GENERATION AND CHARACTERISATION OF MESENCHYMAL STEM CELL (MSC)-LIKE CELLS FROM INDUCED PLURIPOTENT STEM CELLS (IPSCs) ALONG WITH THEIR CO-CULTURE EFFECTS ON LUNG CANCER CELL LINE, H1975

By

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A dissertation submitted to the Department of Pre-clinical Sciences,

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ABSTRACT

GENERATION AND CHARACTERISATION OF MESENCHYMAL STEM CELL (MSC)-LIKE CELLS FROM INDUCED PLURIPOTENT STEM CELLS (IPSCs) ALONG WITH THEIR CO-CULTURE EFFECTS ON LUNG CANCER CELL LINE, H1975

LOH JIT KAI

Mesenchymal stem/stroma cells (MSCs) application in therapeutic treatment has increased attention, especially in regeneration, anti-inflammation, tumour suppression, and drug delivery treatments. However, the MSC interaction studies with tumours have shown both tumour-promoting and suppressing effects. One setback of MSC is the need for more homogenous supplies of cells for consistent treatment results and the limitation in obtaining large amounts of MSC cells from donors in a single extraction or culture expansion. To overcome this issue, induced pluripotent stem cells (iPSCs) technology has been used to derived MSCs (iMSC) that may provide alternative MSC sources for therapeutic treatment. In this study, iMSC was differentiated from peripheral blood mononuclear cell (PBMC)-derived iPSC. The generated 9iMSC was further characterised according to the International Society for Cell & Gene Therapy (ISCT) MSC 2005 guideline. Upon confirming its MSC-like properties and trilineage differentiation status, the iMSC was used to evaluate its inhibitory effects on H1975 lung cancer cells using an indirect co-culture approach involving either iMSC conditioned medium (iMSC-CM) treatment or iMSC transwell coculture (iMSC-TC) treatment. The effects of iMSC-CM on lung

cancer cells demonstrated a significant increase in EMT-associated gene expression as compared to that of the parental cancer cells whereas no difference in EMT gene expression was observed between iMSC-TC treated cancer cells and that of the parental cancer cells. The migration assays also showed a high increase in migrated cells from H1975 cancer cells treated with iMSC-CM while H1975 cancer cells treated with iMSC-TC were similar to that of the control. Cytokine and chemokine assays revealed a number of releasing factors (CXCL1, CXCL12, GM-CSF, CCL2 and SDF-1) which were altered in the transwell coculture media when compared to the original culture media of iMSC and cancer cells alone. These observations indicated the effect of stroma microenvironment and cell-to-cell interaction by which the presence of both iMSC and H1975 cancer cells may influence the paracrine secretion profile of iMSC in its interaction with H1975 lung cancer cells resulting in suppression of its metastatic potential effects.

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APPROVAL SHEET

This dissertation entitled "**GENERATION AND CHARACTERISATION OF MESENCHYMAL STEM CELL (MSC)-LIKE CELLS FROM INDUCED PLURIPOTENT STEM CELLS (IPSCs) ALONG WITH THEIR CO-CULTURE EFFECTS ON LUNG CANCER CELL LINE, H1975**" was prepared by LOH JIT KAI and submitted as partial fulfilment of the requirements for the degree of Master of Medical Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF DISSERTATION

It is hereby certified **LOH JIT KAI** (ID No: **20UMM06206**) has completed this dissertation entitled **"GENERATION AND CHARACTERISATION OF MESENCHYMAL STEM CELL (MSC)-LIKE CELLS FROM INDUCED PLURIPOTENT STEM CELLS (IPSCs) ALONG WITH THEIR CO-CULTURE EFFECTS ON LUNG CANCER CELL LINE, H1975"** under the supervision of Professor Dr. Alan Ong Han Kiat (Supervisor) from the Department of Pre-clinical Sciences, M. Kandiah Faculty of Medicine and Health Sciences, Academician Emeritus Professor Dr. Cheong Soon Keng (Co-Supervisor) from the Department of Pre-clinical Sciences, M. Kandiah Faculty of Medicine and Health Sciences, and also Professor Dr. Chiou Shih Hwa from the Institute of Pharmacology, College of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC.

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Yours truly,

(LOH JIT KAI)

DECLARATION

I, LOH JIT KAI hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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Date: ___03 OCTOER 2023

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LIST OF ABBREVIATIONS

°C	Degree Celsius
2D	Two dimensional
3D	Three dimensional
ALK	Anaplastic lymphoma kinase
AP	Alkaline phosphatase
AT	Adipose tissue
AT-MSC	Adipose tissue derived mesenchymal stem cell
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BM-MSC	Bone marrow derived mesenchymal stem cell
BRAF	V-raf murine sarcoma viral oncogene homolog B1
c-Myc	Avian myelocytomatosis viral oncogene
CCL2	Chemokine (C-C motif) ligand 2
CD	Custer of differentiation
cDNA	Complimentary deoxyribonucleic acid
CM	Conditioned medium
CXCL1	CXC chemokine ligand 1
CXCL12	CXC chemokine ligand 12
CXCL16	CXC chemokine ligand 16
CXCL8	CXC chemokine ligand 8
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EPCAM	Epithelial cellular adhesion molecule
ESC	Embryonic stem cell
EV	Extracellular vesicle
FA	Fanconi anaemia
FA-iMSCs	Fanconi anaemia derived induced pluripotent stem cell derived mesenchymal stem cells
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMPs	Good manufacturing procedures
GROa	Growth-regulated alpha
hBM-MSCs	Human bone marrow derived mesenchymal stem cells
HER2	Human epidermal growth factor receptor 2
hESC	Human embryonic stem cell
HGF	Hepatocyte growth factor
HGPS	Hutchinson-Gilford progeria syndrome
HGPS-iMSCs	Hutchinson-Gilford progeria syndrome derived induced pluripotent stem cell derived mesenchymal stem cells

hiPSC	Human induced pluripotent stem cell
HLA-DR	Human leukocyte antigen – DR
hPL	Human platelet lysate
hPSC	Human pluripotent stem cell
IF	Immunofluorescence
IgG1	Immunoglobulin G type 1
IL-6	Interleukin 6
IL-8	Interleukin 8
iMSC	Induced pluripotent stem cell-derived mesenchymal stem/ stroma cell
iMSC-CM	Induced pluripotent stem cell-derived mesenchymal stem/stroma cell conditioned medium
iMSC-TC	Induced pluripotent stem cell-derived mesenchymal stem/stroma cell transwell coculture
iPSC	Induced pluripotent stem cell
ISCT	International society for cell & gene therapy
Klf4	Kruppel-like factor 4
KRAS	Kirsten rat sarcoma viral oncogene homolog
LCA	Leber congenital amaurosis
M	Molar
MCAF	Monocyte chemotactic and activating factor
MCP-1	Monocyte chemoattractant protein 1
MEK	Mitogen-activated protein kinase
MEM	Modified eagle medium
MET	Mesenchymal-epithelial transition

MIF	Macrophage migration inhibitory factor
miRNA	MicroRNA
MMPs	Matrix metalloproteinases
MSC	Mesenchymal stem/ stroma cell
NANOG	Homeobox protein NANOG
NSCLC	Non-small cell lung cancer
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1
OCT4	Octamer-binding protein 4
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGF-AB	Platelet-derived growth factor alpha polypeptide b
PET	Polyethylene terephthalate
PIK3CA	P110 alpha (p110α) protein
PNSHL	Progressive non-syndromic sensorineural hearing loss
PSC	Pluripotent stem cell
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROC	Republic of China
ROS1	C-ros oncogene 1
RPM	Rotation per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SCLC	Small cell lung cancer
SD	Standard deviation

SDF-1	Stromal cell-derived factor 1
SERPIN E1	Serine protease inhibitor (serpin) protein
SOX2	Sex determining region Y – box 2
SRPSOX	Scavenger receptor for phosphatidylserine and oxidized low density lipoprotein
SSEA4	Stage-specific embryonic antigen-4
STBR	Stirred tank bioreactor
T790M	Thr790Met
TC	Transwell Coculture
TCP	Tissue culture plastic
TGF-β	Transforming growth factor beta
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TRA-1-60	T cell receptor alpha locus
TRA-1-81	Tumour-related antigen-1-81
UC	Umbilical cord
UC-MSC	Umbilical cord derived mesenchymal stem cell
UTAR	Universiti Tunku Abdul Rahman
VEGF	Vascular endothelial growth factor
WT	Wild type

CHAPTER 1

INTRODUCTION

A remarkable reputation has developed around mesenchymal stem/stroma cells (MSCs) because of their ability to regenerate stem cells, anti-inflammatory properties, suppress tumours, deliver drugs, and combination of MSCs with other therapies that are reported to enhance the treatment overall efficacy and success rate. (Valkenburg, et al., 2018; Hmadcha, et al., 2020). Previous studies have reported that MSCs have shown both promoting and suppressing effects against cancer cells or can affect cancer cell resistance and sensitivity against chemotherapy drugs (Shih and Yang, 2011; Liu, et al., 2017; Lee and Hong, 2017). To date, there are a total of 367 completed MSCs clinical trials with 27 at Phase 3 and Phase 4 towards various diseases, while 196 more studies are still recruiting in ClinicalTrials.gov using the keyword "mesenchymal stem cell" (Accessed on 1st March 2023). Currently, over 300 registered MSC-related clinical trials show promising results (Zhou, et al., 2021). In some clinical settings, MSCs demonstrated promising therapeutic benefits and exhibited tolerable safety profiles, leading to regulatory approval in a few jurisdictions (Zhou, et al., 2021). This information and findings illustrate the potential benefits of stem cell therapy using MSCs. However, the use of MSCs as therapeutic sources has been limited by the donor-to-donor immune-rejection effects, donor's age, gender and site of origin which have shown different efficiency in treatment and cell-to-cell interaction due to the differences in the releasing paracrine factors from various source of MSCs (Lin, et al., 2019).To

overcome this limitation, the need for a homogeneous cell source and a constant supply of quality MSCs are crucial (Goodarzi, et al., 2019). This conventional method of using human-extracted MSC has led to different batches and quality of adult MSC for effective treatment as the use of different sources of MSCs on a single disease or treatment often leads to inconsistent treatment outcomes. The shortcoming in quality control and the inability to maintain consistent characteristics of MSCs like immune-compatibility, genetic stability, cell heterogeneity, differentiation and migration capacity remains challenging in clinical trials and for feasible therapeutic applications (Haga, et al., 2015). However, an alternative approach to tackle the issue involves the differentiation of induced pluripotent stem cells (iPSCs) into MSC-like cells or induced MSC (iMSC) may provide a promising prospect due to its ability to propagate at a large scale and undergo prolonged passage, provide a more homogeneous and genetically stable cell population as well as demonstrate similar biological characteristics and therapeutic profiles identical as that of their MSC counterparts (Takahashi et al., 2007; Hass, et al., 2011; Goodarzi, et al., 2019).

There is, however, a lack of clarity and controversy about the mechanisms that allow MSCs to interact with cancer cells. It is important to recognise that epithelial-mesenchymal transition (EMT) is reactivated during cell damage and regeneration, as well as during cancer progression. While multiple pathways that lead to EMT can be activated, cancer cells use it to promote cancer metastasis and progression, resulting in poor clinical outcomes (Wang, et al., 2017). According to studies and research, patients with non-small cell lung cancer (NSCLC) have poor overall and metastasis-free survival due to the

upregulation of EMT transcription factors, while current treatment is not effective (Schliekelman, et al., 2011; Wang, et al., 2017).

Previous studies have shown that various sources of MSC and treatment methods resulted in different effects on NSCLC cells and other types of cancer cells despite the use of the exact origin of MSC. Furthermore, the condition of direct and indirect cell-to-cell contact, or in vitro and in vivo interaction of MSC and tumour cells have also shown the involvement of various cell-to-cell interactions and activation of different pathways or mechanisms. As such, mimicking a stromal environment in which signalling factors such as multiple growth factors, metabolites, and cytokines, are released without direct contact between the host and the subject via co-culture studies may provide a clearer picture of the interaction between MSC and cancer cells (Wang, et al., 2010; Kim and Kim, 2019).

In short, the use of MSC has shown promising effects mainly due to its homing ability to the tumour site and paracrine factors that play a role in cancer treatment that suppresses or inhibits cancer properties such as slowing down the proliferation or preventing metastasis. However, the use of MSC for treatment and research still experiences cut short due to the cost of extracting it from donors, the amount of MSC that can only be removed from single donors is insufficient for the treatment of large groups as well the heterogeneous conditions of such sources. Therefore, it is crucial to find alternative sources of MSC that can be produced or obtained in large quantities with consistent quality at a lower cost without the constant need to find donors. By taking advantage of

iPSC technology, the development of iMSC is made possible. The biological properties of iMSC and native MSC have been compared by previous studies that resulted in nearly similar properties or more significant benefits in terms of cell regeneration, more tumour-tropic and less tumour promoting that can be observed in iMSC (Zhao, et al., 2014; Soontararak, et al., 2018). The high pluripotency and proliferation rate of iPSC has allowed a high number of cells to be differentiated into iMSC at a time, producing high batches of homogenous cells for treatment usage. Though numerous studies are showing the effects of MSCs on cancer cells as either inhibiting or promoting cancer cell growth and invasiveness, the interaction of iMSC with cancer cells is limited let alone with lung cancer in particular,

The purpose of this study is to differentiate PBMC-iPSCs into MSC-like cells as a potential replacement for adult MSCs in H1975 non-small-cell lung cancer through two different indirect methods of treatment that will provide an overview of the differences in MSC-like cells' effects on lung cancer cell in the tumour microenvironment (iMSC-transwell coculture treatment) and the supportive stromal environment (iMSC-conditioned medium treatment) where two conditions of non-cell-to-cell interaction and present of cell-to-cell interaction can be observed. The observation from the study has shown a promising direction of using iMSC-transwell coculture treatment (iMSC-TC), where the subjected cancer cell of H1975 was shown to be suppressed from entering the EMT stage and undergo dormancy. Furthermore, the cell-to-cell interaction between H1975 and iMSC resulted in crosstalk that altered the paracrine factors released by iMSC, eventually leading to the cease expression

of cancer promoting cytokines and chemokines while a new factor emerged, the SDF-1, that is known to promote migratory properties of MSC towards cancer cell (Bouillez, et al., 2017; Kalimuthu, et al., 2017; Jin, et al.,2018; Salamon, et al., 2020; Lan, et al.,2021). These observations showed the opposite as in iMSC conditioned medium treatment (iMSC-TC), where the tumour properties are rather upregulated.

Objective 1: To differentiate PBMC-iPSC into iMSC using spontaneous different methods using an MSC differentiation medium.

Objective 2: To characterise iMSCs generated using morphological evaluation, MSC-specific lineage markers and trilineage differentiation.

Objective 3: To evaluate the paracrine effects of iMSC on H1975 lung cancer properties using conditioned medium and co-culture treatment.

CHAPTER 2

LITERATURE REVIEW

2.1 Pluripotent Stem Cell (PSC)

Pluripotent stem cells (PSCs) possess infinite proliferation ability and are capable of differentiating into cells of all three germ layers (endoderm, ectoderm, and mesoderm) (Yamanaka, 2020). These unique properties are the basis for cell therapy applications in regeneration as well as diseases induced and tissue or organ injuries.

There are two types of human PSCs (hPSCs) that are being researched for clinical purposes: human embryonic stem cells (hECSs) and human induced pluripotent stem cells (hiPSCs). The first ever hESCs were reported by James Thomson's group in 1998 (Thomson et al., 1998) while the first iPSCs were generated by Shinya Yamanaka's group in 2007 (Takahashi et al., 2007; Yu et al., 2007). However, there are concerns with the usage of hESCs for clinical purposes, namely the ethical issues in using human embryos as a stem cell source and the potential immune rejection upon transplantation. The act of extracting hESCs or culturing hESCs from human embryos has been deemed to be an unethical action as the human embryos are considered to be a new living

organism. At the same time, the possible risk of immune rejection in its application has brought about questions of safety concerns. Multiple research teams have tried to overcome the above issues through the nuclear transfer of a patient's somatic cells to generate hESCs with a successful case by Tachibana's group in 2013 (Tachibana et al., 2013). However, the method of human nuclear transfer for hESCs generation remains technically challenging up to now, while various other methods are being explored that may be more sustainable in the longer term with a lower cost of production, higher efficiency of its efficacious effects and ethically sound practices.

2.2 Generation of iPSC

Induced pluripotent stem cells (iPSCs) possess similar biological characteristics as ESCs, including pluripotency and stemness. The generation of iPSC has offered an alternative for ECSs and created a new possibility in clinical research and a better understanding of disease modelling, drug discovery, toxicity testing, autologous cell-based therapeutic applications, and cancer study (Pietronave and Prat, 2012; Young, et al., 2012; Ferreira, et al., 2013; Singh, et al., 2015). Having the similar properties of ECSs of indefinite expansion capability, the ability to generate into three germ layers and differentiation capability to almost all known cell types or organ-specific cells such as tissue and 3D organoids, iPSCs have provided a whole new platform for clinical research while overcome immunological and ethical concerns and technical issues to generate safe human stem cells for research. While the

benefits of being capable of obtaining a homogeneous line and low cost have been favoured by many research labs and industries.

From a technical perspective, the generation of iPSCs can be conducted through adult somatic stem cell genetic reprogramming. The technique of reprogramming involves the use of forced expression of pluripotency transcription factors through integrated or non-integrative systems, via viral or non-viral delivery vector systems to induce somatic cells into iPSCs. The integrated systems are composed of a few types of viral integrated vectors such as retrovirus and lentivirus; while non-viral integrated vectors comprise plasmid DNA, linear DNA, or transposons such as piggybac. The initial reprogramming effort began with 24 pre-selected genes commonly expressed in ESCs and were meticulously combined into different sets of transcription cocktails by Yamanaka's group (Yamanaka, 2020). The cocktail combination eventually narrowed down to the famous OSKM factors known today as Oct4, Sox4, Klf4 and c-Myc which were sufficient for a complete reprogramming to take place. The OSKM factors have been intensively used in iPSCs reprogramming research and opened more options for cell sources for reprogramming and differentiation into specific tissues or organoids for disease model studies.

2.2.1 Type of iPSC Technology

To achieve successful reprogramming, several factors must be considered, including the type of cell, the method of reprogramming, and the delivery efficiency of the pluripotency vector into the host cell (Rao and Malik, 2012).

Reprogramming techniques for iPSCs generation have constantly evolved in the past decade due to technological advancement. Multiple integrative and non-integrative approaches are reported for generating iPSCs from different tissue-derived somatic cells (Hu, 2014; Saha, et al., 2018). Reprogramming via viral integration remains the most efficient reprogramming method. However, for clinical applications, integration-free iPSCs are needed (Li et al., 2014). It was previously demonstrated that non-integrating lentivirus-based expression of microRNAs (miRNAs) could be used to enhance the production of iPSCs in the absence of the Yamanaka factors. The study reported the use of a miR302/367 cluster targeting Oct4 and Sox2 which was able to achieve a reprogramming efficiency of up to 11.6% (Onder and Daley, 2011). Currently, much research is still trying to strife for the most effective reprogramming and cost-efficient methods to produce iPSCs.

2.3 Integrating Vector and Non-Integrating Vector

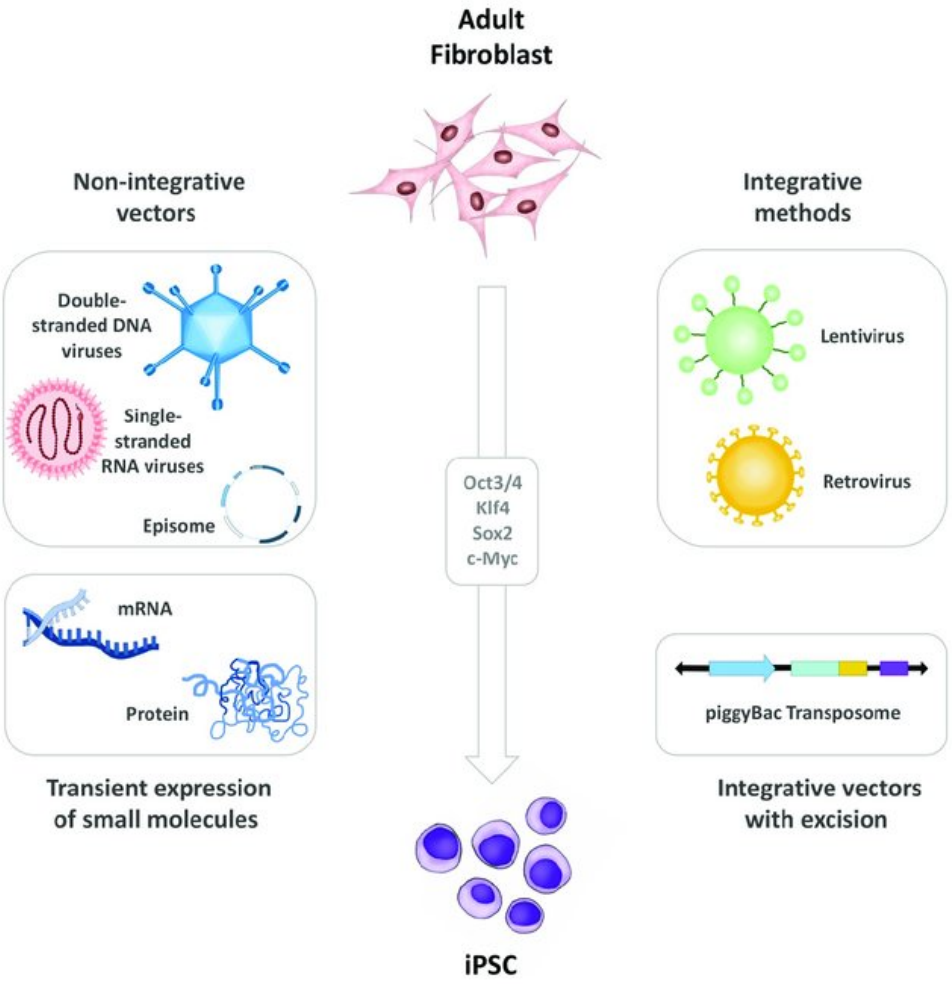


Figure 2.1 Methods of iPSC reprogramming from somatic cells. A preview of using non-integrative vectors and integrative vectors that utilise different components for reprogramming an adult fibroblast into iPSC. (Martínez Falguera, et al., 2021)

The conventional integrative methods using retrovirus and lentivirus vectors are extremely efficient and rapid but the integration of the virus vectors into the host genome will result in permanent genomic modifications. These techniques are associated with the risk of insertional mutagenesis and tumourigenesis

(Okita and Yamanaka, 2007; Ben and Benvenisty, 2011). Transgene silencing and activation are unpredictable, affecting iPSC differentiation potential and raising concerns about the use for clinical purposes (Sommer, et al., 2012; Kadari, et al., 2014). Hence, non-integrative reprogramming approaches are developed with little or no genetic modification. Using DNA-free reprogramming techniques such as the Sendai virus, recombinant proteins, microRNAs, and synthetic messenger RNA has allowed the generation of integration-free iPSCs suitable for clinical and biomedical applications.

Human iPSCs with the absence of genome-integrating DNA elements have been produced using a variety of reprogramming methods. There are several methods of delivering genetic information to cells, including the use of episomal vectors, adenoviral vectors, Sendai viral vectors, plasmids, synthetic mRNA, miRNA, protein transduction, and small molecules (Okita, et al., 2011; Yu, et al., 2011; Hou, et al., 2013; Goh, et al., 2013; Kang, et al., 2015). Some of these methods, such as serial transgene delivery, are limited to certain types of somatic cells, like fibroblasts, or have low reprogramming efficiency (Schlaeger, et al., 2015). As a reagent, episomal vectors are one of the most appealing of all the non-integrating reprogramming methods since they are easy to manipulate and have been shown to perform relatively well. Therefore, improved episomal vectors from past researchers' work are capable of generating human iPSCs that are efficient, free of transgene integration, and represent a major step forward in stem cell therapy for autologous and allogenic tissue (Kang, et al., 2015). For human iPSC to be used in a clinical setting, its genomic stability is crucial. As

genetic aberrations have been strongly linked to cancer, IPSCs prepared for clinical application should be free of cancer-associated genetic alterations.

2.4 Application of iPSC

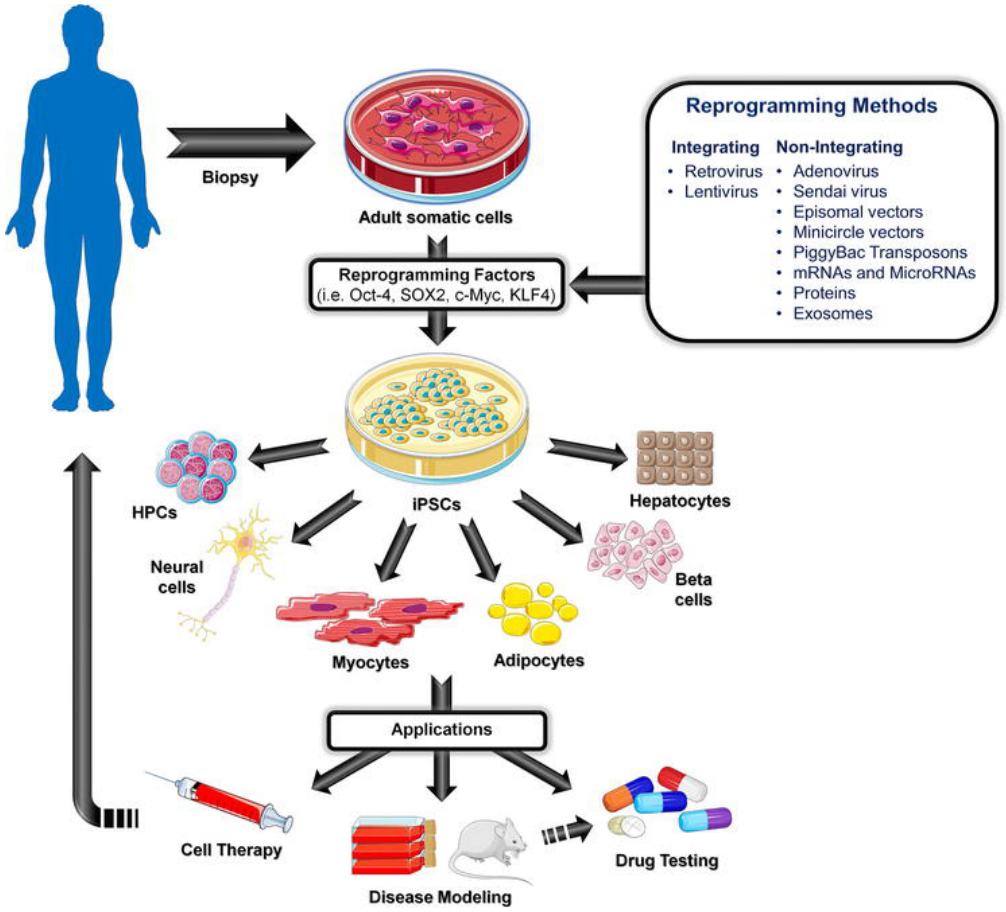


Figure 2.2 Application of iPSC. A brief understanding of its reprogramming methods, differentiation capability and its application in the therapeutic field. (Belviso, et al., 2021)

The application of iPSC technology in clinical research has intensively increased over the years since the successful reprogramming of hiPSCs in 2006. Its pluripotent characteristic was a particularly useful platform for the modelling

of diseases, drug resistance screening, tissue regenerative and bioengineering as well as gene therapy, which brings new and exciting approaches for future clinical applications (Lu and Zhao, 2013). The use of iPSC has provided far more insight and understanding of certain diseases and challenges faced using animal models. Similar to cancer cells, iPSC has unlimited proliferative ability; however, iPSC has the advantage of differentiating into almost all known types of cells in the form of 2D monolayer cell culture or 3D organoids that provide a more well-structured functioning system and with significant mechanism and pathology or mature functioning cells that can be used for a variety of research. Moreover, the cost of cell reprogramming for obtaining iPSC have significantly decreased over the years from $800,000 to $10,000; about 80 times decrease in cost and far better efficiency as compared to the past, making it an ideal subject for various type of research (Jacquet, et al., 2013; Huang, et al., 2019).

2.4.1 Disease Model

Pluripotent stem cells are an important characteristic of early mammalian development, responsible for giving rise to all mature cells. As a result of the insufficient expandable cellular sources available from patients, primary cell-based disease models are limited, particularly for cardiomyocytes, neuronal cells, pancreatic beta cells, and other clinically relevant cells from organs other than skin and peripheral blood (Doss and Sachinidis, 2019). Therefore, the use of iPSC through in vitro culture provides the much needed platform to generate all types of cell lineages with the capacity to generate all known somatic cell types such as cardiomyocytes, endothelial cells, smooth

muscle cells, and neuronal cells (Cyranoski, 2018). Further application of iPSC via in vitro differentiation for the generation of 2D monolayer disease models or 3D organoid disease models have been currently used for the study of treatment of many degenerative diseases such as ischemic heart failure, Parkinson's disease, Alzheimer's disease, diabetes, spinal cord injuries and age-related macular degeneration (Cyranoski, 2018). One example is the study of mitochondria disease that affects retina cells, Leber's hereditary optic neuropathy (LHON) by comparison of iPSC derived from patient-derived cells, carrier-derived cells and healthy donor-derived cells. The produced iPSC from the three different cell sources were differentiated into retinal ganglion cells (RGCs) for LHON to take effect. Characterisation between these cells at the RNA level and deep sequencing can be conducted while narrowing down potential medication for effective cure in further preventing neuron damage in RGC by LHON disease (Yang, et al., 2022).

2.4.2 Drug Screening

In the study of disease biology, in vitro disease modelling, and the development of new drugs, iPSCs and iPSC-derived differentiated cells have been beneficial as tools for drug screening and toxicity testing. As such, iPSCs can be used for drug discovery and development, which reduces the costs of creating new medications (Sirenko, et al., 2014). The ability of iPSCs to self-renew makes them a valuable source of cells for drug testing. In addition, human iPSCs can mimic the characteristics of human ESCs in all respects, so that polymorphic variants can be represented in donor panels that can be used to

understand drug response variability within a population or even personalised medicine. Through the use of iPSCs, a nearly unlimited supply of differentiated cells can be derived from this stem cell technology that benefits researchers by providing sustainable and high-throughput drug screening platforms, 3D organ development and allowing the study of diseases in vitro, the development of cell-based therapies like cell transplantations, extracorporeal tissue devices, and implantable bioengineered constructs containing cells (Khet ani, et al., 2015). This provides a consistent quality of cells, allowing for a more precise drug screening for individual purposes. The use of iPSC technology also allows for the development of a library of cell lines that could represent genetic and epigenetic variations of a broad demographic range (Volpato and Webber, 2020). An animal model cannot accurately characterise a drug's toxicity for humans (Wilding and Bodmer, 2014). The use of animal models has not been able to accurately mimic or represent drug reactions in the human body as well as reactions like allergy, maximum dosage for humans before becoming lethal, minor side effects or adverse side effects (Wilding and Bodmer, 2014; Van, 2019). One example is Isuprel which was used for treating asthma and resulted in more than 3,500 deaths as reported in Great Britain due to the safe dosage measured through animal models like rats, guinea pigs, dogs and monkeys have all far exceeded what the human body can handle (Van, 2019). The safety dosage deemed by all these animals cannot be applied to humans which makes animal models unreliable for drug toxicity testing. Thus, patient-specific iPSCs have been developed for the screening of therapeutic agents and validation of their pharmacokinetic and safety properties (Ko and Gelb, 2014). While the results for validating pharmacokinetic and safety properties have been proven

to be almost similar or up to standard to be applied to patients (Ko and Gelb, 2014). The methods of using patient-specific iPSCs have been categorised into two different ways, the 2D monolayer culture methods and 3D organoids.

Currently, the 3D organoid research trend has been increasing over the past recent years due to its unique biological properties such as higher stability, higher expansion rate, multiple cell types in a single organoid, and the capability to show a more complex mechanism over 2D monolayer culture (Centeno, et al., 2018; Ardalani, et al., 2019; Altmaier, et al., 2022; Ergir, et al., 2022; Cuesta-Gomez, et al., 2023). In the past, iPSC-based studies have resorted to traditional 2D monolayer culture methods that are effective in modelling cell intrinsic deficits but need more important elements present in organs causing relevant and crucial disease-relevant information to be missed. The biologically significant information in organ structure may provide insights into endogenous signalling and intercellular interactions that affect the disease pathogenesis in a direct manner that cannot be observed in 2D monolayer culture (Liddelow, et al., 2017). Although it is impossible to fully recapitulate the human tissue or organ system, the physiological relevance of 3D organoids is greater than that of 2D cell systems. Nevertheless, 3D organoids have revealed more details with closer representations of the human disease models in drug screening studies and their mechanisms compared to that of a 2D cell culture system (Yin, et al., 2016). In the case of in vivo cell development, the structure is generated via cell self-assembly that mimics morphogenesis, embryogenesis, and organogenesis that cannot be seen in 2D monolayer culture but certain processes can be observed in 3D organoids (Kang, et al., 2021). Several studies have evaluated

the formation of 3D in vitro models in different physiological environments to recapitulate in vivo events such as cell growth, differentiation, and morphogenesis (Baraniak and McDevitt, 2012; Kim and Ma, 2013; Law, et al., 2021). These studies have shown that 3D organoids are a better candidate to be used as disease models or even as a platform for research compared to the traditional 2D monolayer culture methods due to the properties and mechanisms present in 3D organoids. More details and observations can be made in 3D organoids that were missed in the past when 2D monolayer cultures were used as the subject for research.

2.4.3 Gene Therapy

An autosomal dominant disorder with genetically heterogeneous genotypes for precision medicine poses significant challenges and opportunities (Doudna and Charpentier, 2014). In the field of gene therapies, gene augmentation for recessive disorders has gained the most attention, being the subject of numerous clinical trials (Lam, et al., 2018; Cukras, et al., 2018; Russell, et al., 2017) and FDA approval for one ocular condition (Ledford, 2017). A perceived need to eliminate the deleterious effects of the mutant allele prevents gene augmentation from being used as a stand-alone treatment for dominant disorders. It is evident that gene editing strategies hold great promise in this regard (Bakondi, et al., 2016; Tsai, et al., 2018; Li, et al., 2018) but for diseases that are highly mutational, testing the safety and efficacy of each mutant-allele-specific genome editor is a practical and economic challenge. Furthermore, gene editing might not be able to target all mutations (Bakondi, et

al., 2016; Pattanayak, et al., 2013; Courtney, et al.,2016) and could cause off-target mutations, especially in the case of wild-type (WT) allele or more serious adverse effects that can cause further harmful of unknown condition (Cromer, et al., 2018). In addition to phenotypes and/or genotypes that are relevant to human disease, preclinical model systems are another important consideration for gene therapy development. For genome editing strategies that utilise sequence-specific tools, this requirement is particularly challenging, as human model systems are required to undergo safety and efficacy evaluation (Lessard, et al., 2017). Besides, humanised animal models provide limited information regarding genome-wide off-target analysis and lack the ability to fully mimic the patients' condition (Maeder, et al., 2019). It has been observed that induced pluripotent stem cells have some residual epigenetic memory from their somatic parent cells. These cells have biased differential potentials depending on the donor cell source because non-CpG methylation patterns were not completely reset during reprogramming (Kim, et al., 2010; Boland, et al., 2014). Therefore, the use of iPSC from patient-derived sources provides a better research platform and understanding of genetic diseases. The use of iPSC in gene therapy research has shown promising results on several diseases like Parkinson's disease (Ng, et al., 2021), Progressive non-syndromic sensorineural hearing loss (PNSHL) (Nourbakhsh, et al., 2021), cystic fibrosis (Lee, et al., 2021), optical disease like Leber congenital amaurosis (LCA) (Kruczek, et al., 2021) and Choroideremia (Patrício, et al., 2018).

2.5 Mesenchymal Stem/ Stroma Cell (MSC)

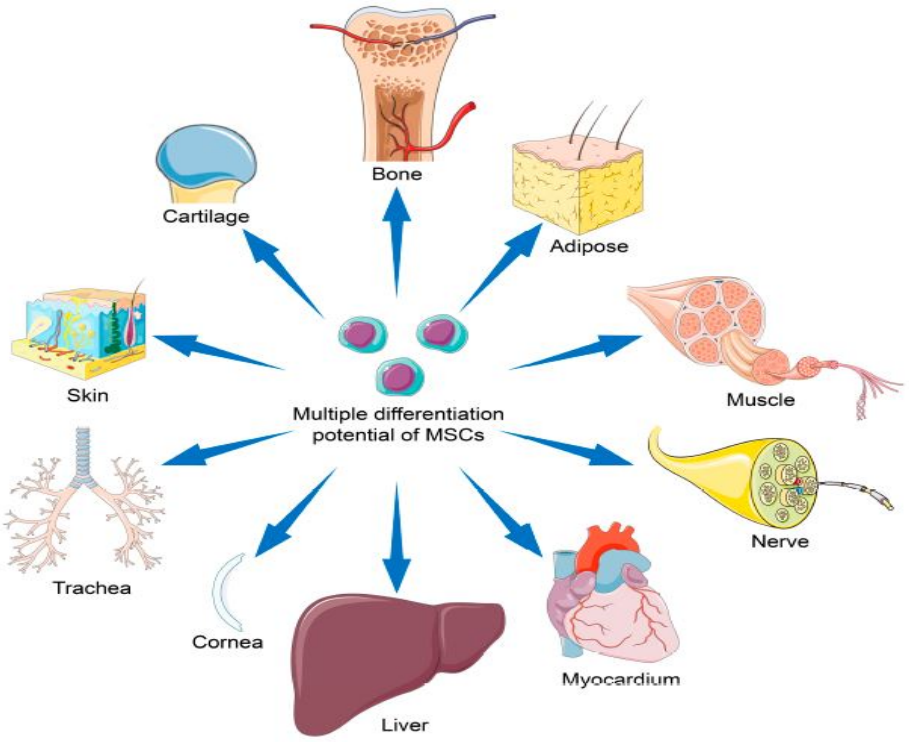


Figure 2.3 MSC Multipotent Differentiation Potential into Different Types of Cells. A preview of the differentiation potential of MSC as a multipotent cell into other cells and tissues. (Han, et al., 2019)

Mesenchymal stem/stroma cell (MSC) has been used widely over the past few decades due to its various biological properties and clinical potential in cell therapies. The use of the term “stem” in mesenchymal stem cells has often led to confusion in terms of the actual cell type it represented and was often used to

represent cells that may not meet specified stem cell criteria (Dominici, et al., 2005; Viswanathan, et al., 2019; Markov, et al., 2021). As such the International Society for Cell & Gene Therapy (ISCT) updated the nomenclature in which cells that show fibroblast morphological and plastic-adherent features, be termed as "mesenchymal stromal cells", while "mesenchymal stem cells" are to be used for cells that meet specified stem cell criteria namely with stringent evidence of stem cell functionality or proper characterisation methods that can indicate the stemness or relationship with stemness (Galipeau, et al., 2019; Viswanathan, et al., 2019). Nevertheless, the acronym "MSC", remains a common usage for both cell types.

The technology for in vivo MSCs culture obtained from the human body has been well established like animal origin MSCs, making it easily accessible for research or clinical purposes. Unlike iPSCs, MSCs have fewer ethical concerns. MSCs also possess self-renewal and immunomodulatory properties that make them an even more attractive candidate for clinical studies (Samadi, et al., 2020). MSCs can be readily cultured in vitro upon extraction, having multipotent differentiation potential (Figure 2.3), and are known to release various paracrine factors including exosomes, growth factors, chemokines and cytokines that are beneficial to other cells. The first isolation and cultivation of human bone marrow MSCs (hBM-MSCs) were conducted by Haynesworth's group in 1992 (Haynesworth, et al., 1992) followed by transplantation into patients by Lazarus's group in 1993 (Lazarus, et al., 1995).

Currently, at least 385 completed MSCs clinical trials are known with 32 at Phase 3 and Phase 4 towards various diseases found in ClinicalTrials.gov. (Accessed date: 15th March 2023) In addition, MSC transplantation has shown excellent safety profile records, with over 1000 registered MSC-related clinical trials with the FDA up to date. The commonly used MSCs sources are bone marrow (Lazarus, et al., 2015), adipose tissue (Halvorsen, et al., 2000), umbilical cord, and placenta (In't Anker, et al., 2004). Despite the multiple sources available for MSC isolation, different batches, and conditions under which MSCs were being extracted have shown inconsistent outcomes in their regenerative efficiency due to the heterogeneity of MSCs because of donor-to-donor variation such as donor's age, gender and cell of origin. As such, there exists a constant challenge in obtaining homogeneous and consistent quality supplies of MSC for more stable and reliable clinical applications (Lin, et al., 2019; Goodarzi, et al., 2019).

2.5.1 Source of MSC

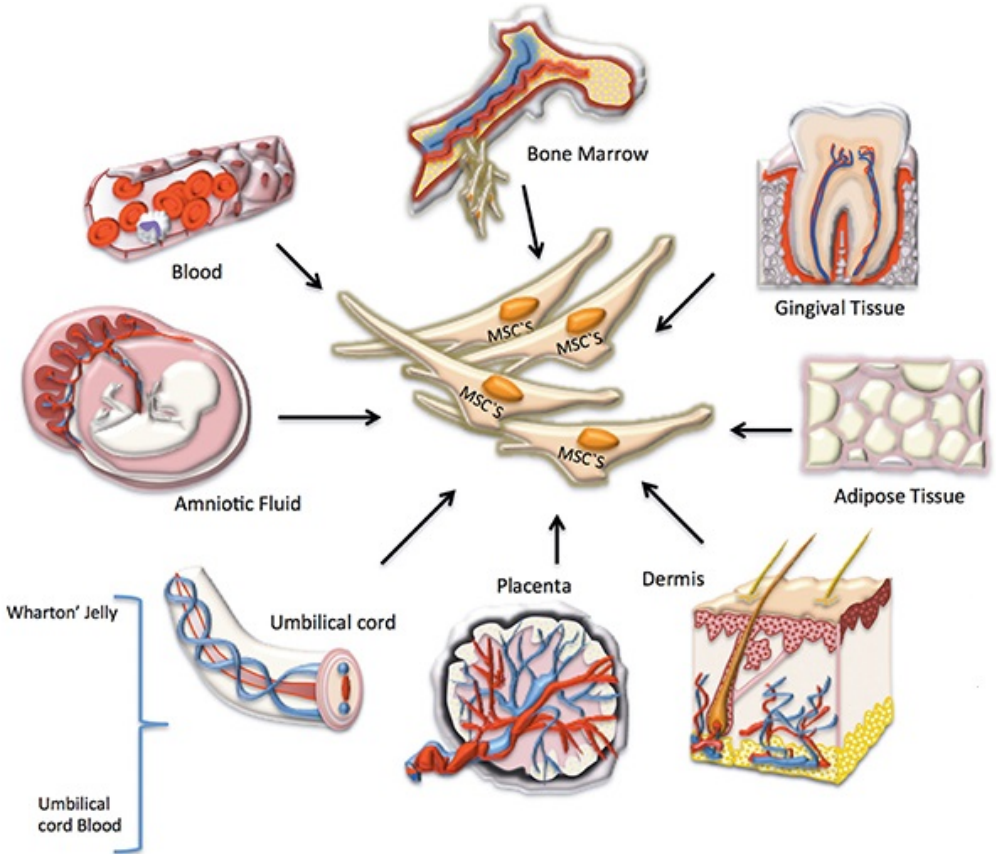


Figure 2.4 Sources of MSC from Different Sites, Tissues, and Organs. Information regarding the harvesting of MSC from various sources. (Merino-González, et al., 2016)

MSCs can be obtained from various sites, tissues, and organs in the human body, such as bone marrow (BM), adipose tissue (AT), amniotic fluid,

placenta, dermis, umbilical cord (UC) or Wharton' jelly, dermis, gingival tissue and more (Han, et al., 2019) (Figure 2.4). To date, BM-MSC has been one of the most common preferred sources of MSC being studied and applied in clinical trials.

BM-MSC was first discovered by Friedenstein's group in 1976 and labelled as undifferentiated MSCs in 1987 (Friedenstein, 1976; Friedenstein, et al., 1987). However, it has then become the primary source of MSC. The extraction of BM-MSC is still a painful procedure that requires high doses of anaesthesia with a risk of infection, while the cell yield and multipotent potential differ with donor age (Berebichez-Fridman, et al., 2017) making it unfavourable to be extracted continuously from the same donor or an older aged donor.

Besides BM-MSC, there are other sources like AT-MSC (Choudhery, et al., 2014), UC-MSC (Gang, et al., 2004), placenta MSC and amniotic MSC (Koike, et al., 2014) that are isolated without pain or through complicated surgery as they are by-products of cosmetic, therapeutic liposuction, birth, and surgical procedures. These by-products only must go through a proper and clean extraction procedure to be readily cultured in vitro for cell expansion and to be applied for therapeutic purposes or research. Although the morphology, phenotypic characteristics, and biological functions of these different sources of MSCs are similar to that of BM-MSC, studies have shown that differences exist in the cytokines and growth factors profiles as well as the differentiation potential among the different sources of MSCs (Han, et al., 2019). Previous studies have shown that different isolation sites, donor's conditions like age,

gender, health condition and genetic profile extracted MSC demonstrated different proliferation activity and trilineage differentiation potential such that the clinical function may vary from one another (Choudhery et al., 2014; Guadix, et al., 2017; Liu, et al., 2017). Also, the use of conventional isolation methods to obtain MSCs has been limited in terms of scalability to provide a large amount of clinically ready MSCs in a short time while inter-donor variability exists (Galipeau, 2013). There are only a limited amount of MSCs that can be extracted from donor bone marrow and there is a risk of pain and infection during the procedure. This has led to the inability to constantly obtain quality MSC for patients in an instant whenever required. To provide a safe cell-based clinical treatment (Guadix, et al., 2017), alternative sources of cells that possess similar biological characteristics as that of MSC, are homogenous, cost-effective and readily scalable would be required for treatment and research usage.

2.6 MSC Scale-Up Production

Despite the challenges in obtaining large amounts of MCSs from a single donor, scaling up the production of clinical-grade MSCs poses another viable option. Generally, there are two distinct methods to scale up extracted MSCs namely by using 2D cultivation technique or 3D bioreactors. There are several crucial factors that need to be ascertained in scaling up MSC production such as cell senescence due to aged cell types, the cost of cultivation and the time taken to achieve a substantial amount needed for treatment as well as the health of the cultivated MSCs must be at optimal conditions at the time for

patients to receive them. It is essential to obtain the best condition of MSCs with minimal costs for industry or even affordable treatment for patients.

In 2D cultivation conditions, undifferentiated MSCs are cultivated and expanded in low density on a flat culture surface allowing a cell to adhere to a wide surface culture plate or large-size culture flat (Fang and Eglan, 2017). While 2D cultivation conditions are lower in cost and the knowledge of the technique is well established, methods like utilising multi-layer flasks or cell stackers have been used to increase the number of cell batches that are expanded at the same time (Jayaraman, et al., 2021). The number of cells and time of confluency are adjustable in 2D cultivation techniques by adjusting the size of the container, the surface area and the stacking system which can range from 10 to 40 layers with different commercialised cell stackers and culture flasks. The use of a stacking system is commonly known as horizontal expansion where the surface area and container size fixes the maximum expansion of cells. However, this feature limits further expansion into larger or industry-level scales as that will require a larger surface area, a longer time for confluency as the surface area increases, the higher usage of medium and manpower for each medium change, passaging and cell harvesting (Campbell, et al., 2015; Merten, 2015). The use of 2D cell culture is also labour-intensive and susceptible to contamination because of its open nature and the need for numerous passages to obtain sufficient cells for its use, while the lack of tools to monitor and control the culture condition makes it an unfavourable option to obtain high-quality cells (Jossen, et al., 2018).

In 3D cultivation conditions, bioreactor systems like stirred tanks and dynamic reactors are commonly used. These devices are relatively easy to operate and monitored through computer control interphase with physical or online input using a mobile phone, laptop or specific device by remote control. The conditions that a bioreactor system can monitor, and control range from temperature, pH, dissolved oxygen, carbon dioxide, stirrer speed, input and output of growth medium and waste medium, glucose level, time-controlled input of serum or growth factors, cell confluency and live image that are all essential to providing a suitable condition for healthy cell growth (Tsai and Ma, 2016; Stephenson and Grayson, 2018; Wongsa, et al., 2022). A stirred tank bioreactor (STBR) consists of cylindrical vessels that are installed with impellers, sparger, baffle plate, jacket, input and output valve and multiple sensors probe. The use of STBR has been long used for the amplification of MSC for cell-based therapy, homologous and heterogenous cell therapy, drug production and fermentation technology that required a large number of cells to be produced at a shorter time than 2D cultivation but with a higher cost to be invested in the facility. The differences between 2D cultivation and 3D cultivation are that 3D cultivation like bioreactor tends to amplify cells through suspension cultivation and constant fluid motion by the impellers is applied to obtain an even distribution of growth medium and beneficial factors to all the cells in the vessels (Odeleye, et al., 2014; Wongsa, et al., 2022). While bioreactor allows the amplification growth of aggregate cells or the use of microcarriers for cells to attach, 2D cultivation only allows monolayer adherent cells to expand. Previous studies have shown that MSCs cultivated in bioreactors could retain their phenotype to a stable degree without

differentiating into other types of cells (Caron, et al., 2012). While cell suspension that aggregates or forms spheroid by nature can better simulate the human in situ condition such that the cell morphology resembles more to actual body tissues as compared to a 2D monolayer (Edmondson, et al., 2014). However, for certain cells, there is a need for external factors to induce cell aggregation, spheroid formation or cell adherent in microcarrier in this constant fluid-flowing vessel such as the introduction of adhesion molecule factors like laminin, integrin, E-cadherin or vitronectin into the system (Badenes, et al., 2016). With recent advancement in technology, human or clinical grade factors are available that allows the use of bioreactor-produced cells to be used for clinical treatment but at a relatively high cost. Microcarrier beads have provided a larger surface area for cell attachment in a similar given volume compared to that of the 2D cultivation technique, allowing adhered MSC to grow better. Regardless of all the benefits and cost control involving 2D or 3D cultivation to amplify MSC cells, more research is still required for a comprehensive understanding of the clinical efficacy of industry-scale produced cells and the necessary optimisation to provide the best outcome and cell qualities for treatment purposes.

2.7 Application of MSC

In therapeutic treatment, MSCs can be applied through two different approaches namely direct or indirect methods. The direct method involves the direct injection of isolated or cultivated MSC in the form of transplantation on the site of injury or treatment for proximate cell-to-cell interaction and

signalling to activate its therapeutic purposes through its paracrine factor release. Whereas the indirect method comprises the collection of MSC exosomes extraction or filtered conditioned medium of MSC or collection of specific paracrine factors followed by the injection to the site of treatment, allowing the collected factors to activate desired signalling or pathways for enhancing or inhibiting specific activities. In the past decade, it has been widely used for various therapeutic research such as disease modelling (Zhao and Ikeya, 2018), tissue regeneration (Xu, et al., 2019), cell-based therapy (Zhao, et al., 2015), degenerative disease (Xin, et al., 2017) and autoimmune disorders (Laso, et al., 2018) which have all showed favourable results. The use of MSCs for cancer treatment, however, had mixed results, showing either cancer-promoting effects (Nishikawa, et al., 2019; Mao, et al., 2017) or cancer-suppressing effects (François, et al., 2018; Wu, et al., 2016); depending on the type of cancer and the source of the MSC used. The inconsistent results from various results have brought concern to the use of MSCs in treatment, resulting in the need for a stable and homogeneous cell source that is more reliable to use.

2.7.1 iMSC Disease Model

2.7.1.1 From iPSC to iMSC

Upon reprogramming of somatic cells using Yamanaka's OSKM factors, the reprogrammed iPSC can be further differentiated into MSC by several methods resulting in induced pluripotent stem cell-derived MSC (iMSC). The

differentiated iMSC was commonly characterised using ISCT guidelines and minimal criteria (Dominici et al., 2006) to gauge its MSC-like properties.

The guideline from the year 2006 indicate that (1) the cell should be tissue culture plastic adherent; (2) presence of positive surface antigen markers CD73, CD90, CD105 and CD166 with expression of ≥95%; (3) detection of negative for CD11b (monocytes and macrophages) or CD14, CD19 (B cells) or CD79α, CD34 (hematopoietic and endothelial cells), CD45 (pan-leukocyte) and HLA-DR with expression of ≤2%; (3) Able to be induce into trilineage differentiation to adipocytes, chondroblasts, and osteoblasts (Dominici et al., 2006). The guideline emphasises the detection of surface markers on adult MSCs and ensures no presence of iPSC surface markers to indicate successful differentiation. Then, to focus on multipotent capability and basic biological characteristics of adult MSCs such as adherent on plastic surfaces and fibroblast-like morphology.

However, in the year 2019, the ISCT guidelines and criteria have been revised. The new guideline and criteria have the addition of specifying tissue origin of cells, using the term stromal cell unless stemness is demonstrated through rigorous experimental evidence, as well as functional assays to justify the therapeutic mechanisms (Viswanathan, et al., 2019). However, the acronym MSC remains acceptable for both stromal and stem cell types (Viswanathan, et al., 2019).

2.7.1.1.1 Different Methods of iMSC Differentiation

In most cases, iMSCs are produced by reprogramming somatic cells into iPSCs, which are then differentiated into iMSCs through the deprivation of pluripotent signals in the culture medium, resulting in spontaneous differentiation (Hynes, et al., 2014). Spontaneous differentiation of iPSC to iMSC can be achieved with the use of a different mixture of growth factors such as platelet-derived growth factor alpha polypeptide b (PDGF-AB)/ basic fibroblast growth factor (bFGF) and different culture conditions with adjustment to the carbon dioxide level and cell density (Hynes, et al., 2014). Besides, the generation of iMSCs can also be achieved through coating material like type I collagen that mimics the structure of physiological collagen for iMSC formation (Liu, et al., 2012). Studies have demonstrated that iPSCs can be induced to produce mesenchymal-like cells or iMSCs that exhibit similar genotypic and phenotypic characteristics to primary MSCs and that these cells are stable over prolonged passage without showing abnormal chromosomes (Steen, et al., 2017; Loh, et al., 2022). The advancement of methods to derive iMSCs as a potential alternative source for MSCs in replacement for therapeutic purposes has been slowly progressing over time such that there are various mature and stable methods in the market and widely used in laboratory research that have undergone clinical trial and approval.

In the past, tissue culture plastic (TCP) has been used to differentiate iPSCs into MSCs (iMSCs). A human platelet lysate (hPL) (Frobel, et al., 2014) or fetal calf serum (FCS) (Diederich & Tuan, 2014) supplement is used to induce differentiation towards this lineage. In vitro, expansion of MSCs can be

facilitated with the use of hPL, which contains a broad spectrum of cytokines, growth factors, and mitogenic compounds (Doucet, et al., 2005; Hemeda, et al., 2014).

Three-dimensional (3D) microenvironments can mimic the extracellular matrix properties of native tissue as another critical parameter (Shao, et al., 2015). Hydrogels made from natural components (e.g., collagen (Sheu, et al., 2001) or fibrin (Noori, et al., 2017)) support the adhesion and migration of cells through integrin binding sites (Frantz, et al., 2010). Additionally, different physical and biochemical cues have been shown to affect MSC differentiation in 3D scaffolds. One of the common bioactive materials used, like hydrogels are bioactive materials that may also influence iPSC differentiation (Tsou, et al., 2016), but 3D scaffolds have yet to be examined for their utility in generating iMSCs (Shou, et al., 2017). Despite the number of different methods and technique for the generation of MSC from iPSC such as the MSC switch that switch the iPSC culture medium to MSC medium, embryoid body formation, specific differentiation, pathway inhibitor, and platelet lysate methods, there are yet to have a fixed Good Manufacturing Procedures (GMPs) as each strategy holds its advantage and limitation in term of the differentiation efficiency, duration of differentiation, the number of cells capable to differentiation at a time, the cost of differentiation and the biological properties of differentiated iMSC (Dupuis and Oltra, 2021). However, some methods are widely used compared to other methods by researchers due to their convenience and low cost or the ability to scale up the differentiation. Cost to performance is one of the essential factors for the industry to profit in clinical treatment while

providing more advanced technology and research findings through the scale-up of differentiation for various research to be conducted at the same period.

For the development of novel therapeutic strategies for genetic diseases, it is imperative to understand the pathological mechanisms underlying human diseases. The lack of appropriate animal models and the limited availability of patient tissue make research on these genetic disorders challenging. These diseases can be better understood by using patient-specific iPSC models. The differentiation of iMSCs from patient iPSCs makes them ideal not only for pathologic research but also for drug screening, drug discovery and toxicity testing (Zhao and Ikeya, 2018).

The use of patient-derived iMSCs has allowed a better study of certain diseases. A study by Liu and colleagues using a rare disease modelled of Fanconi anaemia (FA). FA is a recessive disorder that can be identified in cells by its genomic instability, congenital abnormalities, cancer predisposition and BM failure in patients otherwise the irregular or weakened response of cells towards DNA damage. With the use of patient-derived iMSCs, Liu and colleagues derived FA-iMSCs that showed impairment in maintenance and proliferation in cells that are similar to MSCs from an animal model of Fancg-deficient mice (Liu, et al., 2014). With the properties of iMSCs not limited to the MSCs model, the use of FA-iMSCs has been validated as a suitable platform for the studies of FA mechanism and drug screening purposes (Liu, et al., 2014).

To study the pathology of Hutchinson-Gilford progeria syndrome (HGPS), Zhang and colleagues prepared iMSCs from patients. Progerin, a truncated and farnesylated form of Lamin A, is responsible for HGPS, a segmental premature ageing disease that affects mesenchymal lineages. Several abnormalities are observed in HGFP-iMSCs, including nuclear dysmorphology, DNA damage, and accumulation of calponin-staining inclusion bodies, which are similar to those seen in HGPS fibroblasts. In vitro and in vivo, HGFP-iMSCs were found to be less viable under stress, especially under hypoxia. HGPS-iMSCs could resist hypoxia when progerin levels were reduced by shRNA (Zhang, et al., 2011).

The use of iMSCs as disease models has gradually increased over the years to replace MSCs. Patient-derived cells are reprogrammed into iPSCs and further differentiated into iMSCs to serve as a new platform for disease models. The results of patient-derived iMSCs are similar to that MSCs and the high proliferative capability has allowed a wide range of drug screening and studying of the disease mechanism under different conditions (Chow, et al., 2017; Soontararak, et al., 2018).

2.7.2 iMSC Application in Regenerative Medicine

Research studies of MSC secretomes like extracellular vesicles (EV), exosomes, growth factors, cytokines and chemokines have shown promising potential in regenerative. Furthermore, the biological characteristics of MSCs namely high expandability, readily cultured in vitro, low immunogenicity and

multipotency ability, make them a suitable candidate for tissue repair purposes. Many reports suggested that MSCs releasing secretomes also known as paracrine factors have demonstrated medicinal usage as the bioactive molecules have immunoregulatory and microenvironment modulatory effects on the site of injury (Zhao and Ikeya, 2018). Analysis and profiling of MSCs and iMSCs comparison have shown highly significant likelihood between one another, however, further analysis shows that iMSCs possess lower immunomodulatory properties as their capacity in suppressing T cell proliferation is lower than that of adult or native MSCs which makes iMSCs a potential substitute for MSCs in regenerative medicine (Frobel, et al., 2014).

2.7.2.1 Application of iMSC Implantation in Regenerative Medicine

Several animal studies have used iMSCs as a replacement for MSCs to test the efficiency and efficacy of tissue regeneration and tissue damage repair. The results show that iMSCs show a significantly higher recovery rate and fewer side effects than MSCs. Hynes'a group shows that iMSCs have the capability to promote periodontal regeneration and formation of new mineralised tissue through the demonstration of iMSCs implantation in a periodontal defect rat model (Hynes, et al., 2013). Lo Cicero's group demonstrated that iMSCs-induced osteoblast significantly improves the recovery of bone formation by implanting the induced osteoblast into calvaria defect mice (Lo Cicero, et al., 2016). Another study done by Meow's group compared the implantation effects of iMSCs and BM-MSCs in a hindlimb ischemic mouse model that shows the result of iMSCs significantly reduced the

ischemic damage more than that of BM-MSCs (Miao, et al., 2014). Several groups of scientists compared the recovery effects of iMSCs, BM-MSCs and UC-MSCs in the myocardial infarction and dysfunction mouse model and the outcome showed similar results, all of which supported the use of iMSCs, as this cell type demonstrated a more significant recovery in the cardiac wound and promoted proangiogenic potency when compared with BM-MSCs and UC-MSCs (Zhang, et al., 2015a; Liang, et al., 2017; Zhang, et al., 2015).

In one of the studies by Deyle and colleagues, iPSC was used as the primary source of cells to counter osteogenesis imperfecta (OI). This disease is caused by a genetic bone disorder caused by dominant mutations in type 1 collagen (Deyle, et al., 2012). Patients with OI were given transplantable iMSCs differentiated from iPSCs genetically free from collagen mutations. For the treatment of defects in skeletal tissue caused by OI, these iMSCs could act as bone-forming cells as they are absent of collagen mutations to regenerate the skeletal tissue.

In a previous study, MSCs showed an interesting phenomenon of mitochondria transfer or mitochondria donation in which MSCs demonstrated the transfer of its mitochondria to nearby damaged cells, which provided the damaged cells with new functional mitochondria resulting in the prevention of cell degeneration and eventually led to cell recovery. Similarly, this phenomenon was also found in iMSCs. A study of Jiang and colleagues ~~have~~ conducted a study on mitochondrial complex I defect-induced degeneration in retinal ganglion cell (RGC), a common effect in Leber hereditary optic

neuropathy (LHON) patients. The research group performed intravitreal injection of iMSCs into the vitreous body of Ndufs4-/- mice that experience retinal degeneration. To better observe if there is mitochondria transfer, the iMSCs were pre-stained with human GFP-mito staining before the injection. The result from the experiment shows that the mice injected with iMSC have increased survival rate of RGCs and improved retinal function when compared to the control while the GFP-labeled human mitochondria were shown to be detected in the mouse retina after iMSC injection (Jiang, et al, 2019). Interestingly, mitochondria transfer of MSC and iMSC have also been observed towards other cell types other than RGC. Moreover, Zhang's study has demonstrated that the mitochondrial transfer efficiency of iMSCs was shown to be higher than that of BM-MSCs, with findings indicating that mitochondrial transfer efficiency may be directly related to the expression of intrinsic Rho GTPase 1 (MIRO1) (Zhang, et al., 2016).

2.7.3 Application of iMSC-Extracellular Vesicles (EV) in Regenerative Medicine

The use of EVs from iMSCs and MSCs has also generated much attention in the field of regenerative medicine. Kim's group compares the efficiency of skin regeneration of EVs from iMSC and UC-MSCs in human keratinocytes and dermal fibroblasts that show similar effects in improving collagen secretion and fibronectin expression. However, the use of iMSC-EVs has a more significant increase in proliferation rate and release of fibronectin that aids in regeneration compared to UC-MSC-EVs. Further tests of applying

iMSC-EVs and UC-MSC-EVs directly on the wound sites in rat models have resulted in better reduced scar widths, a higher ratio of re-epithelization and collagen deposition by iMSC-EVs (Kim, et al., 2018).

In the study of regenerative medicine in cardiovascular disease using iMSC-EVs, the application results in vivo and in vitro models have been promising and beneficial. The use of iMSC-EVs was shown to activate angiogenesis-related molecule expression, improve the proliferation of endothelial cells, improve deposition of collagen, improve microvessel density, improve cell migration, promotion of vessel tube formation, reduction of brain tissue loss and enhance overall disease condition (Hu, et al.,2015; Du, et al., 2017; Liu, et al., 2017; Xia, et al., 2020; Feng, et al., 2020).

The effects of iMSCs transplantation and iMSC-EVs in regenerative medicine have provided similar or more significant beneficial results as MSCs without reports of side effects up to now (Hu, et al.,2015; Du, et al., 2017; Liu, et al., 2017; Kim, et al., 2018; Xia, et al., 2020; Feng, et al., 2020).

2.8 Potential Application of MSC in Cancer Research

There are a number of studies done with MSCs on cancer treatment, however, the results are controversial among different groups of researchers. Wang's group conducted a study with the use of MSC as a delivery vector, a death receptor ligand TNF-related apoptosis-inducing ligand was delivered to xenograft lung tumour models, inhibiting tumour growth and preventing

metastasis in the year 2009 (Wang, et al., 2009), whereas in another study conduct by Liu's group using MSC found to inhibit lung cancer development in a rat model when chemical carcinogens were delivered by MSC in the year 2017 (Liu, et al., 2017). The paracrine factors released by the MSC via cell-to-cell interaction with cancer cells play an essential role in the inhibition of cancer properties (Wang, et al., 2009; Liu, et al., 2017). Nevertheless, the mechanisms and pathways involved in the interaction between MSCs and cancer remain unclear. It is important to note that epithelial-mesenchymal transitions (EMT) are reactivated in adults during times of cell damage and regeneration, as well as during cancer progression. As a consequence of EMT, cancer cells undergo tumour metastasis, causing poor clinical outcomes. There is a poor overall and metastasis-free survival rate in patients with non-small-cell lung cancer (NSCLC) who have upregulated EMT transcription factors (Wang, et al., 2017). There has been some research indicating that targeting specific proteins and genes involved in the EMT pathway, may result in mesenchymal-epithelial transition (MET) that provides effective treatments for NSCLC patients. (Schliekelman, et al., 2011; Tang, et al., 2011)

It has recently gained much attention to study the effects of MSC in a stromal environment on tumour progression. EMT is significantly influenced by paracrine factors released by stromal cells like MSC under TME such as transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), or matrix metalloproteinases (MMPs) (Kalluri and Weinbery, 2009; Ding, et al., 2011). The EMT process enhances

the ability of tumour cells to migrate and invade by transforming them from an epithelial state to a mesenchymal state. MSCs have been shown to inhibit tumour growth or metastasis in numerous studies by immune responses suppression, including inhibition of angiogenesis, suppression of serine/threonine Kinase 1 and wingless-related integration site signalling, arrest of the G0-G1 phase of the cell cycle, or induction of apoptosis (Rhee, et al., 2015; Lee and Hong, 2017).

2.8.1 Lung Cancer

Reports from GLOBOCAN 2020 show that lung cancer leads to new cases of 2.2 million (11.4%) and estimated death of 1.8 million (18%). It is ranked second highest for new cancer cases and the highest for cancer death. While Asia contributed to the highest incidence of 59.6% overall cases in 2020. A high prevalence of lung cancer was observed in developing countries while higher contributions from the middle-income and low-income countries (Sung, et al., 2020).

Statistic shows that more than two-thirds of lung cancer-related death globally are attributed to smoking while the rest are due to environmental and genetic factors. Other factors including occupation hazards, family history, lifestyle, dietary and patients accepting chest radiation therapy also affect lung cancer incidence. Lung cancer is still deemed to be a lethal and common cancer even in this modern era. Despite the advancement in technology, an effective

cure for lung cancer is still not well established and lung cancer patients are still identified with poor prognoses.

Based on GLOBOCAN 2020, the global comparison and ranking by new cases of lung cancer for Taiwan have been categorised as rank 1 (815563 new cases combined with China statistics) while Malaysia has been categorised as rank 2 (5139 new cases) (Sung, et al., 2020). This has called for proper treatment as from the statistics, an observation of 18% estimated death from the overall cases. The lack of effective non-chemo treatment due to resistant mechanisms has posed trouble and resulted in the declining survivability of lung cancer patients, thus, the need to consider a new idea of treatment is required.

With an observed 18% estimated death from the overall cases and the lack of effective non-chemo treatment due to resistant mechanisms lung cancer patients' survivability.

2.8.2 Type of Lung Cancer

Lung cancer can be generally categories into two types, 15% of total lung cancer cases are small cell lung cancer (SCLC) and 85% of total lung cancer cases are non-small cell lung cancer (NSCLC) and histologically sub-classified into adenocarcinoma (40%) and squamous cell carcinoma (30%), and large cell carcinoma (15%). The majority of cases of lung cancer patients are diagnosed with NSCLC.

2.8.3 Non-Small Cell Lung Cancer (NSCLC)

NSCLC has been known to dominate the majority of lung cancer cases and the poor prognosis for patients in the category due to the lack of effective treatment up to now. NSLCL has been identified to possess multiple genetic mutations (KRAS, ALK, MET, ROS1, HER2, BRAF, MEK, PIK3CA, and NTRK1) (Chu, 2020). The first most known mutation of echinoderm microtubule-associated protein-like 4 (EML4) anaplastic lymphoma kinase (ALK) (EML4-ALK) followed by the second most known mutation is in the tyrosine kinase domain (TKD) with the activation of epidermal growth factor receptor (EGFR) (Sasaki, et al., 2010; Zhang, et al., 2019). Patients with EML4-ALK eventually develop resistance towards ALK inhibitors that render ALK inhibitor drugs like crizotinib and lorlatinib to be less effective (Pailler, et al., 2019). Unlike EML4-ALK, this EGFR-TKD mutation has resulted in the NSCLC being sensitive to tyrosine kinase inhibitor (TKI) that serve as a potential platform for TKI-drug treatment for NSCLC patients (Wu, et al., 2013)

2.8.4 NSCLC Drug Treatment

The presence of exon 19 deletion and L858R point mutation (Exon 21) are the major EGFR mutation subtypes identified in NSCLC patients. These patients have higher survival rates as compared to other mutations due to the cancer cells responding well to first-generation (gefitinib and erlotinib) and second-generation (afatinib) EGFR-TKI drug treatment (Jiang, et al., 2019). However, over time the effectiveness of first- and second-generation EGFR-

TKI drugs decreased significantly due to the mechanism of resistance through the activation of a secondary mutation, T790M in TKD (Takeda & Nakagawa, 2019). The secondary mutation was overcome with the introduction of third generation (Osimertinib/AZD9291) EGFR-TKI drug that possesses high efficacy in patients with T790M mutation (Jiang, et al., 2018). Yet, long-term treatment of 8-12 months shows sign of cancer cell-acquired resistance that again, decrease the efficiency of EGFR-TKI treatment (Roper, et al., 2020). Therefore, there is a need for an alternative treatment to counter the cancer cell-resistant mechanism.

2.8.5 Applications of iMSC in Cancer Research

It has been shown that MSCs play a variety of roles in cellular functions, including the ability to differentiate into different types of cells for repairing and regenerating damaged tissues (Park, et al., 2018; Barzegar, et al., 2019). Furthermore, current evidence suggests a paracrine signalling mechanism involving the release of several trophic factors into the surrounding cell environment, such as cytokines, chemokines, extracellular matrix proteins, and microRNAs. Paracrine signals have been associated with immunomodulatory abilities, angiogenesis, anti-apoptosis, anti-oxidation, and anti-inflammatory effects (Barzegar, et al., 2019). MSC migration to tumour sites was observed in certain studies whereby either suppression or promotion of tumours within the tumour microenvironment (TME) was observed in response to paracrine signalling (De Becker and Riet, 2016; Galland and Stamenkovic, 2020). CXCL12/CXCR4, CCL2 in breast cancer, and SDF-1 in colorectal, prostate,

and breast cancers are signalling molecules that facilitate MSC migration and activate the MSC homing properties towards the tumour site (Kalimuthu, et al., 2017). The recruitment of MSC to the cancer site can also be achieved via the secretion by cancer cells that result in the interaction of MSC with certain cytokines responsible for angiogenesis (IL-8, TGF-β, and VEGF). By cleaving and activating PAR-1, MMP-1 also helps stimulate MSC homing to the cancer site.

2.9 The Differences Between Adult MSC and iMSC

Due to biological limitations such as population heterogeneity, low passage number, donors' condition, cell senescence, and inability to obtain high amounts of cells in a short period when using MSCs as the sole sources for therapeutic treatment. Many scientists have tried to find alternative sources to get MSCs. As such, intensive research has been done using iPSCs as the sources to differentiate into MSC-like cells with almost similar biological properties to adult MSCs. This has attracted much interest in studying the differences and similarities between MSCs and iMSCs in terms of their biological properties and clinical value.

In a previous study done by Zhao's group in 2015, bone marrow mesenchymal stem cells (BM-MSCs) and MSC-like cells derived from iPSCs (iMSCs) were compared in terms of their interaction and cancer cell properties with the use of two cancer cell models: LoVo colorectal cancers cells and MDA-MB231 breast cancer cells. The finding of the study showed that iMSC has

nearly similar morphology, pluripotent markers expression, MSC surface markers expression and trilineage differentiation potential as that of BM-MSC. However, iMSC showed a higher CFU count at a later passage, higher telomere activities and lower senescence compared to BM-MSC (Zhao, et al., 2015; Rajasingh, et al., 2021).

It was also found that iMSC, like BM-MSC, showed homing properties towards tumours and a lower expression of TGF-β signalling and the presence of tumour-related factors. The study concluded that iMSCs may have lesser potential in promoting epithelial-mesenchymal transition (EMT) than BM-MSCs, thus, iMSCs tend to have a lower potential in enhancing cancer cell invasion and expansion upon interaction with these cancer cell lines (Zhao, et al., 2015).

In addition, another interesting finding from Liu's group in 2017 showed that slight modification on iPSCs before differentiation to iMSC allowed a more excellent inhibition capability in vivo tumour xenograft. Liu's group optimised In combination with a promoter-less neomycin resistance cassette and a cytomegalovirus (CMV) enhancer cassette, the rDNA targeting plasmid (pHrn-IL24) was integrated into the 45S pre-RNA gene. The modification on iPSCs did not affect its efficiency from differentiating into iMSCs. When compared between modified iMSCs and non-modified iMSCs, the earlier showed a smaller size tumour formation, a slower tumour growth curve and tumours with lighter weight in the in vivo cancer study model. These findings may provide an

interesting alternative to genetically modifying the iPSCs into desired biological properties before differentiating them into iMSCs.

Another study by Wang's group in 2022 also showed a modification in iPSCs before differentiation which showed no effects on the differentiation efficiency of iMSCs and exhibited inhibiting properties on cancer cells. The team used a non-viral gene-targeting vector minipHrneo-ILZ-sTRAIL (isoleucine zipper and short TRAIL fusion protein). The group compared BM-MSCs, iMSCs and TRAIL-iMSCs and four different kinds of cancer cells (A375, A549, HepG2 and MCF7) by intravenous injection into a male BALB/cByJ nude mice on the xenograft cancer site. The results from harvested cancer cells showed TRAIL-iMSCs have the smallest tumour size, followed by iMSCs and MSCs. The TRAIL-iMSCs were able to induce apoptosis by activating caspase-8, and the effects are shown across all four types of cancer cells (Wang, et al., 2022). The finding has provided insight into the modification of iMSC sources before the differentiation process can provide more effective or specific treatment in the later parts.

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview of Methodology

The experiment was divided into two parts, Part I was the characterisation of iPSCs followed by the differentiation of iPSCs to iMSCs using a differentiation medium and Part II was the evaluation of the effects of iMSCs on H1975 lung cancer cells under different treatment methods.

In Part I, we first characterised the PBMC-iPSCs by observing microscopic morphology, alkaline phosphatase expression and pluripotency markers with immunofluorescent. The iPSCs were allowed for expansion in. the culture dish and underwent culture medium differentiation for 38 days. The morphological changes during the differentiation were observed every two days after the medium was changed. Upon maturation and appearance of fibroblast-like morphology on day 38, the MSC-like cells were harvested and characterised using qPCR for detection of pluripotency and MSC markers, flow cytometer based on the surface markers expression and its trilineage differentiation potential towards adipocytes, chondrocytes, and osteocytes.

In Part II, the iPSC-derived MSCs were cultured for conditioned medium (CM) extraction and cell sources for transwell coculture (TC) treatment.

The H1975 growth medium was replaced with membrane-filtered iMSC conditioned medium (iMSC-CM) in 50% and 100% concentration with another part with H1975 growth medium to test on indirect coculture treatment that represents non-cell-to-cell interaction. The H1975 was cultured on the base of a 6-well culture dish with iMSC on top of the transwell membrane above the H1975 in similar wells with their respective growth medium in their chamber to test on cell-to-cell interactive indirect coculture treatment named iMSC-TC treatment in this experiment. After 5 days of treatment, the H1975 from direct and indirect treatments were harvested for qPCR and migration assay to test the carcinogenic and migratory properties. Then, the conditioned medium from the H1975 culture, iMSC culture and iMSC-TC treatment culture was obtained individually and tested for cytokine and chemokine proteomic assay for further analysis at the protein level.

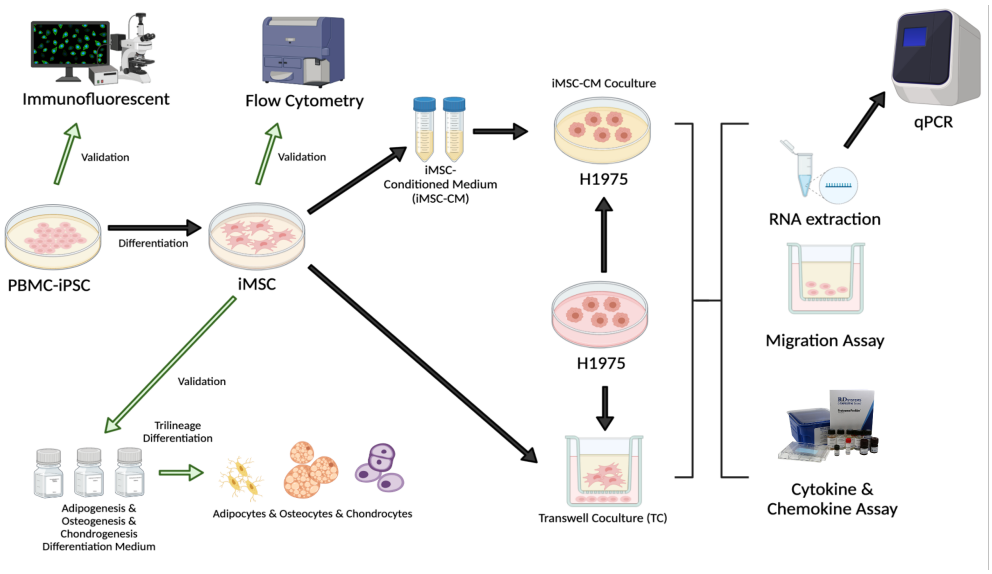


Figure 3.1 Overview diagram of experiment methodology.

3.2 NCI-H1975 Lung Cancer Cell

In this experiment, we use NCI-H1975 (ATTC, United States) as our lung cancer model. NCl-H1975 or H1975 is an epithelial morphology non-small cell lung cancer (NSCLC) that harbours double mutation (EGFR L858R/T790M) (Nambu, et al., 2018). The EGFR mutation allows the study of TKI-drug effectiveness in the lung cancer model.

3.2.1 Cell Culture Maintenance of NSCLC H1975

The H1975 cell line vial was removed from the liquid nitrogen tank and thawed in a 37°C water bath. The thawed vial was sprayed with 70% ethanol as an aseptic technique performed in a bio-safety cabinet (Esco, Singapore). Thawed cells from the vials (approximately 5 x 10^5 cells) were transferred into a 15 ml conical tube with 9 ml of Roswell Park Memorial Institute (RPMI-1640) growth medium supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Grand Island, NY), 4 mM L-glutamine (Gibco/Invitrogen), and 1% penicillin-streptomycin (Gibco/ Invitrogen). The medium composition of H1975 cell lines growth medium in 500ml is summarised in Table 3.1. The 15 ml conical tube Tubes containing the H1975 were centrifuged at 13,000 rpm for 3 minutes to pellet down the cells prior to seeding. All the medium was discarded without disturbing the pellet formed at the bottom of the tube and the pellet was re-suspended in 10ml of fresh medium via gentle pipetting. The H1975 cells were then subcultured and evenly seeded into 1:4 of a 10 cm cell culture dish. The

seeded cells are maintained in a cell incubator under a 5% CO_2 atmosphere at 37°C. The cells were observed daily under a bright-field microscope to ensure proper cell attachment and potential contamination while the medium was changed every three days. Upon reaching 90% confluency, cells were harvested using 2 ml of 0.25% trypsin-EDTA (Sigma-Aldrich, USA) and subcultured every three days.

Table 3.1 Medium Composition of H1975 growth medium in 500 ML

COMPOSITION	WORKING CONCENTRATION	VOLUME (mL)
RPMI-1640	90%	445
Fetal Bovine Serum	10%	50
L-Glutamine	1%	5
Penicillin-Streptomycin	4mM	0.5
Total		500

3.3 Alkaline Phosphatase (AP) Staining

The iPSC culture dish medium was aspirated and washed with PBS. The iPSCs were fixed in 80% ethanol for 60 minutes at room temperature or overnight at 4°C. After fixation, cells were washed with ddH_2O and incubated in 100 mM Tris-HCl (pH 8.2) at room temperature for 5 minutes. The 100 mM Tris-HCl was prepared before the experiment with pH adjusted and filtered for sterile purposes. After incubation, the 100 mM Tris-HCl was aspirated and the

AP substrate working solution (Vector) was added until the surface was fully covered, and the solution was incubated at room temperature for 20 to 30 minutes in the dark to minimise fluorescence loss. The stained cells were washed with 100 mM Tris-HCl once and soaked in 100 mM Tris-HCl and observed under the light microscope to prevent the culture dish from drying up and pH change.

3.4 Immunofluorescence (IF) Staining

The iPSCs were fixed in 4% paraformaldehyde for 15 minutes followed by permeabilisation with 0.2% Triton X-100 in PBS and incubated for 15 minutes. Following permeabilisation, the cells were blocked with 0.5% bovine serum albumin (BSA) in PBS at room temperature for 1 hour. The cells were incubated with primary antibodies in a blocking buffer for 1 hour at room temperature or overnight at 4°C. After primary antibody incubation, the cells were incubated with secondary antibodies in a blocking buffer at room temperature for 1 hour. This was followed by the incubation of DAPI in a blocking buffer at room temperature for 20 minutes. Finally, the cells were washed with PBS after each step three times at 5-minute intervals The stained cells undergo mounting with a glass slide in mounting buffer containing DAPI and were observed under the fluorescence microscope.

3.5 Generation of iMSC from iPSC

The NTA, a subclone of reprogrammed iPSC cell line vial was removed from the liquid nitrogen tank and thawed in a 37°C water bath. The thawed vial was sprayed with 70% ethanol as an aseptic technique performed in a bio-safety cabinet. Thawed cells from the vials (approximately 5 x 10^5 cells) were transferred into a 15 ml conical tube with 4 ml of StemFlex Medium (Thermo Scientific, Waltham, MA, USA). The 15 ml conical tube containing the NTA-iPSC was centrifuged at 13,000 rpm for 2 minutes to pellet down the cells prior to seeding. All the medium was discarded without disturbing the pellet formed at the bottom of the tube and the pellet was re-suspended in 10ml of fresh medium via gentle pipetting. The NTA-iPSC cells were then subcultured and evenly seeded into 1:3 of 3.5 cm cell culture dish pre-coated with Geltrex Matrix (Thermo Scientific). The seeded cells are maintained in a cell incubator under a 5% CO_2 atmosphere at 37°C. The cells were observed daily under a bright-field microscope to ensure proper cell attachment and potential contamination while the medium was changed every three days. Upon reaching 75% confluency, cells were harvested using 1 ml of Versene (Thermo Scientific, Waltham, MA, USA) and incubated in a cell incubator under a 5% CO_2 atmosphere at 37°C for 3 minutes. The culture dish was removed from the incubator and cells were washed down with 2 ml StemFlex Medium followed by a collection of the cell's suspension in a 15 ml conical tube to be used for seeding in the new Geltrex coated culture dish. The cells are subcultured every three days or upon reaching 75% confluency to prevent differentiation or an unhealthy cell culture environment.

For iPSC to iMSC differentiation, cells were allowed to grow up to 90% confluency and induced with MSC differentiation medium containing alpha minimum essential medium (MEM)-α (Gibco/Invitrogen), 10% fetal bovine serum (Gibco/ Invitrogen), 1% MEM non-essential amino acid solution (Gibco/ Invitrogen), 1% HEPES (Gibco/Invitrogen), 1% sodium pyruvate (Gibco/Invitrogen), 1% L-ascorbic acid (Sigma-Aldrich), 4 mM L-glutamine (Gibco/Invitrogen), and 1% penicillin-streptomycin (Gibco/Invitrogen) at 37°C in the presence of 5% CO_2. The medium composition of iMSC differentiation and growth medium in 500 ml is summarised in Table 3.2. The cells were allowed to be 100% confluent for up to 14 days and passage into a new culture dish and repeated for at least 3 passages with different intervals between each subculture. While the differentiating iPSCs are seeded in Geltrex coated plate for Passage 0 and Passage 1 only. The differentiating iPSCs are seeded without Geltrex coating from Passage 2 and onwards.

Table 3.2 Medium Composition of iMSC Differentiation and growth medium in 500 ML

COMPOSITION	WORKING CONCENTRATION	VOLUME (mL)
Alpha MEM	90%	427
Fetal Bovine Serum	10%	50
L-Glutamine	2 mM	5
Sodium Pyruvate	1 mM	5
Non-Essential Amino Acid	1X	5
HEPES	10 mM	5
Penicillin-Streptomycin	0.5%	2.5
L-Ascorbic Acid	100 μM	0.5
Total		500

3.6 Flow Cytometry

Differentiated cells were harvested with 0.25% trypsin-EDTA (Gibco) and washed with PBS containing 0.5% BSA and then resuspended to 5×10^5 cells in 50 μL of PBS containing 0.5% BSA to rest for 1 minute. Cell samples were then separately labelled on ice with an optimal dilution of fluorescein isothiocyanate-conjugated monoclonal antibodies against positive markers [cluster of differentiation (CD44), CD73, CD90, and CD105] and negative markers [IgG1, T-cell receptor alpha (TRA)-1-81, CD34, CD45, and human leukocyte antigen – DR isotype (HLA-DR)] and an empty control without

antibodies. After 20 minutes of incubation on ice or at 4°C while covered in aluminium foil, the cells were washed with PBS containing 0.5% BSA and subjected to flow cytometry analysis.

3.7 Adipogenic Differentiation Assay

To induce adipogenic differentiation, cells were incubated using the StemPro Adipogenic Differentiation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The Oil Red O solution was prepared in 0.5% final concentration by mixing Oil Red O powder and 100% isopropanol with a vortex until dissolved. Briefly, a cell density of 1 x 10^4 cell/cm^2 was seeded onto a 6-well culture dish and allowed to adhere to an MSC growth medium for 6 hours. Then the cells were washed with PBS once and the medium was changed with adipogenesis-inducing differentiation medium and changed every two days. After 14 days, the presence of intracellular lipid droplets was detected by standard staining with Oil Red O (Diapath, Bergamo, Italy), according to the manufacturer's instructions. The differentiated adipocytes were washed with 1× PBS and underwent fixation with 4% paraformaldehyde at room temperature for 1 hour. Then the adipocytes were washed with 60% isopropanol and left to air dry for 30 minutes. The 0.5% Oil Red O working solution of three parts Oil Red O and two parts of ddH2O was mixed and filtered through 0.22 μm and added onto the adipocytes to incubate in the dark at room temperature for 10 minutes. The stained adipocytes are washed with ddH2O four times to remove any excess Oil Red O staining and observed under a light microscope.

Table 3.3 Composition of 0.5% Oil Red O solution

COMPONENT	VOLUME/ REACTION	FINAL CONCENTRATION
Oil Red O Powder	0.5 g	0.5%
100% Isopropanol	100 mL	99.5%
Total	100 mL	

3.8 Osteogenic Differentiation Assay

To induce osteogenesis, cells were incubated using the StemPro Osteogenic Differentiation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The Alizarin Red S solution was prepared in 2% concentration by mixing Alizarin Red S powder and ddH_2O with vortex until dissolved and undergoing a 0.2 µm filter to remove contaminant and debris from undissolved particles. Then the filtered 2% Alizarin Red S solution pH was adjusted to 4.2 using 1M HCl and 1M NaOH by dropwise methods. Briefly, a cell density of 5 x 10^3 cells/cm^2 was seeded onto a 6-well culture dish and allowed to adhere to an MSC growth medium for 6 hours. Then the cells were washed with PBS once and the medium was changed with osteogenesis-inducing differentiation medium and changed every two days. After 21 days, the presence of calcium deposits was evaluated by Alizarin Red S (Sigma-Aldrich), according to the manufacturer's instructions. The differentiated osteocytes were washed with 1× PBS and underwent fixation with 4% paraformaldehyde at room temperature for 30 minutes. Then the osteocytes

were washed with ddH2O two times. The 2% Alizarin Red S solution was added to the osteocytes and incubated in the dark at room temperature for 30 minutes. The stained osteocytes were washed with ddH2O six times to remove any excess Alizarin Red S staining and observed under a light microscope.

Table 3.4 Composition of 2% Alizarin Red S solution

COMPONENT	VOLUME/ REACTION	FINAL CONCENTRATION
Alizarin Red S powder	2 g	2%
ddH_2O	100 mL	98%
Total	100 mL	

3.9 Chondrogenic Differentiation Assay

To induce osteogenesis, cells were incubated using the StemPro Chondrogenic Differentiation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The Alcian Blue solution was prepared in 1% concentration by mixing Alcian Blue 8GX powder and 3% acetic acid solution by inverting the mixture until dissolved and the pH was adjusted to 1.0 using 1M HCl and 1M NaOH by dropwise methods. Briefly, a cell density of 1.6 x 10^7 cell/mL was seeded with the volume of 5 µL onto a 6-well culture dish and allowed to form a micro mass in MSC growth medium for 3 hours. Then the micro mass was washed with PBS once and the medium was changed with chondrogenesis-inducing differentiation medium and changed every two days.

After 14 days, the spheroid cell formation was harvested and fixed with 4% paraformaldehyde followed by paraffin wax sectioning and evaluated by Alcian Blue (Sigma-Aldrich), according to the manufacturer's instructions. The differentiated chondrocytes were washed with 1× PBS and underwent fixation with 4% paraformaldehyde at room temperature for 30 minutes. Then the osteocytes were washed with ddH2O two times. The 1% Alcian Blue solution was added to the sliced chondrocytes and incubated in the dark at room temperature for 30 minutes. The stained chondrocytes were washed with ddH2O four times to remove any excess Alcian Blue staining and observed under a light microscope.

Table 3.5 Composition of 1% Alcian Blue Solution

COMPONENT	VOLUME/ REACTION	FINAL CONCENTRATION
Alcian Blue 8GX	1 g	1%
3% Acetic Acid Solution	100 mL	99%
Total	100 mL	

3.10 iMSC-CM Coculture of H1975

For non-cell-to-cell interaction of indirect coculture, the iMSC-CM was collected from the supernatant of a 10 cm^3 cell culture dish of confluent iMSC. The collected supernatant was centrifuged at 1000 × rpm for 5 minutes and filtered with a 0.22-μm membrane filter. H1975 was passaged and seeded at 1.5 × 10^5 cells on a 10-cm cell culture dish with three different medium conditions (Control, 50% iMSC-CM and 100% iMSC-CM) while the other part of the medium was topped up with H1975 growth medium to a total of 10 mL. The mediums were changed every two days according to their individual medium conditions. Cell morphological changes were observed every day for 5 days under a light microscope or until at least 95% confluent.

For cell-to-cell interaction of indirect coculture, H1975 was passaged and seeded at the bottom Falcon® 6-well TC-treated Polystyrene Permeable Support Companion Plate (Corning) and allowed to adhere with 2 hours incubation, while iMSC was passaged and seeded onto the 0.4 μm Falcon® Transparent PET Membrane (Corning) and allowed to adhere with 2 hours incubation on an empty well, both H1975 and iMSC were incubated under similar condition under 5% CO_2 atmosphere at 37°C. Upon 2 hours of incubation, the iMSC PET Membrane was placed onto the 6-well culture dish on top of H1975. The lower chamber with adhered H1975 cancer cells was added with 3 mL of H1975 growth medium while the upper chamber with the adhered iMSC was added with 3 mL of MSC growth medium, both chamber

media were changed daily. Cell morphological changes were observed every day for 5 days under a light microscope or until at least 95% confluent.

Table 3.6 The iMSC-CM coculture medium volume and conditions.

Condition	H1975 growth medium (mL)	iMSC-CM (mL)
1. Control	10	0
2. 50% iMSC-CM	5	5
3. 100% iMSC-CM	0	10

3.11 Total Ribonucleic Acid (RNA) Extraction

The iMSC-CM, iMSC-transwell treated and untreated H1975 cancer cells were isolated, harvested and lysed in 1 mL of TRIzol reagent (Invitrogen, CA, USA) and vortex for 3 minutes. The centrifuge machine was allowed to pre-cool to 4°C. Then 200 µL of BCP were added to the lysate and vortex for 2 minutes followed by 5 minutes of incubation at room temperature. The lysate with BCP was then centrifuged at 13,000 RPM for 15 minutes at 4°C. Then the supernatant of 500 µL was transferred into a new microcentrifuge tube and added with 1 part of 100% isopropanol and vortex for 2 minutes followed by 5 minutes of incubation at room temperature. The supernatant with isopropanol was then centrifuged at 13,000 RPM for 15 minutes at 4°C. All the supernatant was removed with visible pellets on the bottom of the microcentrifuge tube.

Then 100 µL of 80% ethanol was added followed by then centrifuge at 13,000 RPM for 10 minutes at 4°C and the supernatant was discarded, the step was repeated twice. Lastly, the pellets at the bottom of the microcentrifuge test tube were allowed to air dry for 20 minutes and dissolve in DEPC H_2O with 10 - 30 µL depending on the pellet size. The dissolved RNA was quantified using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA).

3.12 cDNA Conversion

Reverse transcription polymerase chain reaction (RT-PCR) was performed to convert RNA extracted into cDNA prior to qPCR experiments with a Superscript III kit (Invitrogen, CA, USA). The first PCR reaction was a mixture of 1 µg RNA, 100 ng of random primer, 1 µL of 10 mM dNTP and topped up with RNAse-free water up to 14 µL. The setting for the first PCR reaction was 65°C for 5 minutes followed by incubation of ice for 1 minute. The second PCR reaction mixture was prepared with 4 µL 5x buffer, 1 µL 0.1M DTT and 1 µL RT enzyme. The setting for the second PCR reaction was 25°C for 5 minutes, followed by 50°C for 1 hour and 75°C for 15 minutes and cooling down to 4°C. All the cDNA were kept at 4°C until RT-PCR was set up and for long-term storage they were placed at -80°C.

Table 3.7 The first RT-PCR reaction components.

COMPONENT	VOLUME/ REACTION
RNA	1 µg
Random Primer (100ng/µL)	1 µL
10 mM dNTP	1 µL
ddH_2O	Top-up
Total	14 µL

Table 3.8 The second RT-PCR reaction components.

COMPONENT	VOLUME/ REACTION
Component from 1st PCR reaction	14 µL
5x Buffer	4 µL
0.1M DTT	1 µL
RT Enzyme	1 µL
Total	20 µL

3.13 Quantitative Polymerase Chain Reaction and qPCR

The iMSC-CM, iMSC-TC treated and untreated H1975s were collected and washed twice in PBS, and the total RNA was isolated by TRIzol reagent (Thermo Scientific), according to the manufacturer's protocols as stated in section 3.9. The extracted RNA was quantified with NanoDrop 2000 (Thermo Scientific); one microgram of RNA was prepared for the reverse transcription reaction using SuperScript reverse transcription III (Invitrogen) to synthesise complementary DNA (cDNA) strands. cDNA was used in the following quantitative polymerase chain reaction (qPCR) and reverse transcription polymerase chain reaction (RT-PCR). The final qPCR reaction product was mixed according to Table 3.9 and loaded 10 μL into each well of 0.2 mL volume 96-Well PCR Plate individually in the dark, with three wells of technical replication for each primer set. The loaded 96-Well PCR Plate was labelled and covered with a transparent protective film followed by aluminium and undergo quick centrifugation. Next, the aluminium foil was removed and loaded labelled 96-Well PCR plate was placed into QuantStudio™ 3 Real-Time PCR System Machine for qPCR reaction initiation.

According to the product's instructions, the qPCR reaction was performed using the Fast SYBR Green Master Mix. Briefly, the experiment comprised initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds with annealing at 60°C for 30 seconds and extension at 72°C. The qPCR analysis was performed using three independent biological and technical replicates.

Table 3.9 The qPCR reaction components.

COMPONENT	VOLUME/ REACTION	FINAL CONCENTRATION
2x Fast SYBR Green Master Mix	5.0 µL	1X
Primer 10 µM (Forward)	0.5 µL	0.2 µM
Primer 10 µM (Reverse)	0.5 µL	0.2 µM
RNAse Free Water	3.0 µL	-
cDNA Template	1.0 µL	25.0 ng
Total	10.0 µL	

3.14 Migration Assay

The iMSC-CM, iMSC-TC treated and untreated H1975 were both harvested from their respective culture dish into serum-free H1975 growth medium 50 mL falcon tube. The harvested cell was seeded at a cell density of 1 x 10^4 cells in each FluoroBlok™ cell culture insert (Corning) and topped up to 750 µL, and placed onto a 24-well culture dish with 1 mL of 1% FBS H1975 growth medium at the bottom chamber. The cancer cells were allowed to adhere and migrate for 18 hours. Each cell condition was seeded onto 3 independent inserts on independent wells for each experiment and the assay was triplicate in three technical replicates and independent biological replicates.

After 18 hours, the culture inserts were removed from the 24-well culture dish and washed twice with PBS, followed by fixation through soaking

in 4% paraformaldehyde for 2 minutes and washed twice with PBS. The fixed cells undergo permeabilisation by soaking in 100% methanol for 20 minutes and washing twice with PBS. The fixed permeabilised cells in culture inserts were stained with 1.0% PI dye in PBS and incubated in the dark for 15 minutes and washed twice with PBS. Excess cells remaining in the interior of the culture inserts are gently scraped off with cotton swabs and the cells are viewed under an inverted fluorescent microscope for observation of migrated cells to the exterior of the culture inserts and image capturing. Cell counting was conducted using ImageJ on the captured image based on three different areas on each well with three wells for each set of experiments.

3.15 Statistical Analysis

Data were expressed as mean ± SD, whereas statistically significant differences between two groups or among multiple groups were detected by a paired Student's two-tailed t-test and one-way ANOVA with Tukey's post hoc, respectively, using the SPSS, edition 25, software (Chicago, IL, USA). The criterion for significance was set as $p < 0.05$, and highly significant differences in the statistics were accepted if $p < 0.0001$. All data presented are representative of at least three independent experiments.

CHAPTER 4

RESULTS

4.1 Characterisation of induced Pluripotent Stem Cells (iPSCs)

4.1.1 Microscopic Observation of iPSCs

The obtained iPSCs are reprogrammed human peripheral blood mononuclear cells from a healthy male donor. The morphology of undifferentiated iPSCs is known to be cells with a large nucleus and less cytoplasm that formed in compact colonies that have distinct borders and edges, like ESCs (Nagasaka, et al., 2017). From our observation (Figure 4.1), our reprogrammed iPSCs show compact colonies with distinct borders, edges and large nuclei.

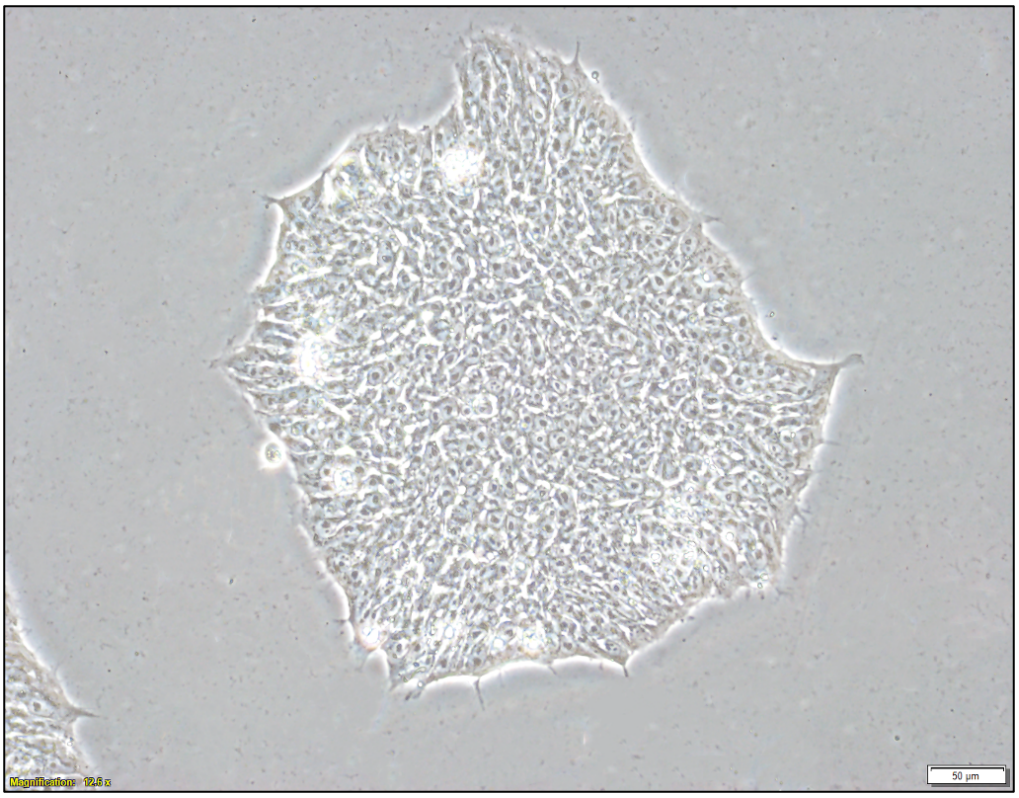


Figure 4.1 Bright-field image showing the representative morphology of iPSCs. Compact colonies with distinct borders, edges and large nuclei can be observed.

4.1.2 Pluripotency and Stemness Characterisation

Alkaline phosphatase is an enzyme found in almost all living organisms. Studies of alkaline phosphatase expression and activity in humans and other mammals have frequently been associated with cell viability during clinical trials or developmental studies. In addition to identifying pluripotent embryonic stem cells, alkaline phosphatase is one of the key markers for identifying pluripotent stem cells like iPSC (Štefková, et al., 2015). However, alkaline phosphatases exist in several isoenzymes and isoforms, which have different tissue-specific expressions and functions. Here, the role of alkaline phosphatase

was used as a stem cell marker for identifying the presence of iPSCs. The observation is shown in Figure 4.2. demonstrated a high amount of alkaline phosphatase expression detected based on the staining, indicating the stemness properties of the derived iPSCs.

In addition, immunofluorescence staining was used to identify further pluripotent markers on key transcriptional factors like OCT4, SOX2, NANOG, and SSEA4; and surface markers like TRA-1-81, and TRA-1-60. Our observation shows the presence of transcriptional factors and surface markers commonly used for pluripotency markers for iPSCs (Figure 4.3) (Yakahashi, et al., 2007; Khudiakov, et al., 2017).

Based on the microscopic examination and immunofluorescence staining results, the iPSCs were found to be stable as these cells have not undergone any form of differentiation and still maintained pluripotency properties after a high number of passages (Passage 29) and long-term cryopreservation of four four-year periods.

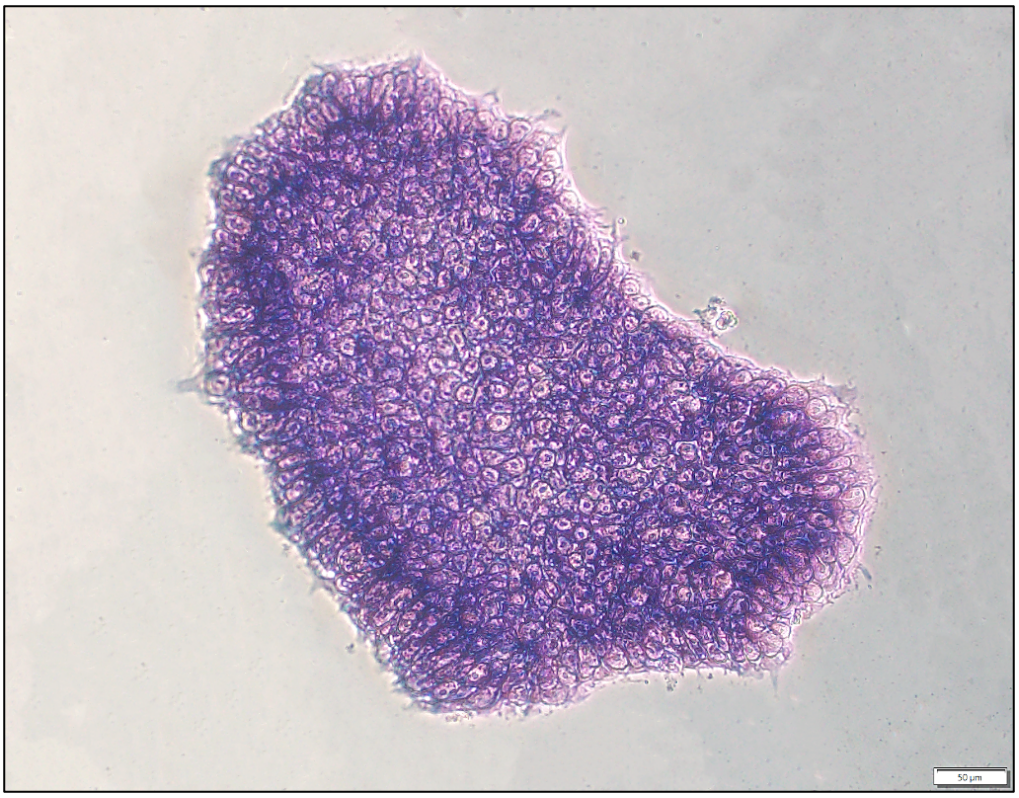


Figure 4.2 Alkaline phosphatase stain of iPSCs showing the high intensity of the positive purple staining (Scale bar, 50 µm). The presence of pluripotent marker alkaline phosphatase can be observed.

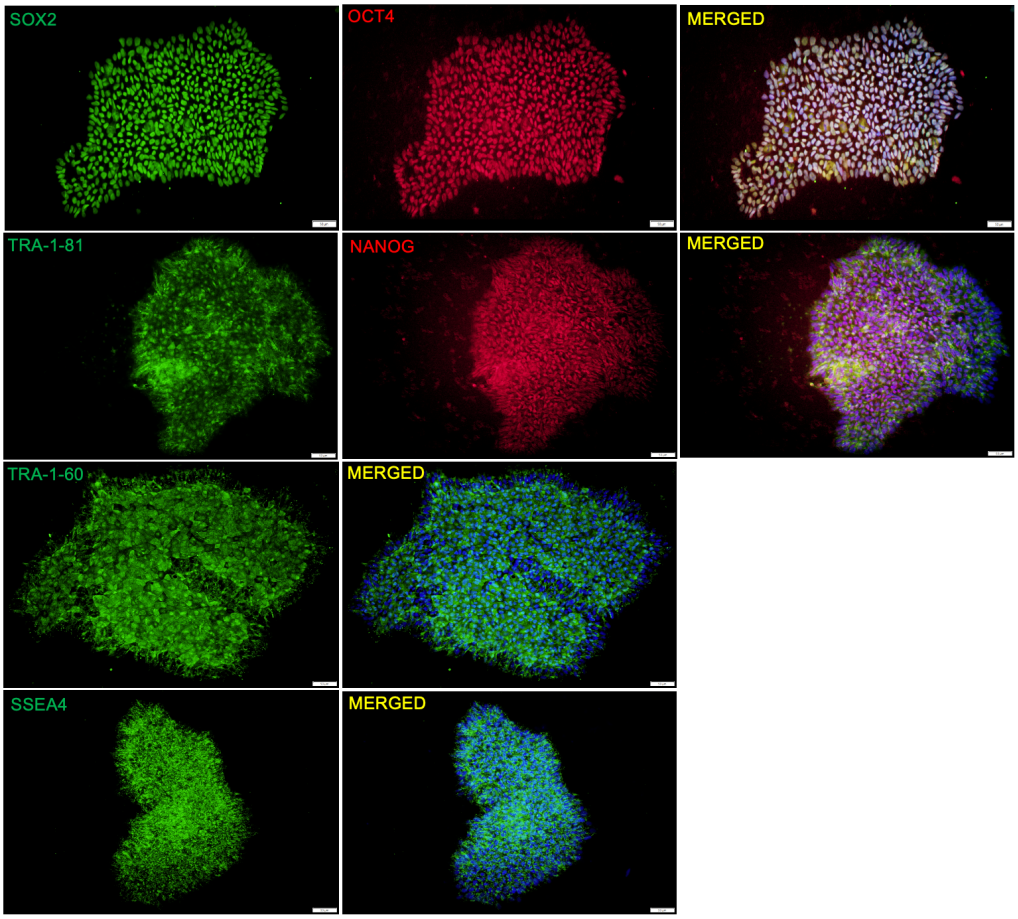


Figure 4.3 Immunofluorescence stain of iPSCs for stemness markers (OCT4, SOX2, NANOG, SSEA4, TRA-1-81, and TRA-1-60; Scale bar, 50 µm). The staining results show high intensity for stemness markers that verified the iPSCs characterisation.

4.2 Differentiation of iPSCs to MSC-like cells (iMSCs)

The differentiation of iPSCs to iMSCs was analysed at a different time point of the differentiation process involving the use of spontaneous differentiation method by using a mesenchymal differentiation culture medium. The iMSC cells generated were subjected to the characterisation of MSC-like

properties using the qPCR method on the pluripotent marker and mesenchymal marker, also the observation of morphological changes.

4.2.1 Differentiation Timeline of iPSCs to iMSCs

A spontaneous differentiation method was conducted, where a mesenchymal differentiation culture medium was used to replace iPSC StemFlex growth medium after the iPSCs had recovered from the thawing process. The mesenchymal differentiation medium was replaced every two days for an overall 38-day duration for a complete differentiation. The differentiating iPSCs were passage on days 14th, 28th, 35th and 38th based on the morphology and confluency (Figure 4.4). From the microscopic observation, the iPSCs were shown to slowly shift loss from their round colonies-forming features to fibroblast-like morphology (Figure 4.5) and require no gelatine coating for attachment on day 28th. The majority of the differentiating iPSCs appeared to be MSC-like morphology after day 28th (Passage 2, P2) and completely appeared to be MSC-like morphology after day 35th (Passage 3, P3). At this point, the differentiated cells were subjected to MSC-like characterisation with qPCR, flow cytometry and trilineage differentiation.

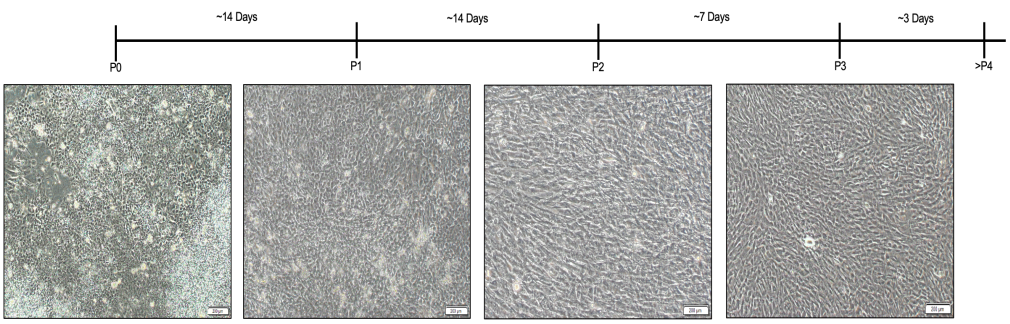


Figure 4.4 Timeline differentiation of iPSC to iMSC over 38 d. The morphology of iPSCs gradually shifts towards a spindle-like sharp that resembles MSCs.

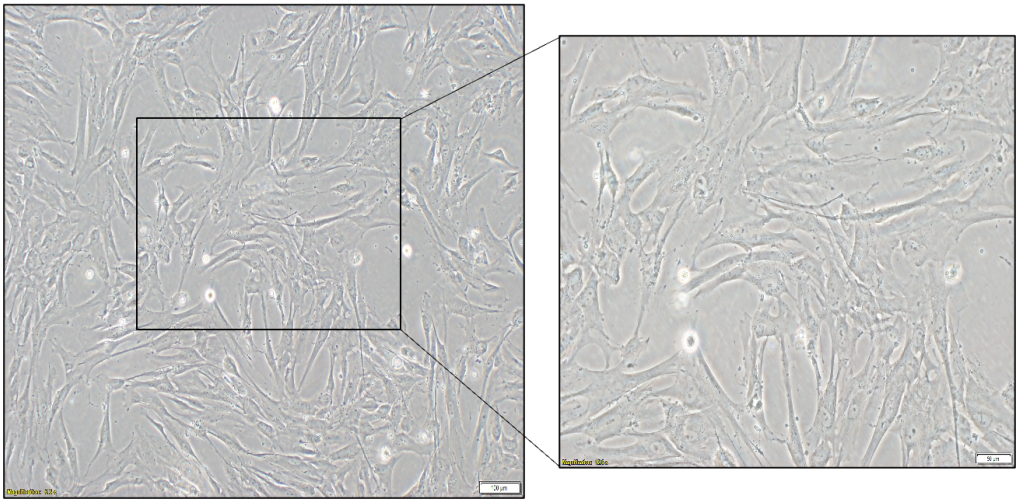


Figure 4.5 Bright-field image showing the representative morphology of iMSCs in spindle-like shape.

4.2.2 qPCR Analysis of iPSC and MSC Markers During Differentiation

The use of pluripotent markers (OCT4, SOX2 and NANOG) and MSC identification markers (CD44 and CD105) were used to identify the degree of differentiation (Figure 4.6). The results showed that main pluripotent markers namely OCT4, SOX2 and NANOG were highly expressed in iPSC cells but reduced significantly to a negligible level at iMSC differentiation stages of P4 and P5 while there were significant increases in MSC identification markers like CD44 and CD105 when compared to that of iPSCs. The qPCR analysis generally indicates the complete differentiation of iPSC to MSC-like cells.

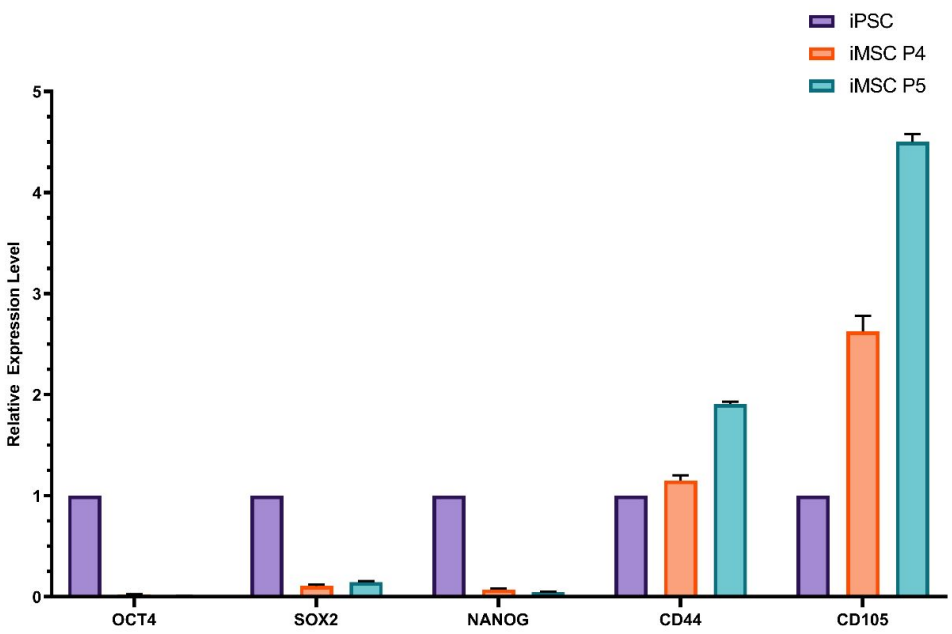


Figure 4.6 Validation of differentiation through qPCR Analysis of iPSC and MSC Markers on iPSC, iMSC P4 and iMSC P5. The results show iPSC markers were suppressed with MSC markers highly expressed over time during differentiation as shown in iMSC P4 and iMSC P5 when compared to the control of iPSC.

4.2.3 Characterisation of iMSCs

Flow cytometry analysis was used to identify the surface markers on the differentiated MSC-like cells. From the analysis, positive surface markers (CD44, CD73, CD90 and CD105) were expressed from 95% to 100%; while negative surface markers (HLA-DR, TRA1-81, IgG1, CD19 and CD45) were expressed <2% (Figure 4.6). The surface marker expression was similar to that of MSC based on the ISCT minimum criteria (2006) for a cell to be referred to as MSC (Dominci, et al., 2006). This finding further indicates the success of the

differentiation methods used for differentiating the iPSC into iMSC in a highly effective manner.

To test the multipotent capability, trilineage differentiation was conducted using a specific differentiation-inducing medium. Adipogenic and osteogenic differentiation was conducted in a 2D monolayer while chondrogenic differentiation was conducted in a 3D spheroid culture. The results obtained have shown that the differentiated cells could differentiate into all three forms (Figure 4.8) but with different efficiency levels. The highest efficiency to lowest was observed from osteogenesis to adipogenesis and then to chondrogenesis.

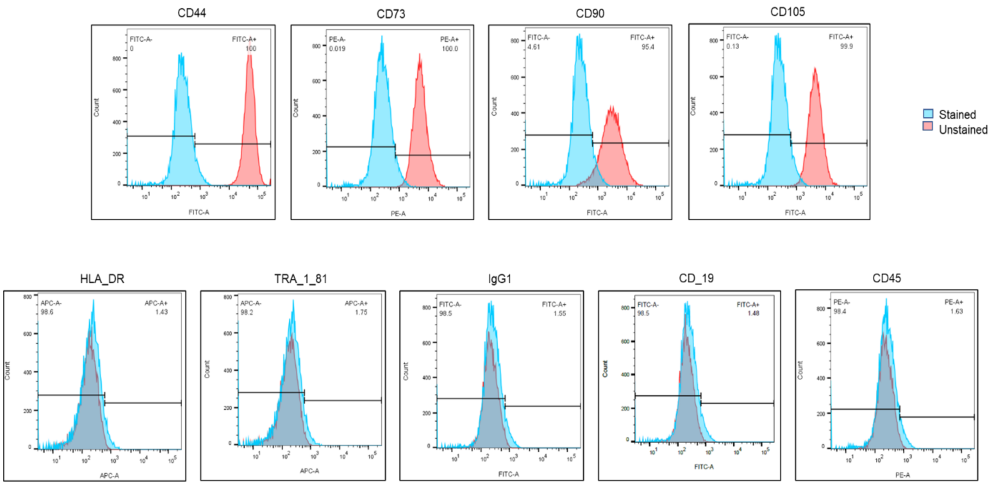


Figure 4.7 Flow cytometry–based analysis of MSC surface marker in iMSCs. For MSC positive surface markers (CD44, CD73, CD90, and CD105) and negative surface markers (HLA-DR, TRA-1-81, IgG1, CD19, and CD45). The light blue colour represents the control, while the red colour represents the iMSCs. The flow cytometry results of iMSCs

show >95% expression for positive surface markers while <2% for negative markers that fulfil the ISCT guideline to classify as MSC-like cells.

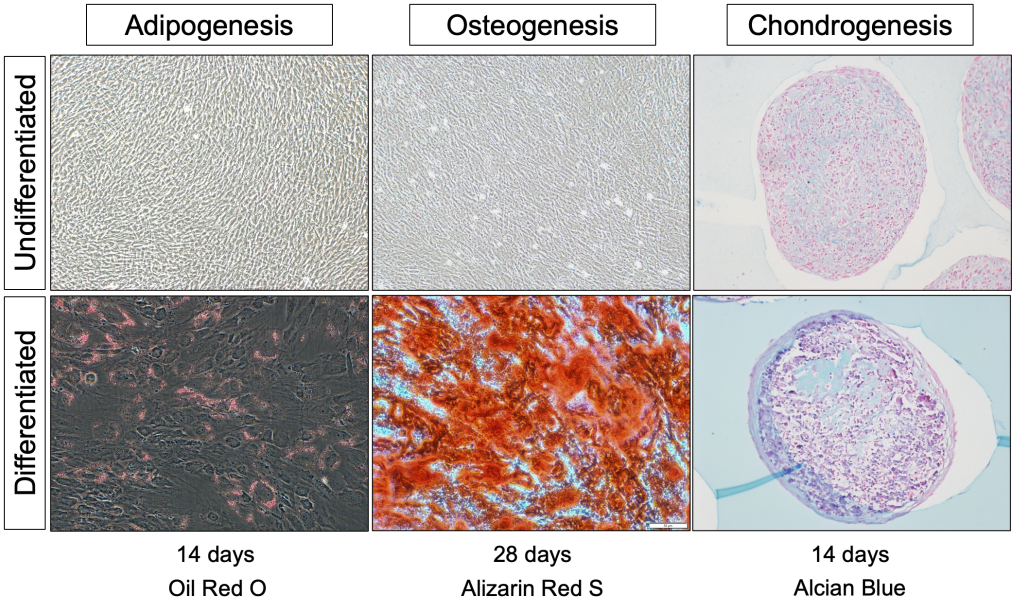


Figure 4.8 Trilineage differentiation of MSC-like cells. iMSCs were adipogenic, chondrogenic and osteogenic differentiated, assessed for up to 21 d, and stained accordingly with their specific stain (Oil Red O, Alizarin Red S and Alcian Blue).

4.3 Evaluation of the effects of iMSC on lung cancer cell properties

4.3.1 Indirect methods of coculture of iMSC with H1975.

The H1975 were treated with iMSC-CM and iMSC-TC for 5 days with medium changed every 2 days. The treated cells were observed under the microscope every day for the detection of any morphological changes. From the observation, there was a slight change in morphology in iMSC-CM treatment regardless of concentration when compared to the control, while TC treatment

did not show any morphology variation from the parental control cell (Figure 4.9). This indicates that iMSC-CM may have different effects from iMSC-TC treatment on H1975. The H1975 cell morphology in iMSC-CM treated was slightly elongated compared to parental control cell and iMSC-TC treatment. The changes in morphology may be a sign of early EMT or environment adaptation of cells in different culture conditions. However, further analysis may be needed to confirm the cause and effects of morphological changes that occurred.

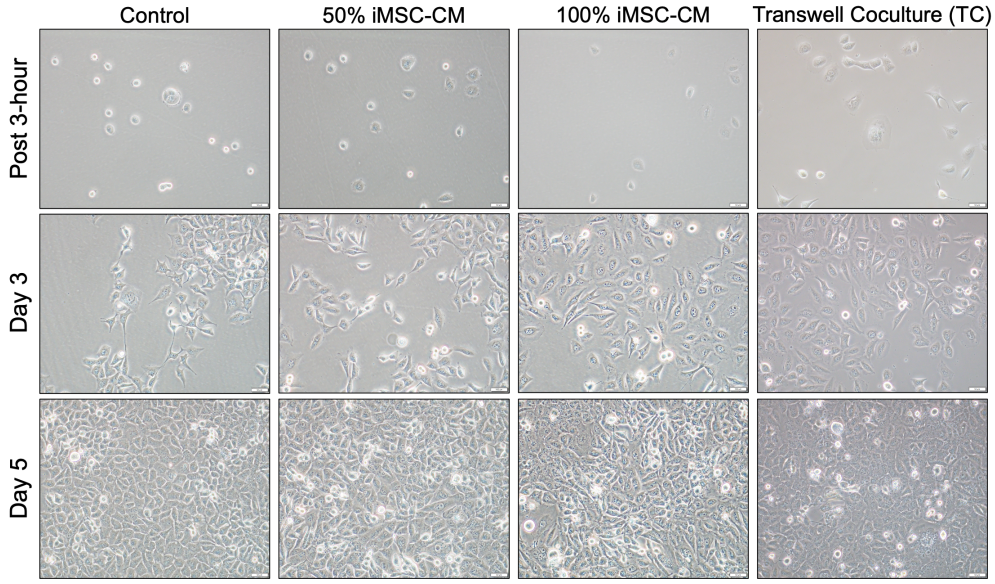


Figure 4.9 Bright-field image of H1975 coculture treatment of iMSC-CM of different concentrations and iMSC-TC. iMSC-CM cocultured morphology of non-small-cell lung cancer H1975 at different time points (post 3 h, days 3 and 5) and compared with control, at different dosage concentrations (50%, 100%) and Transwell Coculture (TC). The morphology using iMSC-CM was shown to be more widespread as compared to iMSC-TC, while iMSC-TC is more similar to its original morphology in control.

4.3.2 qPCR Analysis of Treated and Untreated H1975

The iMSC-CM, iMSC-TC treated and untreated parental H1975 cancer cells were harvested for RNA extraction with TRIzol reagent to analyse its RNA expression in terms of endothelial cell markers (EPCAM), cell cycle markers (P21 and P53) and EMT markers (VIMENTIN, SLUG, SNAI1). The RNA expression of all markers shows significant differences in general through the comparison of control with iMSC-CM treatment and control with iMSC-TC treatment except for SNAI1 in iMSC-CM which remained with no significant differences.

The expression of endothelial markers, EPCAM, has decreased significantly for iMSC-CM and iMSC-TC treatment (Figure 4.9). The expression of cell cycle markers of P21 has shown a significant increase for iMSC and iMSC-TC treatment but a decrease for P53 (Figure 4.10). Then, the EMT markers showed a significant increase for VIMENTIN and SLUG in iMSC-CM but no significant differences for SNAI1 (Figure 4.11). However, for transwell coculture, all EMT markers of VIMENTIN, SLUG and SNAI1 showed a significant decrease for TC (Figure 4.11).

Based on the relative expression level of all the markers, iMSC-CM treatment has been shown to be promoting EMT and able to enhance the migratory properties of the cancer cells while the transwell coculture treatment has been shown to inhibit EMT and decrease the migratory properties of cancer

cells. To further confirm if their migratory properties are affected, a migration assay was conducted.

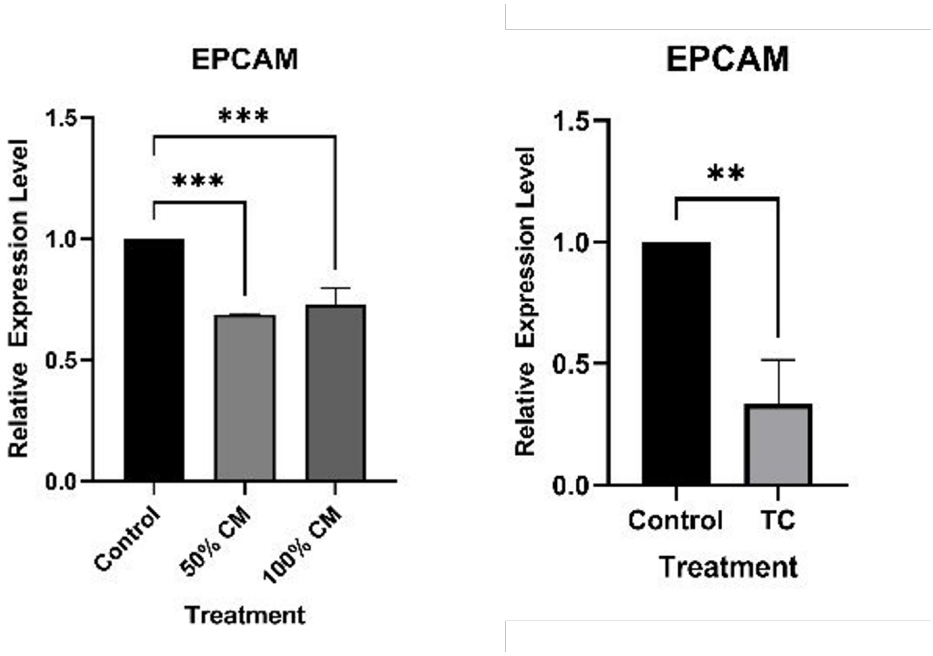


Figure 4.10 Quantitative polymerase chain reaction showing the endothelial cell marker of EPCAM gene expressions in control and treated H1975. The expression of the housekeeping GAPDH gene was used for normalisation. In 50% CM, 100% CM and TC, the EPCAM expression was significantly decreased when compared to control. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, and error bars are the SD.

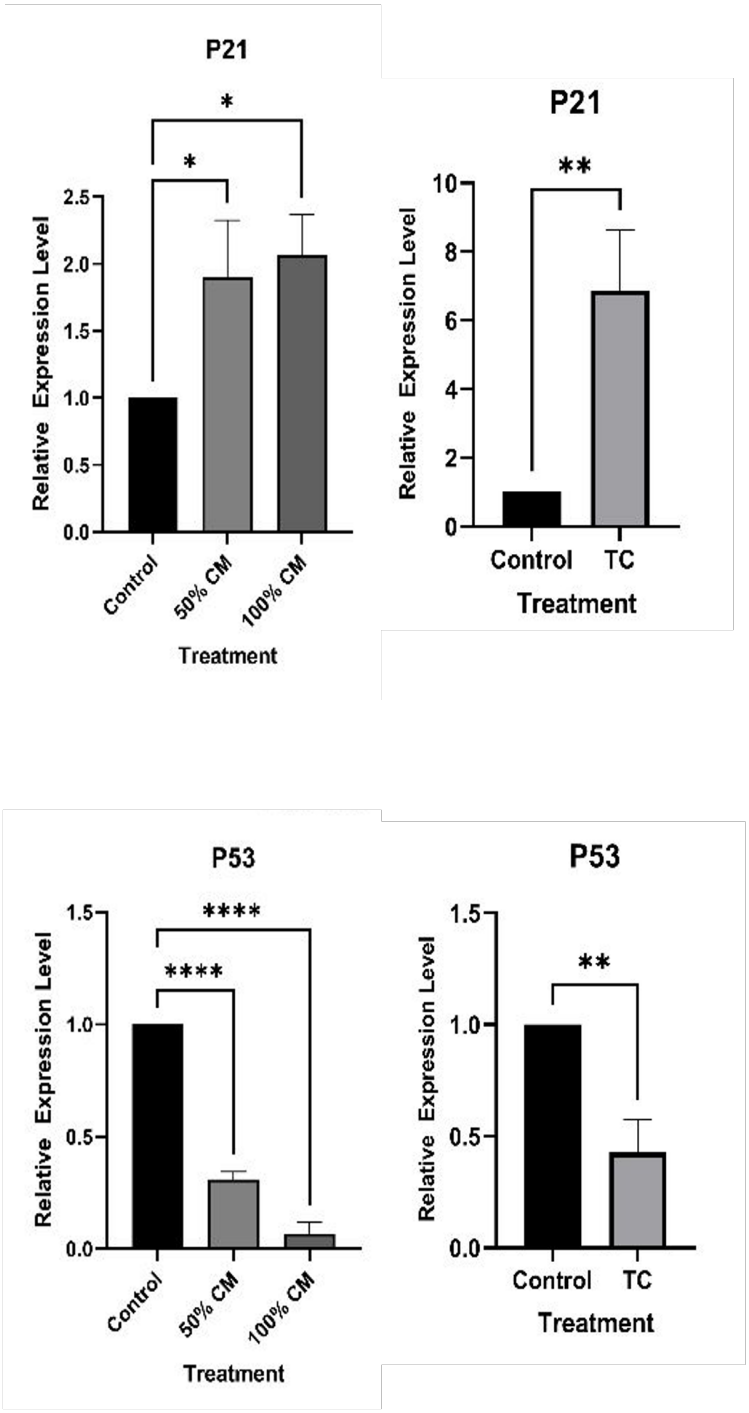
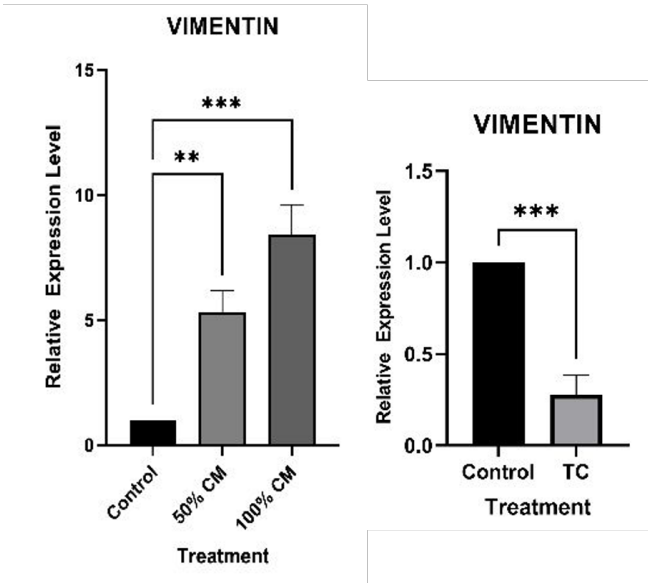


Figure 4.11 Quantitative polymerase chain reaction showing the cell cycle markers of P21 and P53 gene expressions in control and treated H1975. The expression of the housekeeping GAPDH gene was used for normalisation. In 50% CM, 100% CM and TC, a similar trend can be observed with P21 significantly increase while P53 significantly decrease

when compared to control. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, and error bars are the SD.


VIMENTIN
Relative Expression Level
15
10
5
0
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**
Control
50% CM
100% CM
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VIMENTIN
Relative Expression Level
1.5
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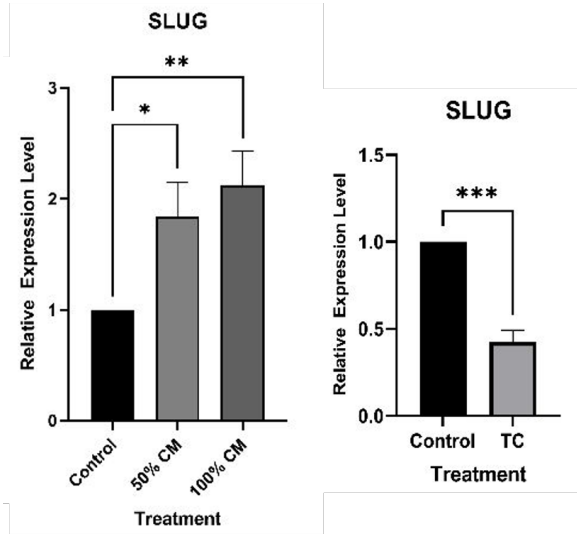
Treatment


SLUG
Relative Expression Level
3
2
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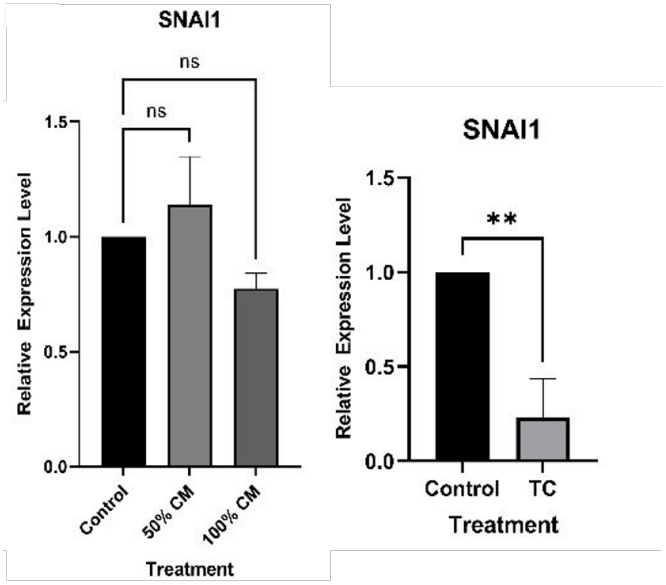


Figure 4.12 Quantitative polymerase chain reaction showing the EMT markers of Vimentin, Slug and Snai1 gene expressions in control and treated H1975. The expression of the housekeeping GAPDH gene was used for normalisation. For EMT markers, in 50% CM and 100CM, the expression of VIMENTIN and SLUG shows a significant increase while TC shows a significant decrease compared to the control. However, for SNAI1, 50% CM and 100% CM show no significant differences while TC shows a significant decrease compared to control. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, and error bars are the SD.

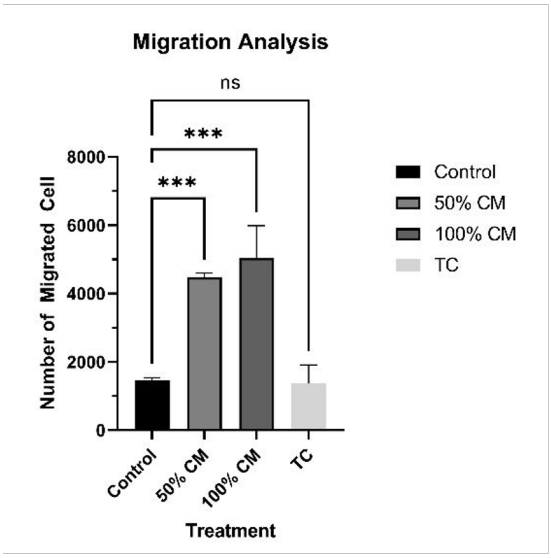
4.3.3 Migration Assay

The ability of live cells to migrate is crucial for normal development, functioning immune system, and disease development or progression such as cancer metastasis and inflammation. Methods to measure or examine cell migration techniques are very useful and essential in biomedical research,

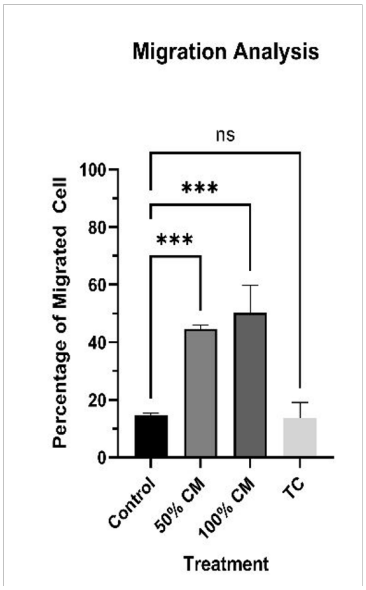
including cancer biology, immunology, vascular biology, cell biology, and developmental biology (Justus, et al., 2014). The understanding of cancer cell migratory capability is essential for the detection of its degree of metastasis and its potential threat in cancer patients. Two standard methods of measuring cell migration are the cell culture wound closure assay or wound healing assay or scratch assay. The migratory rate is measured by the speed of wound closure induced by pipette tips scratch on a confluent cell monolayer or using a wound healing chamber to isolate two confluent colonies with a gap between the colonies. The cells are pre-treated with Mitomycin-C to prevent cell proliferation which may disrupt the results. Inverted microscope snapshots can be used to quantify wound closure rate and cell migration over time by taking multiple snapshots and comparing them. Time-lapse microscopy systems can also document a more detailed closure process. Another method known as the transwell cell migration and transwell invasion assay measures the capacity of cell motility and invasiveness toward a chemo-attractant gradient. In the transwell cell migration assay, the cells are seeded on top of the transwell chamber in serum-free medium or low-concentration serum medium while the lower chamber has a higher concentration serum medium that acts as a chemo-attractant to attract the cells to migrate past the transwell membrane. For transwell cell invasion assay, coatings like Collagen, Geltrex or Matrigel are often used on the transwell membrane to mimic the cell barrier in the human body. Similar to the serum and medium condition in transwell cell migration assay, the cells are observed on their invasive capability by their penetration ability through the barrier to a higher nutrient area.

In this experiment, we used the transwell migration assay, which is one of the commonly used methods to test the migratory properties of a cell line. Based on the observation, iMSC-CM treated H1975 have shown enhanced migratory properties when compared with the control, while iMSC-TC treated H1975 shows no significant differences from the control (Figure 4.12). A higher number of cells have migrated from iMSC-CM treated H1975 compared to iMSC-TC treated H1975. This indicates that the cell treated in iMSC-CM may have to release factors that enhance its migration capability while the cell treated in iMSC-TC does not have releasing factors that enhance migration capability.

Number of Migrated Cells



Percentage of Migrated Cells



Conditions	Control	50% CM	100% CM	TC
Number of migrated cell	1463±76	4466±125	5038±943	1364±546
Percentage of migrated cell	14.6±0.75%	44.7±1.25%	50.4±9.43%	13.6±5.47%

Figure 4.13 Migration assay cell count in control and treated H1975 and quantified table of number of migrated cells and percentage of migrated cells. In 50% CM and 100% CM, the number of migrated cells shows a significant increase while TC shows no significant differences when

compared to the control. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, and error bars are the SD.

4.3.4 Cytokine and Chemokine Assay

Cytokines and chemokines are secreted proteins that play a vital role in cell growth, cell differentiation, specific pathway activation and cell function activation. Cytokines and chemokines are also the key regulators of the interaction for immune responses, immunomodulation, and the immune organs' cellular arrangement (Borish and Steinke, 2003). Inflammation and cancer have been linked since the 17th century (Balkwill and Mantovani, 2001). Intensive study has been done in the past to provide knowledge regarding the cells, cytokines, and physiological processes associated with inflammation and cancer (Marx, 2004; Hagemann, et al., 2007; Yu, et al., 2009; Pyne and Pyne, 2013). It is well known that chronic inflammation can result in certain cancer development, and solid tumours, in turn, can initiate and perpetuate local inflammatory processes that enhance the growth and metastasis of tumours (Bhatelia, et al., 2014; Deng, et al., 2016). This has led to targeting inflammatory pathways in cancer treatment (Munn, 2017).

Based on the observation from iMSC-CM, H1975-CM and transwell coculture conditioned medium (TC-CM), there may be possible interaction between both cell lines of iMSC and H1975 that affects their original releasing cytokines and chemokines (Figure 4.13). The release of cytokines like CXCL1, CXCL12, GM-CSF and chemokines CCL2 were absent after transwell

coculture. However, SDF-1 was present only after transwell coculture. A summary of the absence and presence of original cytokines and chemokines after transwell coculture was tabulated in Table 4.1. All the chemokines and cytokines that were absent after transwell coculture were previously known to be associated with cancer-promoting activities. However, the additional presence of chemokines of SDF-1 is associated with tumour-homing activities (Teicher and Fricker, 2010). It is also worth noting that the secretion of SDF-1 from MSC has been known to promote cardiac endothelial microvascular regeneration in myocardial infarction rat models-(Gong, et al., 2019).

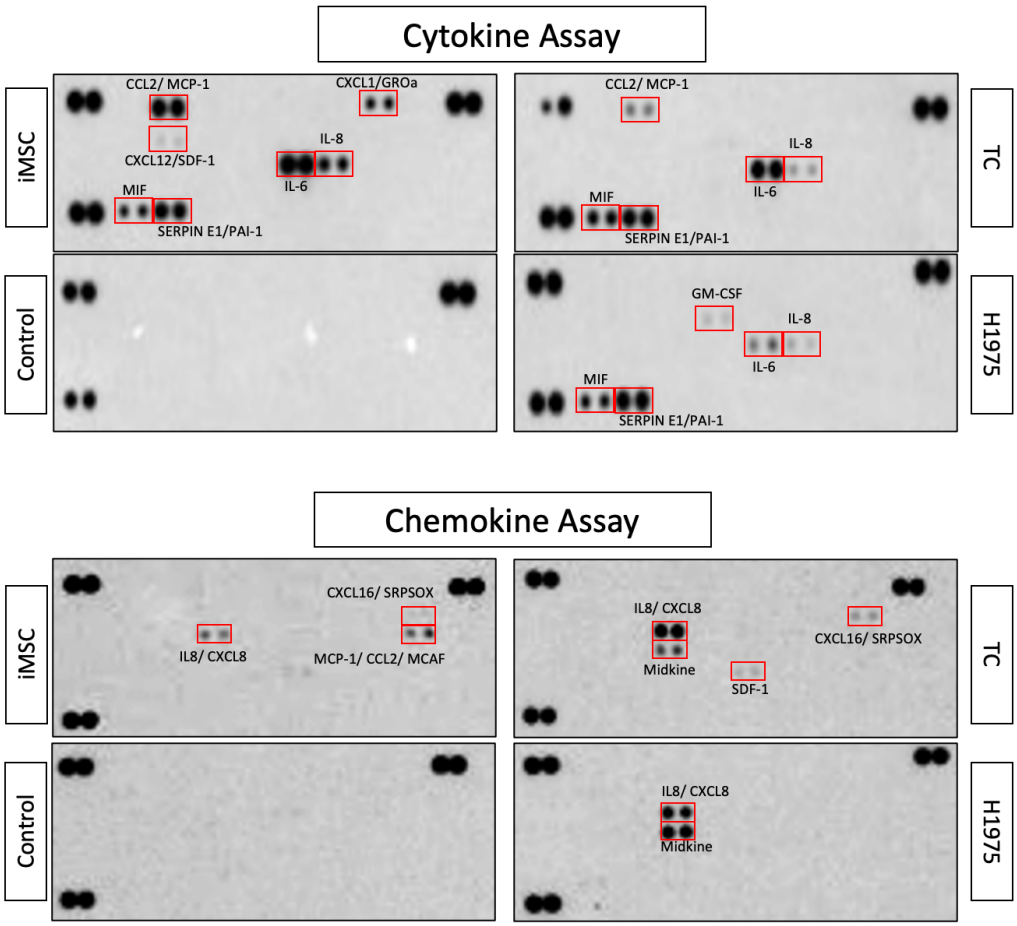


Figure 4.14 Cytokine and chemokine assay using conditioned medium from iMSC, H1975 and iMSC-TC with H1975. The observation shows the

presence of a new chemokine while suppressing the expression of several cytokines and chemokines shown in Table 4.1.

Table 4.1 Summary of cytokines and chemokines in the culture medium of iMSC, H1975 and coculture of iMSC-H1975

Markers	iMSC	H1975	Transwell Coculture
Cytokines			
CXCL1/GROa	√	X	X
CXCL12/SDF-1	√	X	X
GM-CSF	X	√	X
Chemokines			
CCL2/ MCP-1/ MCAF	√	X	X
SDF-1	X	X	√

CHAPTER 5

DISCUSSION

5.1 iMSC Characterisation

5.1.1 Fundamental Biological Properties

The evaluation showed that the iMSC derived in this study possessed the fundamental biological properties mentioned by ISCT in 2006. The generated iMSC has the capability to adhere to a culture dish without the need for a coating that fulfilled the adherent to the plastic surface, showed fibroblast-shape morphology, and was capable of trilineage differentiation into adipocytes, chondrocytes and osteocytes (Dominci, et al., 2006). The majority of the research team has long used the guideline from ISCT as a guideline for differentiating iPSC into iMSC, while most of their products can fulfil the requirements for adherence to the culture dish, morphology, surface markers and trilineage differentiation. However, the trilineage differentiation efficiency for adipocytes, chondrocytes and osteocytes varies among all the researchers' results due to the iPSC origin and methods used (Chen, et al., 2012; Kang, et al., 2015; Xu, et al., 2019; Xia, et al., 2020; Zhu, et al., 2022).

5.1.2 RNA Expression

The MSC markers expression from iMSC further increased from P4 to P5 with no significant increase in pluripotent markers. This indicates a more specific differentiation towards the MSC pathway by the MSC-like cells with no sign of reversing effect back into iPSC. A study by Rajasingh's and colleagues compared the RNA expression with qPCR and fundamental biological properties between iPSC, umbilical cord MSCs (UC-MSCs) and iMSC derived from urinary epithelial iPSCs (UE-iMSCs). The RNA expression trend shows a similar pattern of increase in MSC marker expression and decreases in pluripotent markers for UE-iMSCs with differentiation at D21 with iPSC as control. While the UE-iMSCs expression is similar to UC-MSCs, indicating success in differentiation from iPSC. The authors further compared UCMSCs and UE-iMSC in different passages of passage 7 and passage 18. At the late passage of passage 18, MSC markers of CD73 and CD105 in UE-iMSCs were shown to be significantly higher than UC-MSCs, however, their proliferation rate and morphology show no significant differences despite the differences in CD73 and CD105 expression (Rajasingh, et al., 2021). Where the higher passage of iMSC remains stable in expression level similar to MSCs.

5.1.3 Flow Cytometry Evaluation of Surface Markers

The surface markers of the iMSCs were evaluated based on the absence and presence of surface markers criteria set by the ISCT (Dominci, et al., 2006). With the use of passage 4 iMSC for analysis, the positive surface markers

(CD44, CD73, CD90 and CD105) show more than 95% expression while negative surface markers and control (CD19, CD45, IgG1, TRA-1-81 and HLA-DR) shows less than 2% expression. The surface markers expression has shown a successful differentiation using an MSC differentiation medium that induces spontaneous differentiation. The presence of MSC-identifying markers and the lack of pluripotent markers provide sufficient information that the cells are no longer iPSC and have fully differentiated into MSC-like cells (Ramos, et al., 2016).

Zhu's and colleagues have used similar spontaneous differentiation methods of MSC culture medium derived from Xia's group formulation to differentiate iPSC into iMSC that was successful while fulfilling the ISCT guideline in terms of morphology, adherent capability, surface markers and trilineage differentiation capability. Xia's group used passage 4 of the differentiating iMSC for testing of mesenchymal surface markers results of CD73, CD90 and CD105 that express more than 95% and CD45 and HLA-DR to express lower than 2% while Zhu's and colleague was also able to obtain the similar results using the same formulation of spontaneous differentiation methods from Xia's protocol (Xia, et al., 2020; Zhu, et al., 2022). Similarly, the differentiation iMSC is capable of showing similar results as Xia's group in passage 4 which supported the differentiation reliability.

5.1.4 Trilineage Differentiation

The standard evaluation of MSC multipotent ability has been linked to trilineage differentiation. Trilineage differentiation is known for cells like iPSC or MSC to be able to differentiate into adipocytes, chondrocytes and osteocytes, the multipotent differentiation potential of MSC has brought interest and intensive study on its mechanism and pathway for differentiation into each type of cell (Augello and De Bari, 2010; Ma, et al., 2019; Robert, et al., 2020). In the generated iMSC, the capability to differentiate into adipocytes and osteocytes are confirmed through appropriate staining like Oil Red O which targets lipids droplet and Alizarin Red S for calcium deposits; 3D spheroid chondrocytes confirmed using Alcian Blue staining for the presence of acidic polysaccharides like glycosaminoglycans. From the staining observation, the iMSC derived from our source of NTA-iPSC shows higher staining intensity in osteogenesis while lesser in adipogenesis and chondrogenesis. The differentiation potential of iMSC may be affected by the differentiation methods and sources of origin due to epigenetic memories as seen in previous MSC studies. The trilineage differentiation capability has shown that the differentiation of PMBC-iPSC into iMSC has further potential into transformation into other cell sources for bone regeneration or other treatment usage based on the higher efficiency in osteogenesis compared to adipogenesis and chondrogenesis.

Different scientific research has shown different trilineage differentiation potential results when comparing iPSC-MSCs reprogrammed from fibroblast, ES-MSCs and MSCs. However, a similar pattern was shown

such that the trilineage differentiation potential of each cell differs from one another. The research from Chen and colleagues conducted standard adipogenic, chondrogenic and osteogenic differentiation using iPSC-MSCs, ES-MSCs and fetal MSC. The osteogenic and chondrogenic differentiation degree of iPSC-MSCs and ES-MSCs was relatively high and comparable to fetal MSCs while the adipogenic differentiation degree of iPSC-MSCs and ES-MSCs was lower and smaller lipid droplet size as compared to fetal MSCs under similar differentiation duration and medium condition (Chen, et al., 2012).

Another group of study by Diederichs and colleagues performed reprogramming of BM-MSCs and later differentiated into iMSCs using four different methods (Embryoid bodies (EBs) formation, spontaneous differentiation (SD), coculture (CC) and unconditioned BM-MSC growth medium (GM)). They further conducted adipogenic, chondrogenic and osteogenic differentiation and compared all five cell lines. The results show that iMSC derived from either of the four methods have unstable trilineage differentiation degrees that differ from each other and are lowered in all three forms compared to BM-MSCs (Diederichs and Tuan, 2014). The author later decided to confirm further if epigenetic memories have effects on the trilineage differentiation capability, another cell line of the human amniotic epithelium (hAE) was reprogrammed and differentiated into iMSC in similar methods. The results show that the trilineage differentiation between BM-MSCs derived iMSCs and hAE-iPSC derived iMSCs has statistically significant differences, however, they are generally lower than BM-MSCs (Diederichs and Tuan, 2014). The study by Diederichs's team has provided insight into different sources of

iPSCs and methods used for differentiating iMSCs will affect the biological characteristics of iMSCs.

With refer to a study by Kang and colleagues, normal human dermal fibroblasts (NHDFs) were reprogrammed using mRNA transfection and lentivirus transfection into mRNA-iPSC and lenti-iPSC that were later differentiated using MSC medium into mRNA-iPSC-MSC and lenti-iPSC-MSC. Trilineage differentiation capability was compared among BM-MSC, mRNA-iPSC-MSC and lenti-iPSC-MSC in terms of adipogenic, chondrogenic and osteogenic differentiation. The result shows that calcium staining from osteogenic induction in lenti-iPSC-MSC was higher than BM-MSC with mRNA-iPSC-MSC the lowest among all three-cell lines. Next, the chondrogenic induction shows a similar cartilaginous staining structure among lenti-iPSC-MSC, mRNA-iPSC-MSC and BM-MSC. However, adipogenic induction shows that the efficacy from either lenti-iPSC-MSC or mRNA-iPSC-MSC is lowered and smaller in lipid droplet size when compared to BM-MSC which was similar to Chen's finding (Kang, et al., 2015; Chen, et al., 2012). The methods of iPSC reprogramming may also affect the differentiation potential of iMSCs as seen in osteogenic differentiation results comparing lenti-iPSC-MSC and mRNA-iPSC-MSC. The finding above has shown that the origin of iPSCs, the reprogramming methods of cell lines to iPSCs and the differentiation methods of iMSC have crucial impacts on their biological characteristic. The adipogenic differentiation of iMSCs has consistently shown to be lower than MSCs and smaller in lipid droplet size regardless of the derivation history of the iMSCs. Despite the fulfilment of microscopic morphology and surface

markers by flow cytometry, the biological characteristic of the derived iMSCs may still differ from one another and also from MSCs.

A more recent study by Xu's team compared iMSC and BM-MSC of two different passages at passage 5 and passage 8 of both cell lines to see if the maturity and ageing of a cell affect the biological characteristics. Trilineage differentiation capability was compared between iPSC-derived MSC and BM-MSCs. Similarly, the adipogenic differentiation of iMSC was significantly lowered and smaller in lipid droplet size when compared to BM-MSC at passage 5 and passage 8. Then, the use of toluidine blue on chondrogenic-induced iMSC and BM-MSC shows that BM-MSC at passage 8 have the highest chondrogenic efficacy but is generally higher than iMSC at both passages. However, when comparing in terms of osteogenic capacity, both iMSC and BM-MSC at passage 5 and passage 8 demonstrated equally strong calcium deposits. The author concluded that neither maturity nor ageing from passage 5 to passage 8 significantly affects the differentiation capability of iMSC and BM-MSC as the general pattern has been the same in passage 5 and passage 8 (Xu, et al., 2019).

5.2 Outcome of H1975 NSCLC Treatment with iMSC

5.2.1 Non-Cell-To-Cell Interaction Indirect Coculture Treatment

Cell culture conditioned medium contains paracrine factors or cell secretome from the cell source. The paracrine factors include a mixture of molecules like growth factors, antioxidants, microRNA, proteasomes, lipids,

proteins, and exosomes (Maguire, 2013). In a scenario of injury or disease, the affected area will result in a higher number of paracrine factors secreted by the cells, resulting in a paracrine factor gradient between the affected region and stem cell niches. Consequently, stem cells would be attracted towards the affected organ or tissue, followed by the differentiation of stem cells into tissue-specific or organ-specific cells that result in cell regeneration (Gunawardena, et al., 2019).

The use of cell culture conditioned medium has been widely researched and utilised in the therapeutic field for regeneration and industry for producing products with high concentrations of specific paracrine factors to be used as a supplement or for medical purposes. In a study conducted by Liang's group in 2021, the comparison of iMSC-CM and umbilical cord MSC using a mouse cutaneous wound healing model. Their finding showed that iMSC was able to produce a higher concentration of paracrine factors in the conditioned medium than MSC with a similar number of cells and similar type of paracrine factors released (Liang, et al., 2021). The author concluded that iMSC-CM may be a potential new source for therapeutic treatment as an alternative source of MSC.

In the case of utilising MSC conditioned medium in cancer treatment, controversial results suggest the suitability due to the inconsistent outcome of cancer promotion and cancer inhibition. Previous studies have shown that different sources of MSC-CM have different effects on various types of cancer cells such as inhibition of cell proliferation (Liu, et al., 2018; Pan, et al., 2018), inhibition of cell cycle (Maj, et al., 2017; Yuan, et al., 2018), induction of cancer

cell apoptosis (Sun, et al., 2019), inhibition of migration (Xie, et al., 2018) and inhibition of cell adhesion (Li, et al., 2017; Xie, et al., 2018). On the other hand, several other studies showed the opposite whereby MSC-CM enhance proliferation (Wu, et al., 2016; Pietrovito, et al., 2018), metastasis (Pietrovito, et al., 2018), and reduces cancer cell apoptosis (Pan, et al., 2018).

In the case of the morphology of H1975 cells treated with iMSC-CM morphological changes involving a more flattened shape were observed and this may be due to an adaption phase of cancer cells with external paracrine factors and may have undergone EMT that results in mesenchymal-like shape on Day 5. Previous studies have shown that Osimertinib-treated H1975 cancer cells have morphological changes from epithelial into mesenchymal-like cells with enhanced properties on invasion and migration ability that are associated with metastatic cancer cells (Poh, et al., 2019; Nalini, et al., 2021). As such, similar cases may occur as in the iMSC-CM treated H1975 in which morphological changes were shown to be consistent with its enhancement in invasion and migration ability.

5.2.2 Cell-to-cell Interaction of Indirect Coculture Treatment

The tumour microenvironment is a key factor towards tumour development, which is established by mutated epithelial cells interacting with stromal cells during tumourigenesis (Thiery, 2002). Stromal cells present in the tumour microenvironment unlike cancer cells, are genetically stable and have been a popular course as a potential therapeutic target (Quail and Joyce, 2013).

With the unlikelihood of mutation occurr as compared to cancer cells, stable stromal cells can be easily targeted or modified for therapeutic purposes.

The microenvironment in adult tissues protects slow-cycling and undifferentiated cells (Wang, et al., 2011). This ensures the proper growth and development of undifferentiated pluripotent cells to their specific lineage. But tumour-associated MSCs can secrete inflammatory cytokines, chemokines, and growth factors, forming an inflammatory environment that promotes tumour growth (Sun, et al., 2014). It has been shown in numerous studies that normal tissue derived MSCs can remodel tumour microenvironments rather than just target cancer cells. Therefore, it is essential to understand the effects of iMSC and cancer cell in the presence of tumour microenvironments, using direct coculture methods like transwell coculture was chosen to study the interaction of iMSC derived from normal cell source with H1975 cancer cells to test the effects of iMSC derived from PBMC of a healthy donor.

Our observation shows no morphological differences for H1975 under iMSC-TC treatment against the control throughout the 5 days of treatment. Unlike H1975 treated with iMSC-CM which experiences morphological changes and potential EMT transformation, the transwell coculture H1975 remains morphologically similar to the control cells. The qPCR analysis supports that no EMT changes have occurred through the decrease in all EMT markers expressions and migration assay shows no increase in the number of migrated cells under the same condition for transwell coculture treated H1975 when compared against control. From these observations, it can be deduced that

H1975 has not undergone any form of EMT transition based on the morphological changes as it retains its original form and qPCR analysis showed no difference in the expression level. At the same time, there is no enhancement in metastasis capability based on the migration assay and qPCR analysis. Thus, crosstalk between iMSC and H1975 appears to favour the reduction of metastatic potential as compared to that of using iMSC-CM alone on H1975.

5.3 The Effects of RNA Expression Alteration on H1975 Cancer Cell by Different Treatment Methods

5.3.1 Endothelial Marker

Epithelial cell adhesion molecule (EpCAM) is a major epithelial antigen that can be found on chromosome 2 (2p21) and consists of 14 kb in total. From zebrafish to humans, the gene is conserved across many different species. There is a high degree of amino acid sequence conservation in EpCAM's extracellular domain from fishes to primates, suggesting its functional significance (Pavšič, et al., 2014). It plays an essential role in cell adhesion, migration, metastasis and cell signalling. (Herreros-Pomares, et al., 2018). The EpCAM has been used as a unique tumour or carcinoma marker as it is overexpressed in various human cancers and malignant cancer including lung cancer (Hase, et al., 2011; Wang, et al., 2021). EpCAM has been detected to be associated with survival in lung cancer cells and has been known to be responsible for tumour migration, invasion and progression in various types of cancer cells including lung cancer (Tai, et al., 2007; Zhou, et al., 2015; Keller, et al., 2019). While higher

expression of EpCAM may be related to highly metastatic lung cancer cells and poor prognosis (Zhou, et al., 2015; Wang, et al., 2021). The high expression of EpCAM may be an indicator of lung cancer cells having enhanced migration and invasion properties. However, the high expression has been used by researchers as a target for therapeutic treatment. Where Wang's group have demonstrated an effective immunotherapy strategy that targets polymorphic epithelial mucin (MUC-1) and EpCAM with a combination of bispecific antibody with CD3 (Wang, et al., 2021). The immunotherapy has been shown to promote cytotoxic T-lymphocyte response, cytokine production and the immune environment through increasing $CD8^+$ T cells. The author concludes that the overall results have shown antitumour response and the strategy to target EpCAM may be a promising strategy for anti-tumour therapy (Wang, et al., 2021).

From the results obtained, the RNA expression for EpCAM has shown to decrease for H1975 treated with iMSC-CM but a more drastic decrease can be observed in H1975 treated with iMSC-TC. A study done by Rodriguez-Martinez's group shows that EMT involves the loss of EpCAM expression and an increase of mesenchymal-associated gene expression like VIMENTIN and SNAI1 gene family (Gonzalez and Medici, 2014; Rodriguez-Martinez, et al., 2022). However, these changes may vary depending on tumour types, stages and the treatment (Elizabeth, et al., 2019). This can be observed in the differences in H1975 RNA expression where both direct and indirect coculture methods show a decrease in EpCAM expression but only indirect coculture methods with iMSC-CM show an increase of EMT markers like VIMENTIN

and SLUG but both of these EMT markers are decreased in indirect coculture method with transwell. In line with the results, iMSC-CM may induce EMT transformation in H1975 lung cancer cells while iMSC-TC does not induce EMT.

5.3.2 Cell Cycle Markers

The gene p53, through its unique ability to act as a highly sensitive collector of stress inputs, the p53 tumour suppressor acts as a major barrier to neoplastic transformation and tumour progression (Mantovani, et al., 2019). Besides protecting cellular homeostasis and genome stability, this complex framework also coordinates a variety of effector pathways, however, missense mutations of the TP53 gene are known to be common in human cancers that result in mutation of p53 proteins causing the loss of tumour suppressive activates or pathway by p53 (Mantovani, et al., 2019).

From the observed findings, the effects of both treatments showed similar patterns on both p21 and p53 cell cycle markers. There was a significant increase in p21 expression for both iMSC-CM and iMSC-TC treatment on H1975 cancer cells and a significant decrease in p53 expression for both iMSC-CM and iMSC-TC on H1975 cancer cells.

Past research has shown that p21 expression has been associated with cell senescence and cellular stress, like DNA damage or oxidative stress that may be regulated through a p53-dependent or p53-independent pathway (Abbas

and Dutta, 2009; Qian and Chen, 2010). However, based on a previous study done by Zhao and colleagues on p21 which is a cyclin kinase inhibitor family that mediates G_2/M cell cycle arrest; increasing p21 expression may have been known to be associated with the G_2/M cell cycle arrest through Chk2/p21-cdc25c signalling pathway (Zhao, et al., 2018). Other in vivo and in vitro studies have shown p21 with the ability to induce apoptosis and suppress tumour growth (Gorospe, et al., 1997; Tsao, et al., 1999; Poole, et al., 2004; Jiang, et al., 2014; Ibnat, et al., 2019) while some contradicting studies showed p21 enhancing tumour proliferation and cancer properties (García-Fernández, et al., 2011; Buitrago-Molina, et al., 2013; Ehedego, et al., 2015; Okuma, et al., 2017). While p53 expression has been well known as a tumour suppressor, however, a study by Jung's and a colleague has shown that p53 is associated with EGFR tyrosine kinase inhibitors sensitivity in NSCLC whereby the decrease or knockdown of p53 expression have shown to enhance the sensitivity of H1975 cells towards Osimertinib drug treatment and slow down the cancer cell acquired resistance towards Osimertinib drug (Jung, et al., 2021).

Our observation has shown a similar trend for both iMSC-CM and iMSC-TC treatment on H1975 cancer cells. Both treatments have shown a significant increase in P21 markers but a significant decrease in P53 markers compared to parental control cells. Based on previous studies, the rise in P21 expression may result in cell cycle arrest. This will inhibit or suppress further proliferation of the cancer cell or potentially cause cell hibernation that may bring advantages for treatment as the cancer progression is slowed down, providing patients with a higher chance of survival. The decrease in P53 can

potentially enhance the sensitivity of H1975 cancer cells under drug treatment like Osimertinib drug as P53 has been known to be related to EGFR tyrosine kinase inhibitors sensitivity. As such, it may be a new potential platform to tackle the issue of cancer cell resistance mechanisms that reduce the efficiency of drug treatment over time leading to poor patient prognosis. The findings of iMSC-TC interaction with lung cancer cell represent/mimics the possible influence of iMSC in the tumour microenvironment leading to a reduction of the metastatic potential of the cancer cells that may imply a more favourable treatment response when it is used as a cell therapy option against H1975.

5.3.3 EMT Markers

Following the results obtained from qPCR, it can be observed that using EMT markers (VIMENTIN and SLUG) are highly regulated while SNAI1 remained with no significant changes when compared iMSC-CM treatment to parental cancer cells, however, all EMT markers decreased in iMSC-TC treatment. The qPCR from EMT markers increased expression suggesting that H1975 experienced a shift of EMT linked to the higher migrated number of cells in the migration assay. Previous studies have shown that the increased expression of EMT regulators (SNAI1 and SLUG) and mesenchymal protein (VIMENTIN) may contribute to tumour metastasis and TKI-resistance in EGFR mutant NSCLC cells (Jayachandran, et al., 2016; Tsatsral, et al., 2018).

Our observation from iMSC-CM treatment on H1975 cancer cell has had significant enhancement on EMT-related markers like VIMENTIN and

SLUG but no significant changes for SNAI1 while iMSC-TC treatment have a significant reduction on VIMENTIN, SLUG and SNAI1 when compared to control parental cell. This indicates a potential trigger in EMT when H1975 cancer cells are treated with iMSC-CM only, but the effects are reversed when treated with iMSC-TC. This may provide an insight into a potential platform for direct iMSC interaction with cancer cells to inhibit or suppress the EMT transition for cancer cells that often results in enhanced migratory effects leading to malignant cancer cells that cause poor prognosis of the patient.

5.4 Migration Assay Outcome of Different Treatment

As observed in Figure 4.13, the number of migrated cells is aligned with the qPCR result. As seen in iMSC-CM, the increase in EMT markers known for affecting metastasis capability has shown a significant increase in migrated cells compared to that of the control. On the other hand, iMSC-TC showed a reverse pattern for EMT markers with lower number of migrated cells as compared to that of iMSC-CM and had no significant difference with that of the parental cancer cells. As in previous studies, the strength of EMT marker expression was demonstrated to be proportional to the strength of cancer progression and metastasis capability (Son and Moon, 2010; Huang, et al., 2022).

In a number of in vivo and vitro studies that targeted protein expression through inhibitor and drug treatment with different types of cancer cell lines like lung cancer (A549, H490, H1299 and H1975), breast cancer (MDAMB-231 and MCF7) and oral squamous cell carcinoma (HN12 and HN4), EMT markers like

SNAI1, SLUG and VIMENTIN were strongly related to the regulation of EMT mechanism, that affects the metastasis capability of these cancer cells (Liu, et al., 2016; Luanpitpong, et al., 2016; Wang, et al., 2019; Ni, et al., 2020). The outcome of their studies showed that the lower the expression of these EMT markers, the lower the migration or metastasis capability rate of various types of cancer cells. These trends was consistent with our findings on iMSC-TC which affected the metastatic potential of H1975

5.5 Crosstalk Between the Cell-To-Cell Interaction of iMSC and H1975 in iMSC-TC

The observation from iMSC-TC has clearly shown the suppression of tumour properties based on the qPCR results with the significant decrease in expression of endothelial and EMT markers like EPCAM, VIMENTIN, SLUG and SNAI1 that was previously known to enhance cancer metastasis (Zhou, et al., 2015; Jayachandran, et al., 2016; Tsatsral, et al., 2018). The results are further supported by a cell migration assay where there was no increase in the number of migrated cells in iMSC-TC as compared to the control, whereas in iMSC-CM, a significantly high number of migrated cells was observed. The environment with both cells sharing the same space has allowed crosstalk among each other that eventually shifted the paracrine factors released initially by iMSC into suppressing the tumour properties (Ahmed, et al., 2022). As shown in a previous study by Zhao and his colleagues, iMSC has shown less potential in promoting tumours such as decreased expression level for TGFβ signalling, suppressed EMT transition, enhancement of tumour cell invasion or

migration and tumour cell proliferation rate as compared to native MSC like BM-MSC in the animal study with tumour cells of LoVo, HCC1806 and MCF7 (Zhou, et al., 2014). The team observed changes with similar trends in terms of cytokines and chemokines profile with decreased cancer-promoting factors upon coculturing of iMSC with tumour cells as compared to BM-MSC with tumour cells while tumour-homing properties have increased for the iMSC model allowing for more cells to migrate towards the tumour cells (Zhou, et al., 2014). The immunosuppression and anti-inflammatory of the iMSC model are also found to be relatively higher than native MSC in several studies that make it a potential subject for immune-related treatment (Sánchez, et al., 2011; de Peppo, et al., 2012; Zhou, et al., 2014). This information can be further utilised for future studies with regards to using iMSC and H1975 in a model that has a proper immune system to further test on the immunosuppression and anti-inflammatory as this study has shown that iMSC-TC with H1975 will not enhance its cancerous properties. With the addition of the immune system in the culture environment, there may be a better representation of how the immune system could be triggered as well in suppressing cancer cells.

5.6 Proteomic Chemokine and Cytokine Profile of Different Conditioned Medium

The proteomic cytokine and chemokine profiling assay represents the possible relationship of cell-to-cell interaction such that alteration of the releasing factors (CCL2, CXCL1, CXCL12, GM-CSF, SDF-1) can be observed from both cell lines (iMSC and H1975 cancer cell) that affect the culture

environment of H1975 during transwell coculture with iMSC that differs from the independent culture of both cells. The observation from the assay showed the presence of a new chemokine stromal cell-derived factor-1 (SDF-1) after coculture and the absence of multiple cytokines such as the CXC motif chemokine ligand 1(CXCL1), CXC motif chemokine ligand 12 (CXCL12), granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokine (C–C motif) ligand 2(CCL2). The presence and absence of these cytokines and chemokines have been known to be associated with regulating the cancer cells' properties like proliferation and metastasis.

Previous studies have shown that cytokines and chemokines like SDF-1, CCL2 and CXCL1 may play a crucial role in cancer cell metastasis (Song, et al., 2017; Salamon, et al., 2020; You, et al., 2020). Another experiment by Kim and colleague have shown that CXCL12 is essential for the migration of MSCs towards cancer cell. The author used BM-MSCs and cultured them with H1975 cancer cells in a similar environment to observe BM-MSC movement by altering the presence and absence of anti-CXCR4 affecting CXCL12 expression in the culture environment. The inhibition effects of anti-CXCR4 have been shown to significantly decrease the migration ability of BM-MSCs towards H1975 (Kim, et al., 2018). The presence and absence of GM-CSF have been known to be a key regulator in hematopoietic growth factor and immune modulator, where the presence of GM-CSF stimulate and enhance the immune system especially the production and activation of granulocytes, neutrophils and macrophages (Metcalf, 2010; Bouillez, 2017). The absence of CXCL1 and CCL2 in the transwell coculture suggests the possible reduction of the

metastasis ability of H1975 while the presence of SDF-1 could represent the homing mechanism to an injury or inflamed site (Jin, et al., 2018).

In one of the studies done by Zhao's and colleagues, they compared the relative expression of chemokine of BM-MSC and iMSC like CXCR4, CXCR6, CD44, ITGA6, ITGB1 and VEGFR1 that are known to be the mediator of MCS tumour tropism. The comparison shows that all the expressions are similar with no significant differences except the VEGFR1 expression, which is significantly higher in iMSC. Further experiments to test the effects of BM-MSC and iMSC coculture with LoVo colon cancer cell and MDA-MB231 breast cancer cell shows that BM-MSC highly promotes EMT, invasion properties and cell expansion rate compared to iMSC. While multiple EMT-related genes (NCAD, TWIST1, ZEB1, ZEB2 and VIM) expression increases upon coculture with BM-MSC as compared to iMSC. The harvested coculture cancer cells show bigger tumour mass and higher invasion assay cell count in BM-MSC compared to iMSC. The author indicated that the cell-to-cell interaction and pathway activation of BM-MSC and iMSC with cancer cells were probably different (Zhao, et al., 2014). However, the author did not test the relative expression of chemokine receptors after coculture.

The alteration of releasing factors from both iMSCs and H1975 cancer cells has provided a good indication of cell-to-cell interaction between both cell lines. Collecting conditioned mediums in iMSC-TC containing altered release of cytokines and chemokines may provide a good platform for studying the molecular signals in cell-to-cell interaction between iMSCs and cancer cells and

comparing cells in their individual environment. Nevertheless, further studies would be needed to elucidate the interaction and response of immune cells such as leukocytes, macrophages, dendritic cells, mast cells, T-cells, and B-cells in the body system with iMSC and cancer cells to further discover the possible applications of iMSC to be used as a cell-based therapy against NSCLC as the current information regarding the use of iMSC as an alternative of MSC is still scarce (Lynch, et al., 2017; Galland and Stamenkovic, 2020). However, using MSC and cancer cell studies as references may serve as a guideline and boost for future studies using iMSC and cancer cells. With the hope of this study to further encourage research in the use of iMSC and cancer cells. As cancer has been inclining while the rate of survival has been declining, with lung cancer being the second highest occurring cancer, it is vital to find alternative methods for a way to improve survival rate.

CHAPTER 6

CONCLUSION AND FUTURE RECOMMENDATIONS

6.1 Conclusion

iMSC derived in this study showed the expression of common MSC properties through the microscopic morphology, surface markers analysis with flow cytometry and trilineage differentiation with a specific differentiation-inducing medium which was similar to MSC characterisation standard as reflected in the ISCT guideline (Dominci, et al., 2006). When comparing to the parental control cells, the use of iMSC-CM treatment of H1975 cancer cell showed a significant increase in its cancerous properties and EMT-related gene expression (VIMENTIN and SLUG) that are prone to enhance migratory properties, the proliferation of NSCLC cancer cells. However, in iMSC-TC treatment of H1975 cancer cell, the EMT-related gene expression (VIMENTIN, SLUG and SNAI1) was significantly reduced when compared to parental control cell or iMSC-CM treated cells, which indicated EMT transition had not occurred and a potential reversal effect may have occurred in the cancer cell line as demonstrated in the reduction of migratory effects of the cancer cells upon coculture treatment with iMSCs. The crosstalk between iMSCs and H1975 has significantly altered the paracrine factors released by iMSCs as observed in the differences of iMSC-CM effects and iMSC-TC. The analysis shows that the lack of crosstalk between these cells has caused iMSC to release factors

favouring tumour development. Nonetheless, by placing both cells in the same environment, the cell-to-cell interaction emerged into crosstalk that changed the paracrine factors released by iMSC to be unfavourable to the H1975 cell, eventually suppressing its tumour properties.

From the analysis of cytokine and chemokine assay, the results have provided insight into the crosstalk between iMSC and cancer cells in a transwell coculture system which could cause alteration of releasing factors that may affect the metastatic potential and carcinogenic properties of H1975 cancer cells, while potentially increase the homing capability of iMSC towards tumour cells with the presence of SDF-1 and potentially the recruitment of immune system in a complete body system. Based on the cancer properties of H1975 upon coculture with iMSC and the paracrine profile, the derived iMSC shows immunomodulatory properties that may suppress cancer progressions, and tumour metastasis and provide an alternative source of cell-based therapy for NSCLC.

6.2 Limitations and Recommendations for Future Studies

The current coculture condition used to investigate the crosstalk between iMSC and lung cancer cell line is limited to only one lung cancer cell line which does not provide a good representation of the wider possible paracrine effects of iMSC on other types of lung cancer cells.

Furthermore, as the native MSC was not used in the coculture study, the paracrine profile obtained with iMSC may not be similar or represent MSCs in general when interacting with lung cancer cells.

The full expression of the immunomodulatory effects induced by iMSCs involving the immune system may only be well recapitulated using an animal model.

Therefore, the recommendations for future studies would involve both in-vitro and in-vivo approaches. For in-vitro studies, the use of a wide range of lung cancer cells to interact with iMSC would be necessary for a better understanding of the crosstalk outcome. As previously mentioned, MSCs react differently with different kinds of cancer cell lines. Therefore, it is essential to understand the general trend of iMSC crosstalk effects in the use of lung cancer cell lines.

Besides, the use of native MSC may be a suitable control as a comparison for the paracrine profile of iMSC. It is vital to understand if iMSC and MSC paracrine profiles are similar or different in their original environment, followed by the alteration in their paracrine during crosstalk with tumour cells.

As for the in vivo animal studies, modification for immunocompetent and immunodeficient mice can be done and tested to see the differences between cell treatment and drug treatment that shows a more accurate result of the interaction for cancer cells as compared to the cell culture model (Zitvogel, et al., 2016). Such an approach is important to study the differences between

benign and malignant cancer with the interaction of immune cells in the stroma microenvironment, which cannot be fully replicated in a cell culture model.

In short, using lung cancer-induced animal models treated with iMSC-CM, iMSC injection for coculture and drug treatment may show a more complete and closer interaction between cell-to-cell interaction in the human body. The interaction of iMSC-TC and iMSC injected into the animal model may show different results as iMSC-TC lacks the pan immune system in the animal model. The use of animal models may provide a more precise insight and complete interaction as compared to in vitro cell culture where the measurement or analysis like tumour mass, malignancy or EMT occurrence of tumour, and condition of the animal model can be obtained.

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APPENDICES

APPENDIX A

List of Primers

No.	Primer Name	Primer Sequence (5' to 3')
1	OCT4_F	GCTC ACCC TGGG GGTT CTAT T
2	OCT4_R	TGCC CCTC CCCA CTAG GTT
3	SOX2_F	TCAT CGAC GAGG CTAA GCGG
4	SOX2_R	GCTC GCCA TGCT ATTG CCG
5	NANOG_F	ATGG TGTG ACGC AGGG ATGC
6	NANOG_R	ATTG GGTG CACC AGGT CTGA
7	CD44_F	GCCT CAGC TCAT ACCA GCCA
8	CD44_R	CGTC CCAT GGGG TGTG AGAT
9	CD105_F	CCTG CCAC TGGA CACA GGAT
10	CD105_R	TGCA AGAC TTGT GGGG CTGA
11	EPCAM_F	GCAG GGTC TAAA AGCT GGTG TT
12	EPCAM_R	TCCC TATG CATC TCAC CCAT CT
13	P21_F	GACA GCAG AGGA AGAC CAT
14	P21_R	GCGT TTGG AGTG GTAG AAAT C
15	P53_F	ACAG AGGA AGAG AATC TCCG CA
16	P53_R	TGGT TTCT TCTT TGGC TGGG
17	VIMENTIN_F	AGTC CACT GAGT ACCG GAGA C
18	VIMENTIN_R	CATT TCAC GCAT CTGG CGTT C
19	SLUG_F	CAAC AGAG CATT TGCA GACA GG
20	SLUG_R	AATG TGGA CCTT GGAA CACT GG
21	SNAI1_F	TACT TCAG TCTC TTCC TTGG AGGC
22	SNAI1_R	TTGC AGTT GAAG GCCT TTCG

APPENDIX B

Fold Change of iPSC and MSC Markers in Differentiating Process of iPSC to iMSC to Control by Δ ΔCT Method.

Condition	Gene	(A) Average CT (Target Gene)	(B) Average CT (GAPDH)	(C) Δ CT = (A) – (B)	(D) Δ ΔCT= (C) – ΔCT Control	(E) 2^-(D)
Control (iPSC)	OCT4	8.357	13.160	-4.803	0	1
	SOX2	5.878	13.160	-7.282	0	1
	NANOG	5.798	13.160	-7.362	0	1
	CD44	10.428	13.160	-2.372	0	1
	CD105	9.160	13.160	-4.000	0	1
iMSC P4	OCT4	14.129	13.480	0.649	5.451	0.023
	SOX2	9.430	13.480	-4.050	3.232	0.106
	NANOG	9.953	13.480	-3.527	3.835	0.070
	CD44	10.547	13.480	-2.933	-0.201	1.149
	CD105	8.087	13.480	-5.393	-1.393	2.626
iMSC P5	OCT4	15.595	14.020	2.115	6.918	0.008
	SOX2	8.999	14.020	-4.481	2.801	0.143
	NANOG	10.469	14.020	-3.011	4.351	0.049
	CD44	9.816	14.020	-3.664	-0.932	1.907
	CD105	7.310	14.020	-6.17	-2.170	4.501

APPENDIX C

Fold Change of Endothelial, Cell Cycle and EMT Markers in H1975 by Different Treatment Methods of iMSC-CM 50%, iMSC-CM 100% and iMSC-CT to Control by Δ ΔCT Method.

Condition	Gene	(A) Average CT (Target Gene)	(B) Average CT (GAPDH)	(C) Δ CT = (A) – (B)	(D) Δ ΔCT= (C) – ΔCT Control	(E) 2^-(D)
Control	EPCAM	21.793	18.520	3.273	0	1
	P21	25.364	18.520	6.844	0	1
	P53	19.950	18.520	1.430	0	1
	SLUG	24.945	18.520	6.425	0	1
	SNAI1	22.389	18.520	3.869	0	1
	VIMENTIN	23.780	18.520	5.260	0	1
50% CM	EPCAM	22.097	18.283	3.813	0.540	0.688
	P21	24.226	18.283	5.943	-0.901	1.867
	P53	21.442	18.283	3.122	1.692	0.309
	SLUG	23.845	18.283	5.561	-0.864	1.819
	SNAI1	21.983	18.283	3.700	-0.169	1.124
	VIMENTIN	21.145	18.283	2.861	-2.398	5.272
100%CM	EPCAM	21.485	17.755	3.731	0.458	0.728
	P21	23.563	17.755	5.809	-1.035	2.049
	P53	23.092	17.755	5.337	3.907	0.067
	SLUG	23.102	17.755	5.347	-1.078	2.111
	SNAI1	21.998	17.755	4.243	0.374	0.772
	VIMENTIN	19.954	17.755	2.199	-3.060	8.342

Condition	Gene	(A) Average CT (Target Gene)	(B) Average CT (GAPDH)	(C) Δ CT = (A) – (B)	(D) Δ ΔCT= (C) – ΔCT Control	(E) 2^-(D)
Control	EPCAM	6.890	22.890	-16.000	0	1
	P21	6.114	22.890	-16.776	0	1
	P53	4.245	22.890	-18.645	0	1
	SLUG	9.512	22.890	-13.378	0	1
	SNAI1	8.849	22.890	-14.041	0	1
	VIMENTIN	8.696	22.890	-14.194	0	1
TC	EPCAM	6.042	20.470	-14.428	1.572	0.336
	P21	0.911	20.470	-19.559	-2783	6.883
	P53	3.044	20.470	-17.426	1.129	0.429
	SLUG	8.324	20.470	-12.146	1.232	0.426
	SNAI1	8.529	20.470	-11.941	2.100	0.233
	VIMENTIN	8.696	20.470	-12.338	1.857	0.276