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RNA INTERFERENCE SILENCING OF INTERLEUKIN-6 IN MESENCHYMAL STROMAL CELLS AND ANTITUMOUR EFFICACY AGAINST MULTIPLE MYELOMA CELLS

TEOH HOON KOON

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By

TEOH HOON KOON

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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August 2015

Chair: Associate Professor Chong Pei Pei, PhD
Faculty: Medicine and Health Sciences

Studies demonstrated that mesenchymal stromal cells (MSC) from bone marrow (BM) stroma produced high concentrations of interleukin-6 (IL-6) that promoted multiple myeloma growth. In view of the failure of IL-6 monoclonal antibody therapy to demonstrate substantial clinical responses in early clinical trials, more effective methods are needed to disrupt the favourable microenvironment provided by the BM. In this study, RNA interference (RNAi)-mediated silencing of IL-6 in MSC and the efficacy of these modified MSC on U266 multiple myeloma cell growth inhibition *in vitro* and *in vivo* were evaluated. IL-6 silencing in MSC was induced using two different pathways: (1) direct administration of synthetic IL-6 siRNA using lipofectamine 2000 transfection and (2) vector-based adenovirus vector encoding IL-6 shRNA. Firstly, IL-6 protein in MSC was significantly suppressed to 36.7% and 39.4% post IL-6 siRNA transfection and IL-6 shRNA transduction respectively by 120 h compared to control MSC (100%) ($P<0.05$, T-test with $n = 3$ independent replicates). MSC remained viable and maintained their immunophenotypic profile and trilineage differentiation capacities similar to control MSC indicating no unanticipated phenotypic changes or cellular toxicity post IL-6 silencing. Secondly, *in vitro* and *in vivo* growth inhibition of U266 cells were shown in the presence of MSC transfected with IL-6 siRNA or transduced with IL-6 shRNA. *In vitro* results from three independent replicates showed that MSC transfected with IL-6 siRNA significantly inhibited U266 growth to 52.8% and 66.9% by day three through cell-substrate and cell-cell interactions respectively ($P<0.05$, ANOVA). MSC transduced with IL-6 shRNA also inhibited U266 growth significantly to 53.1% and 74.4% by day five through cell-substrate and cell-cell interactions respectively ($P<0.05$, ANOVA). Results from subsequent *in vivo* study showed significant reduction of U266 average tumour volume to 232.3 mm^3 and 331.7 mm^3 by day 21 in nude mice co-injected with MSC transfected with IL-6 siRNA and MSC transduced with IL-6 shRNA respectively compared to control MSC (1162.9 mm^3) ($P<0.05$, ANOVA with $n = 5$ mice/group). Further histological

analysis also showed increasing presence of lymphocytic infiltrates and significant decrease of mitotic index from 21 in tumours co-injected with control MSC to 15 in both U266 tumours co-injected with MSC transfected with IL-6 siRNA and MSC transduced with IL-6 shRNA indicating reduction of proliferating cells in the treated tumours ($P<0.05$, ANOVA with $n = 3$ slides/group). In conclusion, both RNAi pathways were equally effective in suppressing IL-6 expression in MSC and displayed *in vitro* and *in vivo* antitumor efficacy against U266 cells. These findings support the feasibility of using RNAi as an alternative approach for targeted suppression of IL-6 in MSC to inhibit multiple myeloma cell growth.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PELENYAPAN GEN INTERLEUKIN-6 DALAM SEL STEM MESENKIMA STROMA DAN EFIKASI ANTITUMOR TERHADAP SEL MIELOMA MULTIPEL

Oleh

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Kajian terdahulu menunjukkan sel stem mesenkima stroma (MSC) daripada stroma sumsum tulang menghasilkan banyak interleukin-6 (IL-6) untuk pertumbuhan sel mieloma multipel. Kaedah yang lebih efektif untuk mengatasi persekitaran mikro optimal yang disediakan sumsum tulang diperlukan akibat kekurangan keberkesanan terapi antibodi monoklonal IL-6 dalam menghasilkan respon klinikal yang signifikan. Dalam kajian ini, pelenyapan gen IL-6 MSC berasaskan interferensi RNA (RNAi) dan efikasi MSC yang dimodifikasi ini dalam perencatan pertumbuhan sel mieloma multipel disiasat. Pelenyapan gen IL-6 MSC dilakukan menerusi dua aliran: (1) pemindahan langsung siRNA sintetik IL-6 dengan transfeksi Lipofectamine 2000 dan (2) berasaskan vektor adenovirus yang mengkod shRNA IL-6. Pertama, protein IL-6 berkurangan kepada 36.7% dan 39.4% selepas transfeksi siRNA IL-6 dan transduksi shRNA IL-6 masing-masing selepas 120 jam ($P<0.05$, T-test dengan n = tiga replikat bebas). MSC masih viabel dan mengekalkan profil immunofenotipik serta kapasiti pembezaan tiga "lineage" sama seperti MSC kontrol menandakan tiada perubahan fenotip serta ketoksikan selular selepas pelenyapan gen IL-6. Kedua, perencatan pertumbuhan sel U266 *in vitro* dan *in vivo* ditunjukkan MSC selepas transfeksi siRNA IL-6 ataupun transduksi shRNA IL-6. Hasil kajian *in vitro* tiga replikat bebas menunjukkan MSC, selepas transfeksi siRNA IL-6, merencat pertumbuhan sel U266 dengan signifikan kepada 52.8% dan 66.9% pada hari ketiga menerusi interaksi sel-substrat dan sel-sel masing-masing ($P<0.05$, ANOVA). MSC selepas transduksi shRNA IL-6 turut merencat pertumbuhan U266 dengan signifikan kepada 53.1% dan 74.4% pada hari kelima menerusi interaksi sel-substrat dan sel-sel masing-masing ($P<0.05$, ANOVA). Hasil kajian *in vivo* seterusnya menunjukkan pengurangan signifikan purata isipadu tumor U266 ke 232.3 mm^3 dan 331.7 mm^3 pada hari ke-21 dalam tikus imun kompromi yang disuntik dengan MSC selepas transfeksi siRNA IL-6 dan transduksi shRNA IL-6 masing-masing berbanding MSC kontrol (1162.9 mm^3) ($P<0.05$, ANOVA dengan n = 5 tikus/kumpulan). Analisis histologi selanjut menunjukkan peningkatan infiltrasi limfositik dan pengurangan

signifikan indek mitosis daripada 21 dalam tumor U266 yang disuntik dengan MSC kontrol kepada 15 dalam kedua-dua tumor yang disuntik dengan MSC selepas transfeksi siRNA IL-6 ataupun transduksi shRNA IL-6 menandakan pengurangan sel berproliferasi dalam tumor tersebut ($P<0.05$, ANOVA dengan $n = 3$ slaid/kumpulan). Kesimpulannya, kedua-dua aliran RNAi adalah sama-sama efektif dalam pengurangan ekspresi IL-6 MSC dan mempamerkan efikasi antitumor *in vitro* dan *in vivo* yang setara ke atas sel mieloma multipel. Hasil kajian setakat ini menyokong kebolehlaksanaan penggunaan RNAi sebagai alternatif untuk pengurangan terarah IL-6 dalam MSC untuk perencatan pertumbuhan sel mieloma multipel.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
ALL	Acute lymphoblastic leukaemia
ALS	Amyotrophic lateral sclerosis
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
ApoB	Apolipoprotein B
APRIL	Proliferation-inducing ligand
ASCT	Autologous stem cell transplantation
BP	Base pair
CAM	Cell adhesion mediated
CCD	Charge-coupled device
CCND	Cyclin D
cDNA	Complementary deoxyribonucleic acid
CML	Chronic myeloid leukaemia
c-Myc	v-myc avian myelocytomatosis viral oncogene homolog
CPE	Cytopathic effect
CO_2	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate buffered saline
dsRNA	Double-stranded ribonucleic acid
ECM	Extracellular matrix proteins

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
FBS	Fetal bovine serum
FGFR-3	Fibroblast growth receptor 3
FITC	Fluorescein isothiocyanate
GMP	Good manufacturing practice
HBV	Hepatitis B virus
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia inducible factor 1 alpha
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSC	Haematopoietic stem cells
HTT	Huntingtin
IFN	Interferons
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-15	Interleukin-15
IL-17	Interleukin-17
IL-21	Interleukin-21
IL-6R	Interleukin-6 receptor

JAK/STAT	Janus kinase/signal transducer and activator of transcription 3
JNK	c-Jun-NH_2-kinase
KB	Kilobase pair
K-Ras	Kirsten-rat sarcoma viral oncogene homolog
LDL	Low-density lipoprotein
mAb	Monoclonal antibody
MAPK	Ras/mitogen-activated protein kinase
MDR1	Multidrug resistance 1
MDS	Myelodysplastic syndrome
MGUS	Monoclonal gammopathy of undetermined significance
miRNA	Micro ribonucleic acid
Mitf	Microphthalmia-associated transcription factor
MLL-AF4	Mixed-lineage leukaemia AF4
MOI	Multiplicity of infection
MPN	Myeloproliferative neoplasms
M-protein	Monoclonal protein
mRNA	Messenger ribonucleic acid
MRP1	Multidrug resistance-associated protein-1
MSC	Mesenchymal stromal cells
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
N-Ras	Neuroblastoma-rat sarcoma viral oncogene homolog
ORR	Overall response rate

p18INK4c	Cyclin-dependent kinase 4 inhibitor 2C
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCLS	Poly-Ɛ-caprolactone polymeric scaffolds
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PD-1	Programmed death 1
PDGF	Platelet-derived growth factor
PD-L1	Programmed death ligand 1
PE	R-phycoerythrin
PFU	Plaque-forming unit
P-gp	P-glycoprotein 1
PI3K/AKT	Phosphatidylinositol3-kinase/protein kinase B
pre-miRNA	Precursor micro ribonucleic acid
pri-miRNA	Primary micro ribonucleic acid
RANKL	Receptor activator of nuclear factor kappa-B ligand
RB1	Retinoblastoma 1
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Medium
RRM2	Ribonucleoside-diphosphate reductase subunit M2
RUNX-2	Runt-related transcription factor 2
SCID	Severe combined immunodeficiency
SD	Standard deviation

SDF-1	Stromal-cell derived factor 1
SEM	Standard error of mean
siRNA	Small interfering ribonucleic acid
shRNA	Short hairpin ribonucleic acid
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase
T/C	Tumour growth inhibition ratio
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Overview

Multiple myeloma is a neoplastic disorder of plasma cells, which accounts for more than 10% of all haematological cancers (Howlader *et al.* 2011) . The development of multiple myeloma is a multistep process involving the accumulation of mutations leading to the deregulation of genes controlling cell cycle, apoptosis and the tumour microenvironment interactions. While advances have been made in the treatment and management of multiple myeloma, it remains an incurable disease with an estimated 5-year survival rate of 46.3% (Howlader *et al.* 2011).

Studies have shown that the bone marrow microenvironment plays a crucial role in the pathogenesis of multiple myeloma. The bone marrow microenvironment is composed of extracellular matrix proteins and also a heterogenous population of haemopoietic and non-haemopoietic cells such as mesenchymal stromal cells (MSC), immune cells and cells involved in osteogenesis. In the case of MSC, *in vitro* studies showed that adhesion of multiple myeloma cells induced the transcription and secretion of cytokines that mediated multiple myeloma cells proliferation and migration (Tosi *et al.* 2006). The cytokines secreted include interleukin-6 (IL-6), insulin-like growth factor 1 (IGF-1) and tumour necrosis factor alpha (TNF-α). IL-6, particularly, is a major growth and survival factor for multiple myeloma cells due to its role in activating specific signal transduction pathways, most notably the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT) pathway, to induce proliferation and inhibit apoptosis (Ogata *et al.* 1997; Anderson *et al.* 1989; Kawano *et al.* 1988).

MSC in the bone marrow microenvironment promote the growth of multiple myeloma cells mainly by paracrine IL-6 production (Corre *et al.* 2007; Sahara *et al.* 2006) in addition to a positive feedback loop between MSC and myeloma cells that further enhanced stromal production of IL-6 (Roodman 2004; Dankbar *et al.* 2000). In the 1990s, monoclonal antibody (mAb) targeted therapy against IL-6 began using BE-8 murine-derived IL-6 mAb antibody. Findings from phase I studies showed lowered IL-6 level and disease stabilisation but no significant clinical improvements or patient remission (Moreau *et al.* 2000; Bataille *et al.* 1995; Klein *et al.* 1991). A new humanised IL-6 monoclonal antibody, CNTO 328 is currently undergoing clinical trials. Results from two trials on end-stage, progressive multiple myeloma reported disease stabilisation but again no clinically significant responses (van Zaanen *et al.* 1998; van Zaanen *et al.* 1996). However, combination studies on relapsed and refractory multiple myeloma showed early promising results with bortezomib (Voorhees *et al.* 2007), dexamethasone (Voorhees *et al.* 2009) and melphalan (Hunsucker *et al.* 2011).

Targeted IL-6 mAb therapy is hampered by the lack of clinically significant improvements or complete remission for patients with multiple myeloma. New therapeutic approaches are needed to target IL-6 production especially by MSC in order to disrupt the favourable microenvironment that allows the multiple myeloma cells to thrive.

RNA interference (RNAi), which uses double-stranded ribonucleic acid (dsRNA) to selectively silence messenger ribonucleic acid (mRNA) expression, provides a new approach to target overexpression of IL-6 in the development of new multiple myeloma therapy. RNAi can be triggered in mammalian cells by direct administration of small interfering ribonucleic acid (siRNA) or by using vector-based short hairpin ribonucleic acid (shRNA). Chemically synthesised siRNAs are short, non-coding RNAs introduced intracellularly to silence specific genes. As for the vector-based approach, shRNAs are transcribed from external expression vector, exit the nucleus and are cleaved by Dicer to siRNA before induction of RNAi (Yu *et al.* 2002).

RNAi is widely used now as a powerful genetic tool to study mammalian gene function replacing the laborious and costly gene knockout techniques. RNAi is utilised to silence disease-causing genes in the development of targeted therapeutic approaches for human diseases such as genetic disorders (DiFiglia *et al.* 2007; Ding *et al.* 2003), viral diseases (McCaffrey *et al.* 2003; Song *et al.* 2003) and metabolic disorders (Czech *et al.* 2011; Frank-Kamenetsky *et al.* 2008). In addition, RNAi is also used to silence anti-apoptotic, cell cycle and angiogenic genes in different cancer models including liver and prostate (Yano *et al.* 2004), cervical (Yuan *et al.* 2006) and Ewing's sarcoma (Guan *et al.* 2005). RNAi-based clinical trials have also been carried out on solid tumours such as melanoma (Davis *et al.* 2010) and pancreatic adenocarcinoma (Zorde Khvalevsky *et al.* 2013).

Therefore, the hypothesis of this study is RNAi can be utilised for targeted IL-6 silencing in MSC to inhibit growth of multiple myeloma cells. In this study, RNAi-mediated IL-6 silencing was induced in human bone marrow-derived MSC using two different pathways: (1) direct administration of synthetic IL-6 siRNA into MSC using lipofectamine transfection and (2) vector-based adenovirus encoding IL-6 shRNA (pAd/BLOCK-iT/IL-6). The efficacy of these MSC, post RNAi-mediated IL-6 silencing, on inhibition of U266 multiple myeloma cell growth *in vitro* and *in vivo* were subsequently evaluated.

1.2 Aims and Objectives

The main aim of this study is to investigate the efficacy of RNAi-mediated silencing of IL-6 in human bone marrow-derived MSC on the growth of U266 multiple myeloma cells *in vitro* and *in vivo*. The specific objectives of this study are as follows:

1. To isolate, culture and characterise MSC from bone marrow aspirate of apparently healthy individuals.

2. To induce IL-6 silencing in MSC using synthetic IL-6 siRNA and vector-based recombinant adenovirus encoding IL-6 shRNA.

3. To investigate the viability, immunophenotypic profile and trilineage differentiation capacity of the above MSC post RNAi-mediated IL-6 silencing.

4. To investigate inhibition of U266 cell growth after co-culture with MSC post RNAi-mediated IL-6 silencing MSC *in vitro*.

5. To investigate antitumour efficacy of MSC post RNAi-mediated IL-6 silencing in a murine subcutaneous model of human multiple myeloma.

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