CELLULAR SENESCENCE OF THE LONG TERM CULTURE HUMAN AMNION MESENCHYMAL STEM CELLS (HAMCs)

FIONA MACNIESIA THOMAS

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INSTITUT PENYELIDIKAN BIOTEKNOLOGI

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2019

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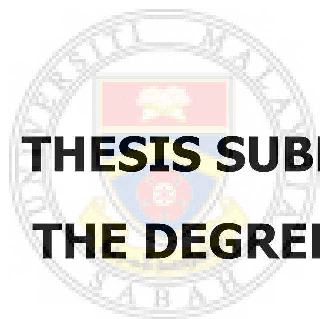

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BORANG PENGESAHAN TESIS

JUDUL: **CELLULAR SENESCENCE OF THE LONG TERM CULTURE HUMAN AMNION MESENCHYMAL STEM CELLS (HAMCs)**

IJAZAH: **SARJANA SAINS (BIOTEKNOLOGI)**

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Disahkan Oleh,

____
FIONA MACNIESIA THOMAS
MZ1421033T

Tarikh: 17 MEI 2019

____
(Tanda Tangan Pustakawan)

____
Prof. Madya Dr. Vijay Kumar A/L Subbiah
Penyelia

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries, references, which have been duly acknowledged.

27th March 2019

....
Fiona Macniesia Thomas
MZ1421033T

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CERTIFICATION

NAME : **FIONA MACNIESIA THOMAS**

MATRIC NO : **MZ1421033T**

TITLE : **CELLULAR SENESCENCE OF THE LONG TERM CULTURE OF HUMAN AMNION MESENCHYMAL STEM CELLS (HAMCS)**

DEGREE : **MASTER OF SCIENCE (BIOTECHNOLOGY)**

VIVA DATE : **6 MARCH 2019**

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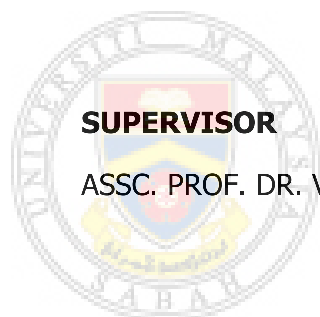

SUPERVISOR	**SIGNATURE**
ASSC. PROF. DR. VIJAY KUMAR A/L SUBBIAH	______________

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27th March 2019

ABSTRACT

A fundamental understanding of senescence in human amnion mesenchymal stem cells (HAMCs) is crucial for its application in cellular therapy. Cellular senescence is characterized by changes in cell morphology and the presence of senescence markers such as SA-β-Gal. Several genes such as p53, p21, pRB, p16 and GADD45 are commonly associated with the cellular senescence pathway. Also, telomerase activity which is important in the regulation of cell proliferation has been linked to cellular aging. At the same time, determining p53 sequence mutation is needed to assess the tumourigenicity risk in long-term cultured HAMCs. Thus, the aim of the study are: i) to determine the level of senescence in HAMCs at passage 5, 10 and 15 (P5, P10 and P15) through morphology changes of cells and the use of senescent-associated β-galactosidase (SA-β-Gal) assay; ii) to determine the expression of senescent-associated gene (p53, pRB, p21, p16 and GADD45) via reverse transcription polymerase chain reaction (RT-qPCR) at P5, P10 and P15; and iii)to determine the DNA damage level in HAMCs during long-term culture using comet assay, telomeric repeat amplification protocol (TRAP) and p53 mutation detection assay. The samples were obtained from amnion placentae of healthy mothers who underwent caesarean section at the Damai Specialist Hospital. After the isolation, HAMCs were cultured *in vitro* up to passage 15. They were assessed at passage 5, 10 and 15 and then analysed to correspond with the objectives of the study. The results show that HAMCs underwent morphological changes – from showing typical MSCs morphology at early passages to flattened and elongated shaped at late passages. The cells viability also decreased in percentage, i. e. 92.94±2.32% at P5, decreased to 87.15±1.48% at P10, and further decreased to 67.24±4.50% at P15. A larger number of cells were also tested positive for SA-β-Gal assay, with increasing percentage of senescent cells from P5 to P15 (P5: 0.03±0.01%, P10: 42.68±0.92%, P15: 82.61±1.40%). From the assessment of gene expression level at P5 to P15; it was found that p53 was up-regulated from 1 to 2.49 (0.27 to 1.3 fold); p21 was up-regulated from 1 to 5.45 (0.27 to 2.45 fold); pRB was up-regulated from 1 to 2.83 (0.38 to 1.39 fold); and p16 was up-regulated from 1 to 11.86 (0 to 0.35 fold). Meanwhile GADD45 was down-regulated from P5 to P15 (1 to 0.49 with 0.24 to 0.88 fold). p53/p21 and p53/pRB signalling pathway were activated in the senescence pathway of HAMCs. Genes expression level increased with increasing passage numbers. Comet assay showed that HAMCs at P15 have higher DNA damage compared to HAMCs at earlier passages (P5: 91±9.54 a.u., P10: 152.33±11.54 a.u., P15: 229±7.94 a.u.). Telomerase activity of HAMCs decreased between P5 to 15 (P5:103.75±37.89, P10: 64.67±34.96, P15: 35.03±13.98). DNA sequencing of p53 gene indicated that mutations had occurred after long-term culture with a higher presence of single nucleotide variants (SNVs) particularly in later passages. Assessment of senescence in HAMCs provided information that HAMCs at early passages have higher proliferative capacity and lower senescent cells. Thus, P5 and P10 are deemed as the most suitable for utilization in cellular therapy. Further study should be performed *in vivo* to investigate if long-term cultured HAMCs could cause malignant transformation.

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ABSTRAK

SELULAR KETUAAN KULTUR JANGKA PANJANG DALAM SEL STEM MESENKIMA AMNION MANUSIA (HAMCS)

Pemahaman asas mengenai ketuaan dalam sel stem mesenkima amnion manusia (HAMCs) adalah penting untuk aplikasinya dalam terapi selular. Selular ketuaan dicirikan oleh perubahan dalam morfologi sel dan kehadiran penanda ketuaan seperti SA-β-Gal. Beberapa gen seperti p53, p21, pRB, p16, dan GADD45 biasanya dikaitkan dengan laluan selular ketuaan. Aktiviti telomerase yang penting dalam pengawalseliaan perkembangan sel juga dikaitkan dengan penuaan selular. Pada masa yang sama, kajian mutasi gen p53 diperlukan untuk mengakses risiko tumorigenicity dalam HAMCs yang dikultur secara jangka panjang. Oleh itu, tujuan kajian ini adalah: i) untuk menentukan tahap ketuaan dalam HAMCs di laluan 5, 10 dan 15 (P5, P10 and P15) melalui perubahan morfologi sel dan penggunaan ujian SA-β-Gal; ii) untuk menentukan ekspresi gen (p53, p21, pRB, p16 dan GADD45) melalui RT-qPCR pada P5, P10 dan P15; iii) untuk menentukan tahap kerosakan DNA dalam kultur jangka panjang HAMCs melalui ujian komet, protocol amplifikasi pengulangan telomerik (TRAP) dan pengesanan mutasi gen p53. Sampel diperolehi daripada amnion plasenta di Damai Specialist Hospital. Selepas pengekstrakan, HAMCs kemudian dikultur secara in vitro untuk jangka masa panjang sehingga P15. HAMCs diakses pada laluan 5, 10, dan 15 dan kemudian dianalisis bersesuaian dengan objektif-objektif kajian. Keputusan menunjukkan bahawa HAMCs melalui perubahan morfologi – daripada morfologi biasa MSCs pada laluan awal kepada morfologi diratakan dan dipanjangkan laluan kemudian. Peratusan viabiliti sel juga menurun, i. e. 92.94±2.32% pada P5, menurun ke 87.15±1.48% pada P10, dan menurun lagi ke 67.24±4.50% pada P15. Sejumlah besar sel juga positif bagi ujian SA-β-Gal, dengan peratusan sel ketuan yang meningkat dari P5 ke P15 (P5: 0.03±0.01%, P10: 42.68±0.92%, P15: 82.61±1.40%). Daripada penilaian tahap ekspresi gen pada P5 sehingga p15: telah dijumpai bahawa ekspresi p53 telah meningkat dari 1 ke 2.49 (0.27 ke 1.3 kali lipat); ekspresi p21 telah meningkat dari 1 ke 5.45 (0.27 ke 2.45 kali lipat); ekspresi pRB telah meningkat dari1 ke 2.83 (0.38 ke 1.39 kali fold); dan ekspresi p16 telah meningkat dari 1 ke 11.86 (0 ke 0.35 kali lipat). Manakala ekspresi GADD45 menurun dari P5 ke P15 (1 ke 0.49 dengan penurunan 0.24 ke 0.88 kali lipat). Laluan isyarat p53/p21 dan p53/pRB telah diaktifkan dalam laluan ketuaan dalam HAMCs. Tahap ekspresi gen meningkat dengan peningkatan laluan. Ujian komet menujukkan bahawa HAMCs pada laluan kemudian mempunyai kerosakan DNA yang lebih tinggi berbanding laluan awal (P5: 91±9.54 a.u., P10: 152.33±11.54 a.u., P15: 229±7.94 a.u.). Aktiviti telomerase HAMCs juga menurun dari P5 ke 15 (P5:103.75±37.89, P10: 64.67±34.96, P15: 35.03±13.98). Penjujukan DNA gen p53 menunjukkan bahawa mutasi telah berlaku selepas kultur jangka panjang dengan kehadiran kepelbagaian nukleotida tunggal (SNVs) terutamanya dalam laluan kemudian. Penilaian terhadap ketuaan dalam HAMCs memberi maklumat bahawa HAMCs pada laluan awal mempunyai lebiih banyak sel proliferatif dan sedikit sel tua. Maka dipercayai bahawa P5 dan P10 adalah paling sesuai digunakan dalam terapi secular. Kajian selanjutnya perlu dilaksanakan secara in vivo untuk mengkaji sekiranya kultur jangka panjang HAMCs boleh mengakibatkan transformasi malignan.

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LIST OF ABBREVIATIONS

AM	-	Adrenomedullin
AMF	-	Amniotic Mesenchymal Fibroblasts
AM-hMSCs	-	Amniotic Membrane Human Mesenchymal Stromal Cells
AMTC	-	Human Amniotic Mesenchymal tissue cells
ATM	-	Ataxia-telangiectasia Mutated
BM-hMSCs	-	Bone marrow-derived Mesenchymal Stem Cells
BRG1	-	Brahma-related Gene 1
CDK	-	Cyclin Dependent Kinase
CDK-2	-	Cyclin Dependent Kinase-2
CDK-4	-	Cyclin Dependent Kinase-4
CDK-6	-	Cyclin Dependent Kinase-6
ColI	-	Collagenase Type I
DDR	-	DNA Damage Response
DMSO	-	Dimethyl sulfoxide
DSBs	-	Double Strand Breaks
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme-Linked Immunosorbent Assay
ERK	-	Extracellular-signal Regulated Kinase
ESCs	-	Embryonic Stem Cells
ETBR	-	Ethidium bromide
F12: DMEM	-	Ham's F12: Dulbecco's Modified Eagle Medium
FBS	-	Fetal Bovine Serum
FGF-4	-	Fibroblast Growth Factor-4
GOF	-	Gain-of-function
HAECs	-	Human amniotic Epithelial Cells
HAMCs	-	Human Amnion Mesenchymal Stem Cells
HAMSC	-	Human Amniotic Mesenchymal Stromal Cells
HGF	-	Hepatocyte Growth Factor
HOXA-9	-	Homeobox Protein Hox-9
HOXA-10	-	Homeobox Protein Hox-10

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HOXA-11	-	Homeobox Protein Hox-11
HSCs	-	Hematopoietic Stem Cells
hTERT	-	Human telomerase reverse transcriptase
IL-1	-	Interleukin-1
IL-10	-	Interleukin-10
iPSCs	-	Induced Pluripotent Stem Cells
IR	-	Ionizing Radiation
Lefty-A	-	Left-right Determination Factors
LMA	-	Low-melting agarose
MAPK	-	Mitogen Activated Protein Kinase
MSCs	-	Mesenchymal Stem Cells
NMA	-	Normal-melting agarose
OCT-4	-	Octamer-binding Transcription Factor
PCNA	-	Proliferating Cell Nuclear Antigen
PGE2	-	Prostaglandin E2
RB	-	Retinoblastoma
ROS	-	Reactive Oxygen Species
RTA	-	Relative Telomerase Activity
SA-β-gal	-	Senescent associated-β-Galactosidase
SCGE	-	Single Cell Gel Electrophoresis
SLE	-	Systemic Lupus Erythematosus
SWI/SNF	-	SWitch/Sucrose Non-Fermntable
SNPs	-	Single Nucleotide Polymorphisms
SOX-2	-	SRY-related HMG-box-2
SSBs	-	Single Strand Breaks
TDGF-1	-	Teratocarcinoma-derived Growth Factor-1
TIMPs-1	-	Tissue Inhibitors of Metalloproteinase-1
TIMPs-2	-	Tissue Inhibitors of Metalloproteinase-2
TIMPs-3	-	Tissue Inhibitors of Metalloproteinase-3
TIMPs-4	-	Tissue Inhibitors of Metalloproteinase-4
TRAP	-	Telomere Repeat Amplification Protocol
TRF	-	Terminal Restriction Fragment
VEGF	-	Vascular Endothelial Growth Factor

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Mesenchymal stem cells (MSCs) are well known for their high capacity to renew themselves and differentiate into any type of cells. Morphologically, MSCs are spindle shaped and have fibroblast-like features prior to differentiation into specialized cells. They are able to differentiate into three germ layers: endoderm, ectoderm and mesoderm; both *in vivo* and *in vitro*. MSCs are found abundantly in bone marrow, placenta, umbilical cord, umbilical cord blood. They also possess characteristics such as low immunogenicity and anti-inflammatory activity (Manochantr *et al.*, 2010).

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Recently, human amnion has been extensively studied as a potential source of stem cells. The human amnion mesenchymal stem cells (HAMCs) are located at the innermost extra-embryonic membrane, distributed in the collagenous stroma underlying the epithelial monolayer of the amniotic membrane. Other than yielding a rich amount of stem cells, HAMCs are found to exhibit MSCs-like characteristics, with ease of handling compared to other sources of stem cells such as embryos. More importantly, there is less ethical concern regarding the use of amnion derived stem cells since its source is usually discarded after delivery and are easily obtained through informed consents from the mother. As such, the usages of these cells are usually non-controversial.

HAMCs' unique characteristics increasingly attract researchers to utilise the full potential for these cells. Many MSCs-related publications show its contributions in cells self-renewal particularly, the medical field. Some applications of these cells

include their use in wound healing treatment, allografting, various surgeries, and even as scaffolds in tissue engineering research (Miki, 2011). Also, HAMCs capabilities are not only limited to differentiation or renewal properties, but extend beyond them to immunomodulatory property, anti-inflammatory property, and has no tumorigenicity. Studies exploring further potentials of these clogenic cells remain on-going, such as the construction of amnion-based cell sheets in skin tissue engineering (Toda *et al*., 2007), neurological disorders treatment in stem cells therapies (Castilo-Melendez *et al*., 2013), and stem cell-derived cardiomyocytes for drug development (Miki, 2011).

Isolation of HAMCs isolation is easily performed and is cost effective, since the fetal membrane is discarded after birth and is available as long as there is an approval from the Ethics Committee and written informed consent from the mothers. The isolated stem cells can be long-term cultured *in vitro* up to several passages (Lindenmair et al., 2012). While there are many protocols that have been published on how to long-term culture stem cells (Tsai *et al*., 2004; Fatimah *et al*., 2013), the success of these methods is measured by the evidence of differentiation of stem cells into different cell types such as hepatocytes, chondrocyte, cardiomyocyte and insulin-producing cells (Toda *et al*., 2007).

Even though HAMCs possess many unique characteristics, the Hayflick *et al*. (1961) theories suggest that stem cells are 'finite' and have limited lifespan when cultured *in vitro*. After long term expansion of stem cells, these cells are believed to reach a state, which is known as 'senescence'. This phenomenon is famously known as cellular replicative senescence, where cells enter irreversible growth arrest phase and can no longer proliferate. This occurrence leads to reduced potency of the stem cells. Some studies reported that senescence is caused by DNA damage (Van Nguyen *et al*., 2007), telomere shortening (Chen *et al*., 2013); and has been associated with organismal aging (Jeyapalan *et al*., 2008).

The pathway of cellular senescence has been associated with several genes such as p53, p21, pRB, p16 and also GADD45. p53 and pRB are both tumor suppressor genes that control cell proliferation in the cell cycle. In addition, they are major mediator of cellular senescence in MSCs. The p21 is a target gene of p53 which play role in p53/p21 senescence pathway. In addition, p16 is a CDK

binding protein that is commonly found in the senescence of MSCs, and is associated with the pRB/p16 senescence pathway. Activation of GADD45 through p53-dependent senescence pathway will cause senescence.

After cells enter the irreversible growth arrest phase, they are known as senescent cells. The cells morphologically change from spindle shaped into enlarged and flattened shape, which is believed to alter its protein expression. At this point, senescence limits the potential of the stem cells to differentiate. Long term culture of HAMCs may experience senescence as early as passage 5. This will limits the potential of HAMCs in regenerative medicine. Thus, there is need to identify the senescence level at early and later passages in HAMCs. In addition, the specific senescence pathway of HAMCs is not yet fully understood and the level of expression of senescent-associated genes greatly confluence the senescence in HAMCs. Furthermore, accumulation of DNA damage in stem cells may cause tumour and HAMCs may acquire DNA damage during senescence.

In the bigger context, cellular senescence is a potential cause of HAMCs limitation in therapeutic application. Thus the study of cellular senescence of HAMCs remains as major goal in the field of stem cells research. Many on-going studies are being done in order to fully understand the mechanism of the senescence in HAMCs. This is because researchers believe HAMCs may offer solution in cancer therapy, by using senescent stem cells to prevent cancer cells from proliferating and differentiating (Chen *et al.*, 2013).

To fully exploit the potential of stem cells, it is crucial to understand the mechanisms of cellular senescence in HAMCs. Therefore, this study focused on determining the cellular senescence of long-term culture in HAMCs. The level of senescence after long term culture of HAMCs was determined via morphological changes of cells and through the use of senescent-associated β-galactosidase assay. The expression of senescent-associated genes such as p53, pRB, p16, pRB, and GADD45 were also determined. These genes were chosen due to its involvement with senescence pathway (Lowe *et al.*, 2004; Rosemary and Richardson, 2009; Rufini *et al.*, 2013) and used to further explore the mechanisms when HAMCs enter senescence upon long term culture. In addition, the level of DNA damage in HAMCs after long-term culture was determined via comet assay,

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telomerase assay and p53 mutation detection assay. The study will provide indications of the impacts of senescence to long-term cultured HAMCs.

1.2 Research Questions

The study produced some research question:

1. Do HAMCS experience cellular senescence and if yes, then at what passage or stage does cellular senescence happen in HAMCs culture? What happened when HAMCs experience cellular senescence?
2. Do genes such as p53, p21, p16, pRB and GADD45 involved in the senescence pathway of HAMCs and what is their level of expression throughout long term culture of HAMCs?
3. Does senescence cause DNA damage and change the DNA structure of the HAMCs?

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1.3 Hypothesis

It is hypothesized that a drastic drop in the cells viability and proliferation as well as an increase in DNA damage occurs when human amnion mesenchymal stem cells (HAMCs) are subjected to long term *in vitro* cultures when passage number increases.