Chondrocytes Isolation and Monolayer Culture 34
3.5 *SOX9* and/or *TERT* Genes Transfer in Chondrocytes 36
3.5.1 Transfection Optimisation 36
3.5.2 Transfection 38
3.6 Fabrication of Microporous Three-Dimensional (3D) PLGA Scaffold 39
3.7 Preparation of Plasma-Derived Fibrin 40
3.8 Formation of Cell-Scaffold Construct 40
3.9 Procedure of Subcutaneous Implantation 42
3.10 Sample Measurement and Analyses 43
3.10.1 Microscopic Observation of Monolayer Cultured Chondrocytes 43
3.10.2 Growth Kinetic Profile Analysis of Monolayer Cultured Chondrocytes 43
3.10.3 Gross Observation of The *In Vitro* and *In Vivo* Cell-Scaffold Construct 44
3.10.4 Scanning Electron Microscopy (SEM) of the *In Vitro* And *In Vivo* Cell-Scaffold Construct 44
3.10.5 Biomechanical Analysis of The *In Vivo* Cell-Scaffold Construct 45
3.10.6 Cell Proliferation Analysis of The *In Vitro* Cell-Scaffold Construct 45
3.10.7 Histology And Immunohistochemistry of Monolayer Cultured Chondrocytes, *In Vitro* and *In Vivo* Cell-Scaffold Construct 45
3.10.7.1 Haematoxylin and Eosin (H&E) Staining 46
3.10.7.2 Alcian Blue/ Fast Red Staining 47
3.10.7.3 Toluidine Blue/ Fast Red Staining 47
3.10.7.4 Safranin O/ Fast Green Staining 47

3.10.7.5 Collagen Type-II and Collagen Type-I Immunohistology 48
3.10.8 Sulphated Glycosaminoglycan Analysis of Monolayer Cultured Chondrocytes, *In Vitro* and *In Vivo* Cell-Scaffold Construct 49
3.10.9 Gene Expression Analysis of Monolayer Cultured Chondrocytes, *In Vitro* and *In Vivo* Cell-Scaffold Construct 50
3.10.9.1 Ribonucleic Acid (RNA) Isolation 51
3.10.9.2 Complementary Deoxyribonucleic Acid (cDNA) Conversion 51
3.10.9.3 Real-Time Polymerase Chain Reaction (RT-PCR/qPCR) 52
3.11 Statistical Analysis 53
3.12 Statistical Analysis 53

CHAPTER FOUR: OPTIMISATION OF TRANSFECTION EFFICIENCY 55
4.1 Materials and Methods 55
4.2 Results 56
4.2.1 Monolayer Cultured Chondrocytes Morphology 56
4.2.2 Transfection Efficiency 57
4.2.3 Cartilaginous Markers Expression of Monolayer Cultured Chondrocytes 59
4.3 Discussions 60

CHAPTER FIVE: EVALUATION OF CHONDROGENIC PROPERTIES OF *SOX9* AND/OR *TERT*-TRANSFECTED CHONDROCYTES IN MONOLAYER CELLS CULTURE SETTING 63
5.1 Materials and Methods 63
5.2 Results 64
5.2.1 Monolayer Cultured Chondrocytes Morphology 64
5.2.2 Growth Kinetic Profile 66
5.2.2.1 Cell Viability 66
5.2.2.2 Cumulative Cell Count 67
5.2.2.3 Growth Rate 67
5.2.2.4 Number of Cell Doubling 68
5.2.2.5 Population Doubling Time 70
5.2.3 Histomorphology and Cartilaginous Markers Expression of Monolayer Cultured Chondrocytes 71
5.2.4 sGAG Content of Monolayer Cultured Chondrocytes 78
5.2.5 Cartilaginous Markers Expression of Monolayer Cultured Chondrocytes 79
5.2.5.1 *ACAN* 79
5.2.5.2 *COL2A1* 80
5.2.5.3 *SOX9* 81
5.2.5.4 *COL1A2* 83
5.2.5.5 *MMP13* 84
5.2.5.6 *TERT* 85
5.3 Discussions 87

CHAPTER SIX:THE FORMATION OF *IN VITRO* CELL-SCAFFOLD CONSTRUCT FORMED USING *SOX9* AND/OR *TERT*-TRANSFECTED CHONDROCYTES 92
6.1 Materials and Methods 92
6.2 Results 93
6.2.1 Gross and Microscopic Morphology of Cell-Scaffold Construct 93
6.2.2 Cell Number of Cell-Scaffold Construct 96
6.2.3 Histoarchitecture and Cartilaginous Marker Expression of Cell-Scaffold Construct 97
6.2.4 sGAG Content of Cell-Scaffold Construct 111
6.2.5 Cartilaginous Markers Expression of Cell-Scaffold Construct..... 112
6.2.5.1 *ACAN* 112
6.2.5.2 *COL2A1* 114
6.2.5.3 *SOX9* 115
6.2.5.4 *COL1A2* 117
6.2.5.5 *MMP13* 118
6.2.5.6 *TERT* 120
6.3 Discussions 121

CHAPTER SEVEN:THE FORMATION OF *IN VIVO* CELL-SCAFFOLD CONSTRUCT USING THE ECTOPIC IMPLANTATION MODEL 128
7.1 Materials and Methods 128
7.2 Results 129
7.2.1 Gross and Microscopic Morphology of Cell-Scaffold Construct .. 129
7.2.2 Mechanical Strength of Cell-Scaffold Construct 131
7.2.3 Histoarchitecture And Cartilaginous Marker Expression of Cell-Scaffold Construct 132
7.2.4 sGAG Content of Cell-Scaffold Construct 139
7.2.5 Cartilaginous Markers Expression of Cell-Scaffold Construct..... 140
7.2.5.1 *ACAN* 140
7.2.5.2 *COL2A1* 141
7.2.5.3 *SOX9* 141
7.2.5.4 *COL1A2* 142
7.2.5.5 *MMP13* 143
7.2.5.6 *TERT* 144
7.3 Discussions 145

CHAPTER EIGHT:SAFETY AND EFFICACY ISSUES OF NON-VIRAL GENE TRANSFER IN CARTILAGE TERM APPROACH FROM THE WORLDVIEW OF ISLAM 150
8.1 Introduction..... 150
8.2 Gene Transfer Definition, Concept and its Application in Cartilage Tissue Engineering..... 152
8.3 Safety And Efficacy Issues: Ethical Concern from Western Perspective 156
8.4 Safety And Efficacy Issues: Ethical Concern from Islamic Worldview .. 158
8.5 Challenges in Gene Transfer for Catilage Tissue Engineering: Way Forward 162
8.6 Conclusion 167

CHAPTER NINE: CONCLUSION 169
9.1 Overall 169
9.2 Limitation of the Study 170
9.3 Future Direction 170

REFERENCES 172

APPENDIX A: RESEARCH ETHICAL APPROVAL 188
APPENDIX B: *SOX9* AND *TERT* GENES SEQUENCES 190
APPENDIX C: LIST OF FULL ARTICLES/ PROCEEDINGS/ ABSTRACTS 202
APPENDIX D: LIST OF OTHER ARTICLES/ PROCEEDINGS/ ABSTRACTS 212
APPENDIX E: AWARD 215

LIST OF TABLES

Table 3.1	The primer sequences for identifying human's SOX9 and TERT genes in the plasmid vector	31
Table 3.2	Plasmid digestion reaction components	32
Table 3.3	The lipofection reagent reactions per well based on manufacturer's recommendation	36
Table 3.4	PCR reaction components	37
Table 3.5	The Lipofectamine® 3000 reagent reaction per well based on the manufacturer's recommendation	38
Table 3.6	The experimental groups in the study	38
Table 3.7	The experimental groups and relevant abbreviation	41
Table 3.8	The rabbit's cartilaginous-specific markers primer sequences	50
Table 3.9	The components of cDNA conversion reaction	52
Table 3.10	The components of RT-PCR reaction	52
Table 4.1	The transfection efficiency obtained from using three transfection reagents	59

LIST OF FIGURES

Figure 3.1	The plasmid vectors map.	30
Figure 3.2	The rabbit's articular cartilage from lateral and medial femoral condyles of knee joint.	35
Figure 3.3	The illustration of silicon mould setup for scaffold fabrication	40
Figure 3.4	The overall experimental methodology.	54
Figure 4.1	The methodology of transfection efficiency optimisation..	56
Figure 4.2	Phase contrast photograph of *SOX9*-transfected chondrocytes and non-transfected chondrocytes (control) at P1, P2 and P3 viewed under 100X magnification.	57
Figure 4.3	Digital fluorescent microscopy images of *SOX9*-transfected chondrocytes viewed using Cytell^{TM} Cell Imaging System (Focus (microns)=1559 and Exposure (msec)=141)	58
Figure 4.4	Gel photos of cartilaginous markers (*SOX9* and *COL2A1*) and *COL1A2* expression.	60
Figure 5.1	The methodology of monolayer cultured cells analysis.	64
Figure 5.2	Phase contrast micrograph of transfected chondrocytes groups and control group.	65
Figure 5.3	Cell viability (%) of the transfected chondrocytes groups and control at P0, P1, P2 and P3.	66
Figure 5.4	The cumulative cell number of the transfected chondrocytes groups and control from P0 until P3.	67
Figure 5.5	Growth rate of the transfected chondrocytes groups and control group from P0 until P3.	68
Figure 5.6	The number of cell doubling of the transfected chondrocytes groups and control group from P0 until P3.	69
Figure 5.7	The cumulative number of cell doubling of the transfected chondrocytes groups and control group from P0 until P3.	69
Figure 5.8	Population doubling time of transfected chondrocytes groups and control group from P0 until P3.	70
Figure 5.9	Cumulative population doubling time taken by the transfected chondrocytes groups and control groups from P0 until P3.	71

Figure 5.10 Microscopic photos of the serially passages transfected chondrocytes groups and control group stained using H&E. 72

Figure 5.11 Microscopic photos of the serially passages transfected chondrocytes groups and control group stained using alcian blue/fast red. 73

Figure 5.12 Microscopic photos of the serially passages transfected chondrocytes groups and control group stained using toluidine blue/fast red. 74

Figure 5.13 Microscopic photos of the serially passages transfected chondrocytes groups and control group stained using safranin O/fast green. 75

Figure 5.14 Collagen type-II immunocytochemistry photos of the serially passages transfected chondrocyte groups and control group. 76

Figure 5.15 Collagen type-I immunocytochemistry of the serially passages transfected chondrocytes groups and control group. 77

Figure 5.16 sGAG content of the serially passages transfected chondrocyte groups and control group. 78

Figure 5.17 *ACAN* expression in transfected chondrocytes groups and control group in serial passages. 80

Figure 5.18 *COL2A1* expression in transfected chondrocytes groups and control group in serial passages. *COL2A1* was downregulated at P3 in all groups. 81

Figure 5.19 *SOX9* expression in transfected chondrocytes groups and control group in serial passages. *SOX9* was upregulated from P0 until P2 and eventually downregulated at P3 in all groups. 82

Figure 5.20 *COL1A2* expression in transfected chondrocytes groups and control group in serially passages. The *COL1A2* was upregulated from P0 until P3 in all groups. 84

Figure 5.21 *MMP13* expression in transfected chondrocytes groups and control group in serially passages. *MMP13* was upregulated in all groups. 85

Figure 5.22 *TERT* expression in transfected chondrocytes groups and control group. *TERT* was upregulated in all groups in serial passages. 86

Figure 6.1 The methodology of 3D constructs formation and analysis. 93

Figure 6.2 Photographs of all *in vitro* constructs in the culture at week-1, week-2 and week-3. 94

Figure 6.3 SEM of PLGA/fibrin and PLGA scaffold without cells viewed under 100X magnification. 95

Figure 6.4 SEM of representative *in vitro* constructs seeded with cells at a) week-1, b) week-2 and c) week-3 viewed under 100X magnification. d) The magnified attached cell on the scaffold's surface viewed under 1000X magnification. 95

Figure 6.5 The number of cells of the *in vitro* constructs at week-1, week-2 and week-3. 97

Figure 6.6 *In vitro* constructs stained using H&E viewed under 200X magnification. 100

Figure 6.7 *In vitro* constructs stained using alcian blue/fast red viewed under 200X magnification. 102

Figure 6.8 *In vitro* constructs stained using toluidine blue/fast red viewed under 200X magnification. 104

Figure 6.9 *In vitro* constructs stained using safranin O/fast green viewed under 200X magnification. 106

Figure 6.10 *In vitro* constructs stained using immunohistochemistry of collagen type-II viewed under 200X magnification. 108

Figure 6.11 *In vitro* constructs stained using immunohistochemistry of collagen type-I viewed under 200X magnification. 110

Figure 6.12 sGAG content of the *in vitro* constructs at week-1, week-2 and week-3. 112

Figure 6.13 *ACAN* expression in the *in vitro* constructs at week-1, week-2 and week-3. 113

Figure 6.14 *COL2A1* expression in the *in vitro* constructs at week-1, week-2 and week-3. 115

Figure 6.15 *SOX9* expression in the *in vitro* constructs at week-1, week-2 and week-3. 116

Figure 6.16 *COL1A2* expression in the *in vitro* constructs at week-1, week-2 and week-3. 118

Figure 6.17 *MMP13* expression in the *in vitro* constructs at week-1, week-2 and week-3. 119

Figure 6.18 *TERT* expression in the *in vitro* constructs at week-1, week-2 and week-3. 121

Figure 7.1 The methodology of 3D constructs implantation and analysis. 129

Figure 7.2 The gross images of the *in vivo* constructs at week-2 and week-4. 130

Figure 7.3 The SEM of the *in vivo* constructs at week-2 and week-4 viewed under 100X magnification. 131

Figure 7.4 The compression stress of the *in vivo* constructs at week-2 and week-4. 132

Figure 7.5 *In vivo* constructs stained using H&E viewed under 200X magnification. 133

Figure 7.6 *In vivo* constructs stained using alcian blue/fast red viewed under 200X magnification. 134

Figure 7.7 *In vivo* constructs stained using toluidine blue/fast red viewed under 200X magnification. 135

Figure 7.8 *In vivo* constructs stained using safranin O/fast red viewed under 200X magnification. 136

Figure 7.9 *In vivo* constructs stained using immunohistochemistry of collagen type-II viewed under 200X magnification. 137

Figure 7.10 *In vivo* constructs stained using immunohistochemistry of collagen type-I viewed under 200X magnification. 138

Figure 7.11 The sGAG content in the *in vivo* constructs at week-2 and week-4. 139

Figure 7.12 *ACAN* expression in the *in vivo* constructs at week-2 and week-4. 140

Figure 7.13 *COL2A1* expression in the *in vivo* constructs at week-2 and week-4. 141

Figure 7.14 *SOX9* expression in the *in vivo* constructs at week-2 and week-4. 142

Figure 7.15 *COL1A2* expression in the *in vivo* constructs at week-2 and week-4. 143

Figure 7.16 *MMP13* expression in the *in vivo* constructs at week-2 and week-4. 144

Figure 7.17 *TERT* expression in the *in vivo* constructs at week-2 and week-4. 145

LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AA	Antibiotic antimycotic
AAV	Adeno-associated virus
ACI	Autologous Chondrocyte Implantation
ADA	Adenosine deaminase
BMP	Bone morphogenetic protein
BSC	Biosafety cabinet
$CaCl_2$	Calcium chloride
CDC	Cartilage-derived cell
cDNA	Complementary deoxyribonucleic acid
CDMP	Cartilage-derived morphogenetic protein
CGTPs	Cellular and Gene Therapy Products
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EPC	Epiphyseal chondroprogenitor cell
F-12	Ham's F-12 nutrient mixture
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GCP	Good clinical practice
H&E	Haematoxylin and eosin
HA	Hydroxyapatite
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IACUC	Institutional animal care and use committee
IREC	Institutional research Ethic committee
IGF	Insulin-like growth factor
IHH	Indian hedgehog
IL	Interleukin
ITS	Insulin transferrin selenium
Lico A	Licochalcone
LB	Luria Bertani
MACI	Matrix-induced autologous chondrocyte implantation
MREC	Medical research and ethics committee
mRNA	Messenger ribonucleic acid
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
Na_2HPO_4	Disodium hydroxyphosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NMRR	National medical research register
OA	Osteoarthritis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic) acid
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
S.E.M	The standard error of the mean
SEM	Scanning electron microscopy
sGAG	Sulphated glycosaminoglycan
SOX9	SRY (sex determining region Y)-box 9
TAE	Tris-acetate EDTA
TERM	Tissue engineering and regenerative medicine
TERT	Telomerase reverse transcriptase
TKR	Total knee replacement

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Osteoarthritis (OA) is a global common health condition. Approximately 85% of the OA burden is contributed by knee OA, worldwide (Hunter & Bierma-Zeinstra, 2019). According to World Health Organization (WHO), it is estimated that 9.6% of men and 18% of women aged above 60 years suffered from OA. The 80% of the sufferers will experience difficulties during movement, another 25% of them are totally incapable to perform their daily routine activities. Besides physical disability, the disease also affects the sufferer's psychological state that may cause several mental health conditions. For instance, it has been reported that the mental conditions such as suicidal ideation and memory loss have been associated with OA (Vina & Kwoh, 2019). Besides that, OA is also known as a one of the factors that contribute to the development of cardiovascular disease (Wang, Bai, He, Hu, & Liu, 2016). The never-ending drawbacks of this disease can threaten the sufferer's life quality, entirely. These possible OA impact lead to the technological advancement in treatment modalities related to cartilage degenerative disease. According to disease severity, the current available treatment modalities such as prescribed medications, total knee replacement (TKR) (Sarda & Alshryda, 2017), autologous chondrocytes implantation (ACI) or matrix-induced autologous chondrocytes implantation (MACI) (Ebert et al., 2017) are being administered to relief the pain. For now, the treatments are able manage the symptoms, but unable to address the root cause of the disease.

The high economic burden of OA is another aspect that affects sufferer's life. Based on the previous reports, the total cost for OA treatment per patient was

estimated to be around RM50,000 (Salmon et al., 2016) or RM61,000 (Dibonaventura, Gupta, McDonald, & Sadosky, 2011) and the number is expected to increase each year.

Therefore, tissue engineering is perceived as a hope to provide an alternative treatment for cartilage-related disease. Tissue engineering field offers organ or tissue replacement to human by replacing the person's own regenerated tissue at the damaged site (known as the autologous implantation) (Langer & Vacanti, 1993). The concept of autologous is described as the use of biological substances taken from the same individual. The use of autologous tissue is able minimise immune responses as compared to the tissue taken from a different individual. This concept has been applied in the ACI and MACI procedures to restore a functioning tissue with minimal immunoreactivity effect. In the ACI procedure, cell culture technique is used to prepare a sufficient number of cells prior to the implantation (Davies & Kuiper, 2019; Ogura, Bryant, Merkely, & Minas, 2019), whereas MACI uses three-dimensional (3D) tissue implants made up of the autologous cells seeded in a scaffold (Erickson, Strickland, & Gomoll, 2018; Jones & Cash, 2019).

The use of tissue engineering principles has been recognised to improve the existing medical intervention. Despite that, the incorporation of other approach such as gene transfer with tissue engineering is being explored in search of a better treatment option. Gene transfer (gene therapy) approach is capable to facilitate the genetic materials delivery into the mammalian cells, plant cells and bacteria. This approach has been around for years since early 1960s and its first clinical trial was performed in 1990. Until now, a numerous research publication combining gene transfer with tissue engineering have been made, showing the incorporation of the approaches is reliable.

1.2 PROBLEM STATEMENT

According to the World Health Organization (WHO), organ transplantation is always the end-state organ failure treatment. Organ shortage is a known health-related issue because the available donated organ could not accommodate the increasing demand from the patients who need the organs. One of the examples of the organ that can be donated is articular cartilage.

The injured articular cartilage has the potential to progress into cartilage degenerative disease and OA if it is left untreated. Several other factors include obesity, ageing and overuse of the joints could also disrupt the cartilage morphology and its function. This painful event could eventually limit the sufferers' physical activity. The available treatment modalities could not completely cure the disease and only promises a temporary recovery effect. Other than that, the emotional state of a patient that receives continuous treatment may be affected by the expensive treatment cost.

Efforts are being made through cartilage tissue engineering application in finding an alternative, non-invasive and less expensive treatment modality. Despite that, the growth of tissue engineering research is also contributed by other technique including gene transfer. The incorporation of gene transfer with cartilage tissue engineering has been practised for years. The researchers have been tested several cartilage related genes such as *SOX9*, cartilage derived morphogenetic protein, (*CDMP*), and bone morphogenetic protein (*BMP*) to find the suitable signalling cues for cartilage repair. This study chooses *SOX9* and *TERT* genes to be transfected into chondrocytes as the genes are directly involving in cartilage formation and maintaining the cells lifespan, respectively. In the previous studies, the transfer of *SOX9* gene in the human osteoarthritic chondrocytes has been tested in the monolayer

culture (Sha'ban, Osman Cassim, Mohd Yahya, Saim, & Hj Idrus, 2011) and 3D culture (Mohamad Sukri et al., 2015). To the best of our knowledge, there has been no study used the combination of *SOX9* and *TERT* genes transfected in chondrocytes. Hence, it is hoped that this study could provide some information regarding cartilage regeneration.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objectives

The study aimed to evaluate the chondrogenic properties of the *SOX9* and/ or *TERT* genes transfected chondrocytes in monolayer culture, *in vitro* 3D culture and *in vivo* ectopic implantation.

1.3.2 Specific Objectives

The study aimed to achieve the following objectives:

1- To optimise the transfection efficiency of three transfection reagents.

2- To evaluate the chondrogenic properties of *SOX9* and/ or *TERT* transfected chondrocytes in monolayer culture.

3- To evaluate cartilaginous properties of the cell-scaffold construct formed using *SOX9* and/ or *TERT* transfected chondrocytes in 3D culture.

4- To evaluate cartilaginous properties of the cell-scaffold construct formed using *SOX9*/*TERT*-transfected chondrocytes implanted in an *in vivo* ectopic implantation model.

5- To review the safety and efficacy issues of gene transfer application in cartilage TERM from the Islamic perspective.