TRANSCRIPTIONAL PROFILING OF SHED-EPITHELIAL DIFFERENTIATION IN CO-CULTURE MODEL

SITI NURNASIHAH BINTI MD HASHIM

UNIVERSITI SAINS MALAYSIA

2025

TRANSCRIPTIONAL PROFILING OF SHED-EPITHELIAL DIFFERENTIATION IN CO-CULTURE MODEL

by

SITI NURNASIHAH BINTI MD HASHIM

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

February 2025

ACKNOWLEDGEMENTS

All praises to Allah S.W.T., The Most Gracious and The Most Merciful. Alhamdulillah, I completed my Doctor of Philosophy and wrote this dissertation, "Transcriptional Profiling of SHED-Epithelial Differentiation in Co-Culture Model."

Firstly, I would like to thank my Supervisor, Assoc. Prof. Dr. Azlina Ahmad, for the trust given to me. I am genuinely thankful for her continuous support and guidance throughout these 12 years. I am also very grateful for her understanding as I juggle my personal life and care for my parents. I also offer the most incredible gratitude to my Co-Supervisors, Assoc. Prof. Dr. T.P. Kannan, Dr. Khairul Bariah Ahmad Amin Noordin, and Dr. Sarahani Harun, who assisted and guided me very well with this project.

My PhD journey has indeed humbled me since my father was diagnosed with multiple myeloma cancer in 2020. His determination to fight his illness has lifted my motivation to never give up and always strive for my future. My parents, Haji Md Hashim Che Mat and Hajjah Salmiah@Yang Yaacob are my strongest pillars. I want to thank them for their endless prayers, moral support, advice, and motivation. They have provided good access to knowledge and education, and I cannot thank them enough. My greatest appreciation goes to my siblings- and-in-law, Zamree/Rahayu, Salha/Shafrizal, Faridah/Dr. Ezzeddin, and Faisal/Afifah for their prayers, concern, and financial support. Not to forget my niblings, Aesya, Rayyan, Lutfi, Hannah, Nazeem, Aina', Riyyad, Luqman, Qayyim, Kasyaf, Afifi, and Qaireen. May my study journey become an inspiration to all of you.

Besides, I would like to express my gratitude to my best buddies, Ain Fatihah, Shazwani, Suraya, and Dr. Marahaini. Not to forget, thank you to my current research team, Mohadese, PG friends, especially Dr. Badr and Dr. Syazana, and my other friends. Thanks for understanding, being helpful, always being by my side whenever I needed it, and believing in me.

I am also thankful to the staff of the Craniofacial Sciences Laboratory, School of Dental Sciences, USM, for their kind assistance throughout my research journey. Lastly, I thank the USM Fellowship for financial support in 2019–2020 and the USM Research University Grant 1001/PPSG/8012214 for the research funding.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

%	Percentage
<	Less than
>	More than
≤	Less than or equal to
≥	More than or equal to
®	Registered
°C	Degree Celsius
h	Hour
s	Second
™	Trademark
U	Enzyme unit
V	Voltage
α	Alpha
β	Beta
Δ	Delta
γ	Gamma

LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
2D	Two-dimensional
3D	Three-dimensional
ADSCs	Adipose-derived stem cells
AKT	Protein kinase B
ALK	Activin receptor-like kinase
ANOVA	Analysis of variance
APC	Allophycocyanin
ASCs	Adult stem cells
BiNGO	Biological Networks Gene Ontology
BMMSCs	Bone marrow mesenchymal stem cells
BMP4	Bone morphogenetic protein 4
bp	Base pair
BP	Biological process
BPE	Bovine pituitary extract
CC	Cellular component
CC HK	Co-cultured HEK001 in KSFM (Transwell insert)
CC SK	Co-cultured SHED in KSFM
CC SKI	Co-cultured SHED in KSFM+A83-01
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
ChIP-seq	Chromatin immunoprecipitation sequencing
CK	Cytokeratin
cm^2	Square centimetre
CO_2	Carbon dioxide
cRNA	Complementary ribonucleic acid
Cy3	Cyanine-3
DAVID	Database for Annotation, Visualization, and Integrated Discovery

DEGs	Differentially expressed genes
DEPC	Diethyl pyrocarbonate
DFPCs	Dental follicle progenitor cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPSC	Dental pulp stem cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithelial Growth Factor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESCs	Embryonic stem cells
et al.	and others
FBS	Foetal bovine serum
FC	Fold change
FDR	False discovery rate
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FSC	Forward side scatter
g	Gram
GEO	Gene Expression Omnibus
GO	Gene ontology
HEK001	Human epidermal keratinocytes
HGF	Hepatocyte growth factor
HK	HEK001 in KSFM
HKD	HEK001 in KSFM+DMSO
HKI	HEK001 in KSFM+A83-01
hPSCs	Human pluripotent stem cells
HSCs	Haematopoietic stem cells
IGF	Insulin growth factor
iPSCs	Induced pluripotent stem cells
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase

K/KRT	Keratin
KEGG	Kyoto Encyclopedia of Genes and Genomes
KSFM	Keratinocyte Serum Free Medium
LB	Lithium borate
LESCs	Limbal epithelial stem cells
MAPK	Mitogen-activated protein kinase
MCC	Maximal Clique Centrality
MCODE	Molecular Complex Detection
MenSCs	Menstrual blood-derived stem cells
MET	Mesenchymal-epithelial transition
MF	Molecular function
$Mg(OAc)_2$	Magnesium acetate
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mm	Milimetre
mM	Millimolar
MSCs	Mesenchymal stem cells
MTT	Thiazolyl Blue Tetrazolium Bromide
NFKB	Nuclear factor kappa B
NGS	Next-generation sequencing
nm	Nanometre
nm	Nanometre
nt	Nucleotide
PBS	Phosphate buffer saline
PDLSCs	Periodontal ligament stem cells
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein complex
PI3K	Phosphatidylinositol 3-kinase
pmol	Picomole
PPI	Protein-Protein Interaction
R^2	Correlation coefficient
RA	Retinoic acid
RIN	RNA Integrity Number

RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Reverse transcriptase-real time polymerase chain reaction
SA	SHED cultured in α-MEM
SAD	SHED cultured in KSFM+DMSO
SAI	SHED cultured in KSFM+A83-01
SCAP	Stem cells from apical papilla
scRNA-seq	Single-cell sequencing
SHED	Stem cells from human exfoliated deciduous teeth
SK	SHED cultured in KSFM
SKD	SHED cultured in KSFM+DMSO
SKI	SHED cultured in KSFM+A83-01
SMAD	Suppressor of mothers against decapentaplegic
SSC	Side scatter
STRING	Search Tool for the Retrieval of Interacting Genes
TGFβ	Transforming growth factor beta
TGFβR	Transforming growth factor beta receptor
UC-MSCs	Umbilical cord-derived MSCs
vs	Versus
WJ-MSCs	Wharton's jelly mesenchymal stem cells
WNT	Wingless-related integration site
x g	G-force
α-MEM	Minimum Essential Medium Alpha

LIST OF APPENDICES

PEMPROFILAN TRANSKRIPSI BAGI PEMBEZAAN SHED-EPITELIUM DALAM MODEL KO-KULTUR

ABSTRAK

Penjanaan tisu epitelium menerusi kaedah terapi sel tunjang telah memberi harapan baru dalam bidang penyembuhan luka. Bagaimanapun, lokasi asal dan potensi sel tunjang perlu diambil kira untuk mencapai proses pembezaan epitelium kerana proses ini penting dalam rawatan luka. Sel tunjang jenis ektoderma iaitu sel tunjang daripada gigi susu manusia yang terkelupas (SHED), telah digunakan dalam penyelidikan ini kerana ia berasal dari lokasi yang sama seperti kulit. Tujuan kajian ini dijalankan adalah untuk mengkaji pemprofilan transkripsi bagi pembezaan SHED-epitelium dalam model ko-kultur. Dalam kajian ini, SHED dikultur bersama dengan sel epidermis keratinosit manusia (HEK001) dalam persekitaran mikro-seperti-epitelium, yang mengandungi medium aruhan, KSFM, untuk membantu pembezaan epitelium. SHED yang dikultur dalam medium asas, α-MEM (SA) dan medium aruhan (SK), serta HEK001 yang dikultur dalam KSFM (HK), merupakan kumpulan kawalan. Kumpulan yang dirawat terdiri daripada SHED ko-kultur dalam KSFM (CC SK) pada hari ke-1, 3, 5, 7, 10, dan 14. Penanda pengekspresan gen epitelium awal disaring menggunakan tindak balas berantai polimerase transkriptase balik selangkah (RT-PCR). Eksperimen seterusnya melibatkan pengekspresan gen *microarray* yang hasilnya dianalisis menggunakan bioinformatik. Kemudiannya, pengesahan hasil eksperimen *microarray* dilakukan menggunakan tindak balas berantai polimerase transkriptase berbalik selangkah masa nyata (RT-qPCR). Hasil kajian bioinformatik mencadangkan tapak jalan TGFβ terlibat dalam pembezaan SHED-epitelium. Oleh itu,

perencat A83-01 (1 μM) digunakan untuk menyekat TGβR1 bagi menghalang aktiviti SMAD2/3 pada tapak jalan tersebut. Kesan perencatan tersebut dianalisis menggunakan RT-qPCR, sitometri aliran, dan immunositokimia. Hasil RT-PCR mendapati gen-gen yang terlibat di awal pembentukan epitelium seperti *CK18* dan *AQP3* diekspres dalam SHED tanpa ko-kultur mahupun dengan ko-kultur. Bagaimanapun terdapat satu tambahan gen, *ΔNp63*, yang hanya diekspres dalam SHED ko-kultur. Analisis bioinformatik kemudiannya telah mengenalpasti *SUSD2*, *CXCL1*, dan *GGT5* sebagai DEG yang diekspres paling tinggi. Secara keseluruhan, hasil analisis bioinformatik menunjukkan DEG pada hari ke-5 dan 7 terlibat dalam aktiviti kitaran sel dan pembezaan epitelium. Selain itu, pemerkayaan DEG yang berkaitan dengan tapak jalan TGFβ menandakan penglibatan tapak jalan ini dalam proses pembezaan SHED-epitelium. Analisis pengekspresan gen menerusi RT-qPCR membuktikan penglibatan tapak jalan ini dalam proses tersebut dengan wujudnya pengekpresan gen yang tinggi pada penanda yang berkait seperti *TGFβ3* dan *BMP8A*. Menariknya, hasil kajian ini turut menunjukkan kesan perencatan tapak jalan TGFβ dalam mengawal pembezaan SHED ko-kultur kepada sel mirip epitelium kelenjar, di mana ianya dibuktikan dengan perubahan sel dan penanda gen yang berkaitan dengan epitelium kelenjar, seperti *SUSD2*, *GGT5*, *PPARG*, dan *COL6A2* dan penanda protein, seperti Muc 1. Kajian ini telah membuktikan peranan tapak jalan TGFβ dalam mengawal pembezaan SHED kepada sel-seperti-epitelium. Walaupun begitu, perencatan tapak jalan TGFβ oleh A83-01 dicadangkan dapat memanipulasi tapak jalan pembezaan sel-seperti-epitelium yang asal kepada sel-seperti-epitelium-jenis-kelenjar. Model SHED ko-kultur kami berjaya menunjukkan pembezaan epitelium, yang mungkin boleh diaplikasikan dalam kajian seterusnya untuk menjana semula tisu epitelium terutamanya dalam bidang penyembuhan luka.

TRANSCRIPTIONAL PROFILING OF SHED-EPITHELIAL DIFFERENTIATION IN CO-CULTURE MODEL

ABSTRACT

Regenerating epithelial tissue through stem cell differentiation gives new hope for wound healing. However, stem cell's origin and potency need to be considered to achieve high efficiency epithelial differentiation, as it is a crucial step in wound healing. In this study, ectodermal stem cells, known as stem cells from human exfoliated deciduous teeth (SHED), were used because they arose from a similar origin to the skin. The aim of this study was to investigate the transcriptional profiling of SHED-epithelial differentiation in a co-culture model. Here, SHED were grown in the epithelial-like microenvironment along with human epidermal keratinocytes (HEK001) cultured in the induction medium, keratinocyte serum free medium (KSFM), to promote epithelial differentiation. SHED cultured in the basal medium, minimum essential medium alpha (α-MEM) (SA) and KSFM (SK), and HEK001 cultured in KSFM (HK) were used as controls. The treatment consisted of co-cultured SHED cultured in the KSFM (CC SK) on days 1, 3, 5, 7, 10, and 14. The epithelial gene expression markers were screened using reverse transcriptase-polymerase chain reaction (RT-PCR). The subsequent experiment used microarray gene expression, subjected to bioinformatics analysis. Then, microarray validation was done using reverse transcriptase-qualitative real-time PCR (RT-qPCR). The bioinformatics analysis suggested that the TGFβ signalling pathway is involved in SHED-epithelium differentiation. Therefore, a downstream TGFβ pathway inhibition study was conducted using A83-01 (1 μM) to inhibit TGFβR1 that blocks SMAD2/3 activity.

The inhibitory effects were analysed using RT-qPCR, flow cytometry, and immunocytochemistry. RT-PCR analysis showed that the genes involved in the early epithelial formation like, *CK18* and *AQP3*, were expressed in SHED with or without co-cultured. Notably, only *ΔNp63* were expressed in co-cultured SHED. Bioinformatics analysis has determined that *SUSD2*, *CXCL1*, and *GGT5* were found to be highly regulated differentially expressed genes (DEGs). The overall result of the bioinformatics analysis suggested that the DEGs on days 5 and 7 were mainly associated with cell cycle activity and epithelial differentiation. In addition, the high enrichment of the DEGs related to the TGFβ signalling pathway marked its involvement in regulating SHED-epithelial differentiation. The gene expression analysis through RT-qPCR confirmed the involvement of the TGFβ pathway in regulating the process due to the high expression of markers associated with this pathway, *TGFβ3* and *BMP8A*. Captivatingly, the result demonstrated that the TGFβ inhibition by A83-01 inhibitor regulated glandular epithelial-like differentiation in the co-cultured SHED, which was supported by the morphological change and expression of gene markers associated with glandular epithelial cells, such as *SUSD2*, *GGT5*, *PPARG*, and *COL6A2*, and protein marker, such as Muc 1. This study proved the regulatory role of the TGFβ pathway in the SHED early differentiation into epithelial-like cells. Nevertheless, the TGFβ inhibition by A83-01 has been suggested could manipulate this pathway to regulate glandular epithelial-like cells. Our co-cultured SHED model demonstrated epithelial differentiation, which holds promise in future studies for application in epithelial tissue regeneration, especially in wound healing.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Regenerative medicine encompasses multiple disciplines, while this study focuses specifically on epithelial tissue regeneration. The human body is mainly composed of epithelial tissue derived from the ectoderm, endoderm, and mesoderm layers (Huysseune *et al.*, 2022). The epithelial tissue is found in the epidermis, cornea, oral cavity, nasal cavity, and anal canal (Muse and Crane, 2018). Generally, the epithelial tissue can be categorised into two types: surface epithelium, which serves as a barrier, and glandular epithelium, primarily responsible for secretion such as hormone and saliva (Chruścik *et al.*, 2021). Epithelial differentiation is the key mechanism responsible for the regeneration of epithelial tissue. Throughout this procedure, the undifferentiated epithelial cells, also known as the epidermal stem cells, located at the basal layer of the epithelium, renewed injured cells and maintained the integrity of the tissue (Deo and Deshmukh, 2018).

Epidermal stem cells are unipotent and can only produce similar cell types within their niche (Çankirili *et al.*, 2020). Various conditions can hinder these unipotent cells from replacing and replenishing cells immediately, delaying the wound healing process, such as lesions caused by diabetes, trauma, vascular insufficiency, and third-degree burns (Díaz-García *et al.*, 2021). In the worst-case scenario, epithelial cells' barrier and secretion function cannot be achieved, especially in patients with autoimmune disorders. These patients may experience hyperkeratosis, caused by delayed wound healing leading to scarring, and xerostomia, a condition of deregulated salivary gland leading to dry mouth (Garcia-Rios *et al.*, 2022; Kim, 2023). Those disorders which are associated with delayed wound healing require a process known as re-epithelialisation.

Wound healing is a complex process that depends on the interplay between the extracellular matrix, growth factors, cells, and endogenous proteins, mimicking the native skin or tissue niche (Ogliari *et al.*, 2014). Chronic wounds often fail to progress through critical stages of wound healing, such as inflammation, proliferation, and remodelling (Niu *et al.*, 2019). Re-epithelialisation is part of the proliferation (Pastar *et al.*, 2014). The main concern during wound healing is closure at the wound site, achievable through complete re-epithelisation. Failure to heal the epithelium in chronic wounds would disrupt multiple biological mechanisms, leading to ischemia, infection (Pastar *et al.*, 2014), deficiencies in protease inhibitors, cytokines, and growth factors, alongside elevated levels of proinflammatory cytokines and proteases (McCarty and Percival, 2013). These conditions eventually lead to prolonged inflammatory response and promote scar formation (Cialdai *et al.*, 2022). During re-epithelisation, the denuded or wounded site requires epithelial cells to restore the tissue barrier of the epithelium (Bártolo *et al.*, 2021; Yang *et al.*, 2021).

The regeneration of epithelial tissue through stem cell differentiation seems promising. Some studies suggest the potential of stem cell usage in the recovery of wound healing (Nguyen *et al.*, 2022; Khadivar and Alipour, 2023). Plasticity enables stem cells to change their fate in response to microenvironments such as extracellular signals (Bonfanti *et al.*, 2012). Epithelial differentiation has been demonstrated in various stem cell types, such as ESCs (Petrova *et al.*, 2014; Zhang *et al.*, 2017), iPSCs (Larribère *et al.*, 2017), and ASCs including menstrual blood-derived stem cells (menSCs) and mesenchymal stem cells (MSCs) (Akhavan-Tavakoli *et al.*, 2017; Hosseinzadeh *et al.*, 2017). Nevertheless, their origin affects stem cell's potency (Zakrzewski *et al.*, 2019).

The primary criteria for stem cell selection are cell harvesting techniques and ethical considerations (Akhavan-Tavakoli *et al.*, 2017). Embryonic stem cells (ESCs) are known for their pluripotency but face an ethical concern due to the use of human zygotes (Ghimire *et al.*, 2021). Although induced pluripotent stem cells (iPSCs) showed a promising outcome, establishing iPSCs can be challenging due to strict quality control from cell acquisition and propagation (Moy *et al.*, 2023). Adult stem cells (ASCs) are another option but in some cases, it is challenging to be propagated (Rao and Vemuri, 2009). Above all, stem cells possess plasticity characteristics, making them a better choice than mature cells (Pirsadeghi *et al.*, 2024). Considering all of the aforementioned facts, some ASCs appear to pose little concern due to their lack of ethical considerations, ease of acquisition, and propagation (Gopalarethinam *et al.*, 2023).

Dental stem cells, a form of ASCs, have lately gained interest, not just because they are easy to obtain and have fewer ethical problems (Aly, 2020) but also due to their cultural characteristics and plasticity (Huang *et al.*, 2009; Sakai *et al.*, 2010). There are many types of dental stem cells, such as dental pulp stem cells (DPSCs), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), stem cells from human exfoliated deciduous teeth (SHED), and dental follicle progenitor cells (DFPCs) (Granz and Gorji, 2020). Among these stem cells, SHED stand out due to their characteristics. SHED are isolated from the dental pulp (Miura *et al.*, 2003; Zainuri *et al.*, 2018) and exhibit a high proliferation rate and cell population doublings (Miura *et al.*, 2003; Chansaenroj *et al.*, 2021). Besides, SHED can be differentiated into numerous types of cells (Sakai *et al.*, 2010; Wang *et al.*, 2010; Hu *et al.*, 2018). Furthermore, SHED originates from ectoderm, similar to epithelial cells of the skin

and salivary glands (Tsai *et al.*, 2015; Muallah *et al.*, 2023). Thus, SHED was selected for this study (Zakrzewski *et al.*, 2019; Aly, 2020).

Based on the properties outlined above, it was postulated that the SHED could be employed to accelerate re-epithelialisation during wound healing. Many studies have utilised SHED in wound healing related to bone repair, renal, and brain injury (Miura *et al.*, 2003; Yamagata *et al.*, 2013; Hattori *et al.*, 2015). A previous study demonstrated that SHED in monoculture could differentiate into epithelial cells with the addition of growth factors (Doğan *et al.*, 2015), while co-cultured SHED were shown to differentiate into corneal epithelium (Tsai *et al.*, 2015). However, those studies did not investigate any signalling pathway involved during the differentiation of SHED into epithelial cells. Although it has been postulated that the molecules from neighbouring cells can trigger stem cell differentiation (Sah *et al.*, 2017; Yang *et al.*, 2020), research on co-culture studies regarding SHED-epithelial differentiation is limited. Hence, this study investigated transcriptomic profiling to elucidate the molecules and pathways that might play a role during the SHED-epithelial differentiation. This study enabled a comprehensive understanding of cellular interactions during co-culture by interpreting the gene changes and cellular function. It is noteworthy that stem cell-epithelial differentiation is known to be regulated by several pathways, including the Wingless-related Integration Site (WNT)/β-catenin signalling pathway (Saari *et al.*, 2022), Notch signalling pathway (Qu *et al.*, 2016), extracellular signal-regulated kinase (ERK), c-Jun N-terminal Kinase (JNK), and Protein Kinase B (AKT) signalling pathways (Zhao *et al.*, 2016), and the Transforming Growth Factor Beta (TGFβ)/ Suppressor of Mothers Against Decapentaplegic (SMAD) signalling pathway (Sah *et al.*, 2017). If the results of this discovery align

with these established pathways, it suggests that the model is suitable for future use in *in vivo* and clinical applications for wound healing.

The present study focused on epithelial tissue regeneration for wounds using a co-culture model in which the SHED were indirectly co-cultured in the epithelial microenvironment with human epidermal keratinocyte (HEK001) in the keratinocyte serum free medium (KSFM), an epithelial induction medium using Transwell. The cell model was utilised to mimic the native epithelial microenvironment. Previous study suggests that whenever cells are cultured in a medium, cytokines and biomolecules related to the cells, known as secretomes, are released into the medium (Sanchez-Guzman *et al.*, 2021). Thus, in the current study, it is hypothesised that secretomes released from HEK001 into the medium could enhance the epithelial microenvironment of the medium and assist SHED-epithelial differentiation.

This study was also meticulously designed to observe the time-dependent changes of the co-cultured SHED. In this study, the SHED-epithelial differentiation was confirmed through gene expression, flow cytometry, and immunocytochemistry. This study also utilised microarray gene expression screening, a comprehensive method to identify genes and pathways that regulate SHED-epithelial differentiation during co-culture. This was followed by a thorough bioinformatics analysis. The gene expression microarray data has been deposited in the NCBI GEO profiles under the accession number GSE165451.

Our preliminary finding identified that the TGFβ signalling pathway corresponded to regulating epithelial differentiation of the co-cultured SHED. The identified pathway was confirmed based on bioinformatics analyses. Since the TGFβ pathway regulates many biological processes, such as cell growth, epithelial-mesenchymal transition

(EMT), and differentiation (Kahata *et al.*, 2018; Liarte *et al.*, 2020), its regulation in co-cultured SHED was further investigated using molecular biology techniques. For this purpose, a chemical inhibitor study using A83-01 was conducted to confirm the involvement of the TGFβ pathway, which showed a high possibility of involvement. A83-01 inhibits several receptors in the TGFβ pathway, such as TGFβR1, type 1 activin, and nodal receptors, by deactivating SMAD2/3 (Gurung *et al.*, 2015).

Following inhibition, both gene and protein expression markers for stem cell-epithelial differentiation were evaluated. The stem cell genes and protein markers assessed included Cluster of Differentiation 73, 90 and 105 (CD73, CD90, and CD105). Meanwhile, the epithelial markers genes markers used were *Type 2-Hydroxybutyrate Dehydrogenase* (*BDH2), Ets Homologous Factor (EHF),* and *Peroxisome Proliferator-Activated Receptor Gamma* (*PPARG)* while the epithelial protein markers used were Pan-Cytokeratin (Pan-CK) and Mucin 1 (Muc 1). Additionally, genes related to the TGFβ pathway, such as *Transforming Growth Factor Beta 3 (TGFβ3), MYC proto-oncogene (MYC), Bone Morphogenetic Protein 8A (BMP8A), Sushi Domain Containing 2 (SUSD2), Chemokine (C-X-C motif) Ligand 1 (CXCL1), Gamma-Glutamyltransferase 5 (GGT5), Collagen Type VI Alpha 2 Chain (COL6A2),* and *Thioredoxin-Interacting Protein (TXNIP)* were also analysed. Lastly, genes related to epithelial-mesenchymal transition (EMT), such as *Snail Family Transcriptional Repressor 2* (*SNAI2)* and Tumour Protein P63 (*TP63)* were also investigated.

1.2 Significance of study

The precise molecules that govern the SHED-epithelial differentiation remain uncertain. Hence, this study examined the genes, proteins, and pathways that play a

role in this process. Creating an epithelial-like microenvironment that closely resembles the native human system would increase the chance of SHED differentiating into epithelial cells. SHED were a compelling candidate due to their highly proliferative and multipotent behaviour. Besides, SHED has a strong potential for differentiation into epithelial-like cells because they share a similar ectodermal origin to keratinised epithelial cells. To enhance the epithelial microenvironment, we utilised an indirect co-culture method. This setup, which includes the presence of epithelial cells and an induction medium, facilitated the release of key molecules that could drive SHED differentiation into epithelial-like cells.

In the current study, the TGFβ pathway was selected based on the preliminary findings from the bioinformatics analysis. The TGFβ pathway regulates many biological processes, including cell cycle, cell fate determination, and differentiation. Given the intricate interplay observed between these processes in our current study, investigating the impact of TGFβ pathway inhibition offers valuable insights into its regulatory role in SHED-epithelial differentiation. This study could improve our understanding of the molecular regulation of the epithelial differentiation process. To summarise, the genes and pathways discovered as regulators of differentiation could be targeted in future research, particularly on epithelial tissue regeneration, such as wound healing.

1.3 Research questions

Does co-culture SHED with HEK0001 cells influence gene and protein expression profiles, including associated pathways, during SHED-epithelial differentiation?

1.4 Hypothesis

Co-culture SHED with HEK0001 cells during SHED-epithelial differentiation alters gene and protein expression profiles, including associated pathways.

1.5 Research objectives

1.5.1 General objectives

To investigate the transcriptional profiling of SHED-epithelial differentiation in a co-culture model.

1.5.2 Specific objectives

1. To determine the expression of epithelial gene markers of SHED after co-cultured with HEK0001 for epithelial differentiation using a one-step reverse-transcriptase polymerase chain reaction (RT-PCR).
2. To identify possible differentially expressed genes (DEGs) and pathways associated with epithelial differentiation based on microarray gene expression of SHED after co-cultured with HEK0001 using bioinformatics analysis.
3. To validate the identified DEGs of SHED after co-cultured with HEK0001 using a one-step reverse-transcriptase real-time polymerase chain reaction (RT-qPCR).
4. To determine the expression of gene markers related to the TGFβ pathway of SHED after co-cultured with HEK0001 treated with and without TGFβ inhibitor, A83-01 using RT-qPCR.
5. To assess the expression of stem cell protein markers of SHED after co-cultured with HEK0001 treated with and without TGFβ inhibitor, A83-01 using flow cytometry.
6. To assess the expression of epithelial protein markers, Pan-cytokeratin, and Muc 1 of SHED after co-cultured with HEK0001 treated with and without TGFβ inhibitor, A83-01 using immunocytochemistry.

CHAPTER 2

LITERATURE REVIEW

2.1 Epithelium

Epithelium is among the major tissues of the human body derived from the three germ layers: ectoderm, mesoderm, and endoderm. The epithelial cells intricately line various organs and structures (Kurn and Daly, 2020). Ectoderm-derived epithelium includes the epithelial cells that line the skin, cornea, mouth, nose, and anus (Muse and Crane, 2018). The endothelial cells, which line blood vessels, represent a specialised epithelium derived from mesoderm (Vargas-Valderrama *et al.*, 2020). Meanwhile, the epithelial cells lining the oesophagus, lungs, stomach, intestines, and major components of many glands are derived from the endodermal epithelium (Chruścik *et al.*, 2021). Their functions are varied and influenced by their specific location and types.

2.1.1 Physiology of epithelium

Generally, epithelial cells are densely interconnected cells that line the external and internal organ surfaces (Larsen *et al.*, 2020). The cell-cell interactions within epithelial tissues are mediated by junctional complexes, which consist of tight junctions, adherens junctions, desmosomes, and gap junctions' proteins. These complexes are crucial in establishing epithelial polarity (Díaz-Díaz *et al.*, 2020). Moreover, they help to distinguish the epithelial polarity such as apical, basal, and lateral regions. The cell's apical surface is oriented towards the external environment, and the structures such as microvilli, cilia, and stereocilia affect cellular function (Kurn and Daly, 2020). Its basolateral (combination of basal and lateral) surface is oriented internally toward the basement membrane (Roig-Rosello and Rousselle, 2020). The basal surface separates the epithelium from connective tissue, while the lateral surface favours cell-cell

interaction (Kurn and Daly, 2020). Figure 2.1 shows the diagram illustrating the physiology of the epithelium.

2.1.2 Type of epithelium

Epithelium can be categorised into two, which are surface epithelium and glandular epithelium (Figure 2.2). The fundamental composition of surface epithelium can be classified according to its shape or the number of layers it carries. The shapes can be either squamous, cuboidal, or columnar. Meanwhile, the layers can be simple, stratified, or pseudostratified (Rehfeld *et al.*, 2017). Single-layered epithelium is termed simple, while multiple-layered epithelium is termed stratified. Therefore, epithelial cells have been classified as simple squamous, simple cuboidal, simple columnar, stratified squamous, and stratified columnar (Shashikanth *et al.*, 2017). Nonetheless, stratified cuboidal and stratified columnar are rare conditions. Such conditions are usually observed in the large gland (Rehfeld *et al.*, 2017) and can also be identified in the anal canal (Tanaka *et al.*, 2012). On the other hand, pseudostratified columnar epithelium is often present in distensible organs like the respiratory tract (Kurn and Daly, 2020).

Meanwhile, the glandular epithelium can be further classified as endocrine and exocrine glands. Figure 2.2 summarises the classification of epithelium using examples from the literature (Rehfeld *et al.*, 2017; Chruścik *et al.*, 2021).

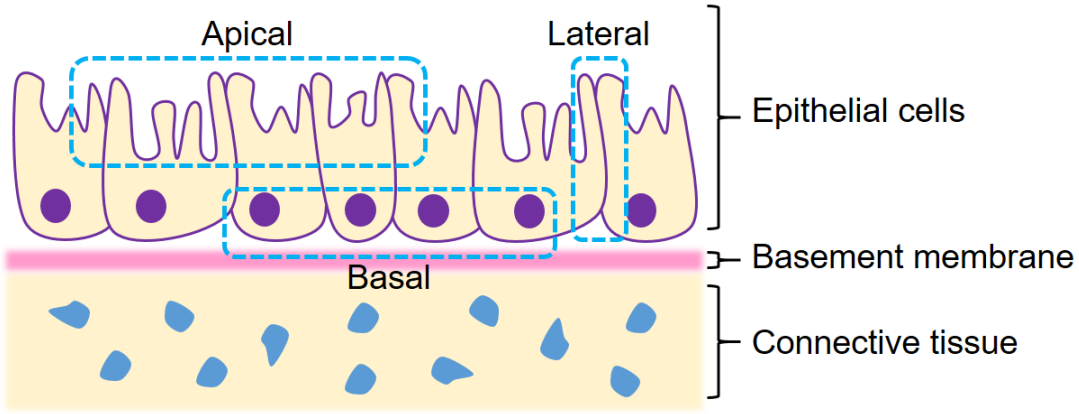

Figure 2.1 Diagram illustrating the physiology of the epithelium, showcasing its distinct polarity: the apical region, basal region, and lateral regions.

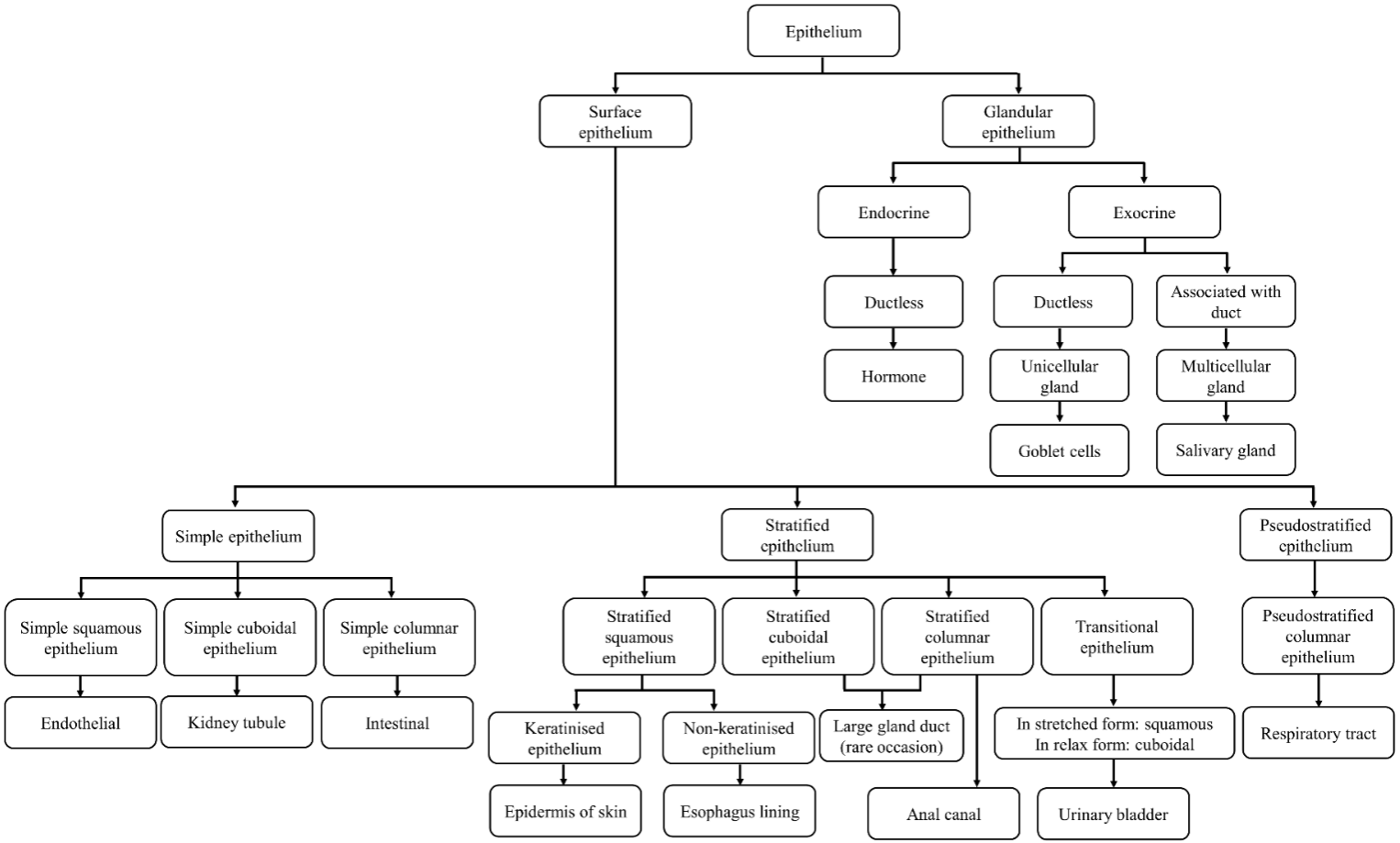

Figure 2.2 Classifications of epithelium. Epithelium can be classified as surface epithelium and glandular epithelium.

2.1.2(a) Surface epithelium

The stratified squamous epithelium covers a large part of the human body, including the skin, cornea, and oesophagus (Pereira and Sequeira, 2021). It protects the body from water loss and foreign pathogens (Larsen *et al.*, 2020). This type of epithelium undergoes a maturation process known as keratinisation or non-keratinisation (Deo and Deshmukh, 2018). The only similarity between the two processes is that they have a basal layer of precursor cells known as epithelial stem cells (Kao, 2020).

2.1.2(a)(i) Keratinised epithelium

Keratinised epithelium can be characterised by the absence of a nucleus and the presence of keratin (Groeger and Meyle, 2019). They can be found in the epidermis of human skin. The epidermis of the skin's outer layer is formed by stratified layers of highly specialised epithelial cells known as keratinocytes (Pereira and Sequeira, 2021). It emerges from the ectoderm after gastrulation (Hu *et al.*, 2018). Epidermal architecture is unique as the epithelial cells at the basal layers undergo a differentiation process to form keratinocyte cells. On top of that, keratinocyte cells are unable to divide (Sanz-Gómez *et al.*, 2020).

Generally, the epidermis has four layers: cornified, granular, spinous, and basal (Figure 2.3). These layers vary in thickness and shape, depending on the location and species, as well as the state of keratinocyte differentiation (Moroki, 2023). Besides, epithelial cells from each layer exhibit different morphology. Proliferative epithelial cells lay at the basal layer. The cells eventually migrate towards the upper epidermal layers until they terminally differentiate to form the dead surface layer of the skin at the cornified layer (Moreci and Lechler, 2020).

Keratinised epithelium can also be found in the oral mucosa, and its formation is similar to that of the epidermis. However, unlike skin cells, which are characterised by keratinised features, the cells in the oral mucosa undergo two distinct maturation processes. Depending on the cell's exact location, it can be keratinised or non-keratinised epithelium (Groeger and Meyle, 2019). The keratinised epithelium of the oral mucosa includes masticatory and specialised mucosa, comprising tongue dorsum, gingiva, and hard palate (Borowiec *et al.*, 2013; Deo and Deshmukh, 2018). Longer time is required to allow complete re-epithelialisation at this keratinised area (Turabelidze *et al.*, 2014). Figure 2.3 shows the layer of keratinised epithelium.

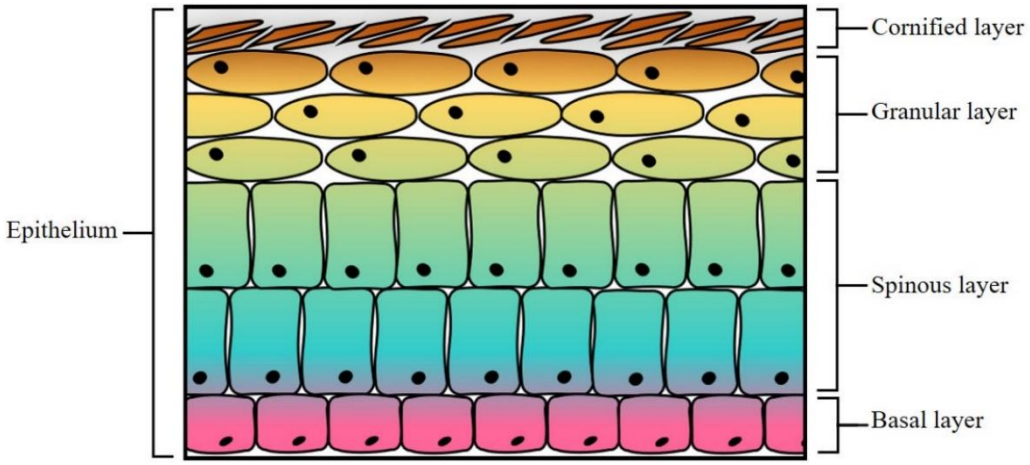

Figure 2.3 The layer of keratinised epithelium consisting of basal, spinous, granular, and cornified layers.

2.1.2(a)(ii) Non-keratinised epithelium

Non-keratinised epithelium differs from keratinised epithelium because it has a nucleus without keratin (Groeger and Meyle, 2019). The tissue can be found in the mucosa lining (Brizuela and Winters, 2021), including the soft palate, cheeks, lips, alveolar mucosa, vestibular fornix, and floor of the mouth (Groeger and Meyle, 2019). It can also be found in the nasal cavity, consisting of ciliated, mucous, and basal cells (Heffler *et al.*, 2018; Upadhye *et al.*, 2018). In the non-keratinised oral mucosa, the stratum filamentosum and the stratum distendum are above the basal layer (Johnston, 2015; Groeger and Meyle, 2019). The oral mucosa wound site heals faster with less inflammation than the epidermal wound (Waasdorp *et al.*, 2021).

2.1.2(b) Glandular epithelium

Glandular epithelium can be categorised into the endocrine and exocrine glands. It comprises one or more epithelial cells that synthesise and excrete chemical substances (Chruścik *et al.*, 2021). The glandular epithelial cells can be found in saliva, thyroid, thymus, pancreas, and liver (Chansaenroj *et al.*, 2021; Chruścik *et al.*, 2021).

2.1.2(b)(i) Endocrine glands

Endocrine glands are ductless and secrete hormones into the bloodstream (Freeman *et al.*, 2019; Chruścik *et al.*, 2021). For example, cells at the anterior pituitary, thymus, adrenal cortex, and gonads secrete hormones (Chruścik *et al.*, 2021). In addition, the pancreas is also comprised of endocrine glands, known as Beta cells, that secrete insulin hormone for glucose regulation (Sha *et al.*, 2020).

2.1.2(b)(ii) Exocrine glands

Exocrine glands comprise acinus and ducts that secrete secretory fluids directly into body cavities or onto external surfaces (Freeman *et al.*, 2019; Khan *et al.*, 2022). The acinus is mainly responsible for cell secretion, while the duct is used for transportation (Freeman *et al.*, 2019). Acinar secretory cells can be divided into serous, mucous, or lipid-enriched acinar (Khan *et al.*, 2022). While serous acinar secrete proteins such as enzymes, the mucous acinar secrete mucin (Freeman *et al.*, 2019). Meanwhile, lipid-enriched acinar is involved in the secretion of meibum (by the meibomian gland, found in the eyelid) and sebum (by the sebaceous gland, found in the skin), mainly for moisturisation and lubrication (Khan *et al.*, 2022).

Exocrine glands can be unicellular or multicellular (Chruścik *et al.*, 2021; Khan *et al.*, 2022). Unicellular exocrine glands comprise individual and standalone cells, such as goblet cells. They are located in the mucous membranes of the small and large intestines and are responsible for mucus production (Chruścik *et al.*, 2021).

On the contrary, multicellular exocrine glands are made up of several cell types to perform specific functions (Khan *et al.*, 2022). The secretion of multicellular exocrine glands can be further categorised based on the three secretion mechanisms: merocrine, apocrine, and holocrine (Khan *et al.*, 2022). Merocrine glands secrete secretory contents via exocytosis without damaging the secreting cell. Meanwhile, apocrine glands work by creating cell buds. Lastly, holocrine glands cause the cells to die by emptying their product into the gland lumen (Freeman *et al.*, 2019; Khan *et al.*, 2022). Serous and mucous glands mainly use merocrine mode of secretion.

In contrast, lipid-enriched glands use the holocrine mode of secretion (Khan *et al.*, 2022). The primary function of exocrine glands varies according to their location.

However, their primary functions revolve around digestion, mucosal protection, thermoregulation, lubrication, and nutrition (Freeman *et al.*, 2019). To regulate these functions, epithelial cells undergo a regeneration process known as epithelial differentiation. Epithelial differentiation is usually controlled by the stem cells within their niche (Wang and Tang, 2021). Other types of stem cells can also regulate it.

2.2 Epithelial tissue regeneration

The primary objective of epithelial tissue regeneration is to repair or replace damaged epithelium structures within organs and tissues in the human body. For instance, epithelial tissue is a type of tissue that needs to be regenerated to maintain its physical barrier function (Chruścik *et al.*, 2021). Epithelial stem cells are crucial in this process, as they continuously replenish epithelial cells and sustain homeostasis within their specific microenvironment or niche (Ruan *et al.*, 2021; Wang and Tang, 2021).

2.2.1 Homeostasis

Cell homeostasis can be achieved by regulating stem cell behaviour, which might involve symmetric or asymmetric cell divisions (Berika *et al.*, 2014; Cockburn *et al.*, 2022). In symmetric cell division, epithelial stem cells give rise to two daughter cells with properties akin to the mother cells and, thus, increase the stem cell pool (Berika *et al.*, 2014). Conversely, asymmetric cell division balances self-renewal and differentiation, contributing to epithelial cell turnover and homeostasis (Cockburn *et al.*, 2022).

During epithelial differentiation, undifferentiated epithelial stem cells or progenitor cells evolve into specialised epithelial cells (Deo and Deshmukh, 2018). Through this intricate process, cells leave the progenitor pool, committing to a specific cell fate and function according to their niche (Capdevila *et al.*, 2021). For instance, specialised

epithelial cells are needed for tissue homeostasis to allow absorptive function in the gut (Iftekhar and Sigal, 2021). Besides, the cells are also required to provide skin barrier function (Dehkordi *et al.*, 2019). These functions can be achieved through complete re-epithelialisation.

2.2.2 Re-epithelialisation

Re-epithelialisation is the process of covering stripped surfaces of epithelial cells and is involved in the repair mechanism of wound healing (Wang *et al.*, 2024). In the skin, for instance, the epidermis is required to provide keratinocytes to cover the wound (Wang and Graves, 2020). Without complete re-epithelialisation, any wound cannot be considered healed. During epithelial tissue repair and regeneration, the wound area necessitates extracellular matrix (ECM), growth factors, cells, and endogenous proteins for complete closure (Niu *et al.*, 2019). Hence, wound healing undergoes critical stages of wound healing, such as inflammation, proliferation, and remodelling (Niu *et al.*, 2019).

During inflammation, the coagulation cascade is activated to reduce blood loss, and at the same time, inflammatory cells such as macrophages are recruited to protect the wound site from debris and pathogens (Carr, 2022). Then, the proliferation phase takes over to facilitate fibroblast migration, collagen formation, angiogenesis, recruitment of cytokine and growth factors, and re-epithelialisation activation (Niu *et al.*, 2019; Carr, 2022). After complete re-epithelialisation, the wound undergoes a remodelling phase, orchestrating the reorganisation and strengthening of collagen and ECM components (Niu *et al.*, 2019; Diller and Tabor, 2022). However, a continuous state of inflammation, including incomplete re-epithelialisation, can disrupt the remodelling phase, leading to dysregulated ECM remodelling, and promote scarring (Qian *et al.*,

2016; Cialdai *et al.*, 2022). Scarring typically happens to diabetic patients who suffer continuous lesions and patients with third-degree burns (Wang and Graves, 2020; Lukomskyj *et al.*, 2022). To achieve complete re-epithelialisation, the epithelial stem cells are required (Yang *et al.*, 2021).

However, a challenge emerged due to the short lifespan of specialised epithelial cells, necessitating immediate replenishment by epithelial stem cells at the basal layer (Kai, 2021). These processes would be challenging due to the unipotency of epithelial stem cells (Zakrzewski *et al.*, 2019). Recently, many studies have explored alternative sources of stem cells for regenerating functional epithelial cells.

2.3 Stem cells

Stem cell therapy is a promising strategy for regulating epithelial differentiation, as suggested in a few studies (Nguyen *et al.*, 2022; Khadivar and Alipour, 2023). Stem cells, characterised by their ability to self-renew and differentiate (Zakrzewski *et al.*, 2019), offer a unique feature in their plasticity. They can transform into a new phenotype based on their potency, responding to external induction (Chagin and Chu, 2023). Stem cells can be categorised based on their differentiation potential, including totipotent, pluripotent, multipotent, oligopotent, and unipotent stem cells (Aprile *et al.*, 2024).

Totipotent stem cells can differentiate into any human cell (Zakrzewski *et al.*, 2019). They have the highest differentiation capability to form complete organisms, such as cells of the zygotes (Pirsadeghi *et al.*, 2024). Meanwhile, pluripotent stem cells can develop into all germ layers, including ESCs and iPSCs (Zakrzewski *et al.*, 2019; Aprile *et al.*, 2024). In contrast, multipotent cells can differentiate into several cell types, such as hematopoietic, neural stem cells, and MSCs (Aprile *et al.*, 2024;

Pirsadeghi *et al.*, 2024). Additionally, oligopotent stem cells have restricted differentiation capability as they only differentiate into the cells of their lineage, for example, hematopoietic stem cells (Zakrzewski *et al.*, 2019). Lastly, unipotent stem cells can differentiate into one cell type that suits their niche, such as epidermal stem cells (Zakrzewski *et al.*, 2019; Pirsadeghi *et al.*, 2024). While potency classification is based on the level of differentiation potential, stem cell types are categorised based on their source and origin.

2.3.1 Type of stem cells

Stem cells can be classified into three types which are ESCs, iPSCs, and ASCs. Since the 1950s, research on ESCs have been carried out using an *in vivo* model. ESCs are pluripotent cells derived from the blastocyst's inner cell mass (Takahashi and Yamanaka, 2006). Initially, researchers stumbled upon the unexpected incidence of teratoma or teratocarcinoma in one of their mouse strains (Stevens Jr and Little, 1954). This unexpected observation eventually led to the discovery of the first embryonal carcinoma cell line, which strongly resembles stem cell pluripotency (Martin, 1981; Andrews, 2024). The monstrous nature of cancer cells makes this cell model inappropriate for human study (Sajjad *et al.*, 2021). As a result of 1998, human ESCs were isolated following a comprehensive investigation of the mouse model (Thomson *et al.*, 1998). Subsequently, human ESC research and application have thrived.

ESCs can be characterised by their ability to self-renew and give rise to any cell type from the three germ layers, including mesoderm, endoderm, and ectoderm (Kim *et al.*, 2023). However, the use of human ESCs has sparked debate over the critical concern of using embryos in scientific settings (Pereira Daoud *et al.*, 2020). Only a few laboratories can investigate ESCs due to the limitations imposed by the countries,

funding, or the International Society for Stem Cell Research's guidelines (Lovell-Badge *et al.*, 2021). Despite these regulations, science has advanced tremendously. In addition to clinical trials (Deinsberger *et al.*, 2020), ESCs have shown extensive differentiation potential, including the development of keratinocytes (Zhong *et al.*, 2020), cornea epithelial-like cells (da Mata Martins *et al.*, 2020), endometrium-like cells (Jiang *et al.*, 2021), and renal lineage (Chow *et al.*, 2020). Completed clinical trials have utilised ESCs-derived retinal pigment epithelium (RPE), administered through injection or topical application for macular regeneration, while ongoing trials are exploring ESC-derived cardiomyocytes, epithelial cells, and MSCs (Deinsberger *et al.*, 2020).

Later, in 1998, iPSCs were established after Yamanaka factors (Oct3/4, Sox2, c-Myc, and Klf4) were introduced in fibroblast cells and exhibited pluripotent behaviour (Takahashi and Yamanaka, 2006). The iPSCs are pluripotent stem cells similar to ESCs. While ESCs can differentiate into various somatic cell types, iPSCs can be directly generated from somatic cells through cell reprogramming, which involves the manipulation of transcription factors and genes associated with pluripotency (Wang *et al.*, 2021). Due to their unlimited cell source and propagation, the iPSCs hold great potential to be used in cell therapy and transplantation (Zakrzewski *et al.*, 2019; Liu *et al.*, 2020). Furthermore, the use of iPSCs raises fewer ethical concerns, as patients make a voluntary choice about their usage. On top of that, autologous transplantation uses an individual's cells, reducing the risk of immunological rejection (Otsuka *et al.*, 2020). To date, iPSCs have shown a potential to differentiate into endothelial-like cells (Cheng *et al.*, 2021), alveolar epithelial-like cells (Müller *et al.*, 2023), and have also been applied in clinical trials (Deinsberger *et al.*, 2020). However, establishing functional iPSCs is lengthy, inefficient, and uncertain (Moy *et al.*, 2023). Additional

drawbacks encompass concerns related to cell efficiency, expansion, and the lineage origin necessary for application in cell differentiation studies (Li and Belmonte, 2016; Moy *et al.*, 2023). Besides, iPSCs retain a memory of their parental cells, displaying a limited potential to differentiate into other cell types (Liu *et al.*, 2020). Furthermore, the techniques used during iPSC isolation are relatively expensive (Huang *et al.*, 2019).

While ESCs and iPSCs are pluripotent, ASCs are mostly multipotent (Zakrzewski *et al.*, 2019). The ASCs, or somatic stem cells, are undifferentiated cells that coexist alongside differentiated cells in specialised niches (Gurusamy *et al.*, 2018). They can be identified as HSCs and non-HSCs. The HSCs are primarily found in bone marrow, peripheral, and cord blood (Ng and Alexander, 2017). Meanwhile, non-HSCs can be found throughout the body upon embryonic development, including in bone marrow, skin, gut, skeletal muscle, central nervous system, heart, and dental tissue (Zakrzewski *et al.*, 2019; Mannino *et al.*, 2022). Their main function is to retain cell homeostasis and growth and be involved in tissue repair (Zakrzewski *et al.*, 2019). In clinical applications, HSCs, for instance, are used to treat multiple myeloma and leukaemia (Chu *et al.*, 2020). Furthermore, the usage of MSCs have been shown could promote wound healing caused by burns, diabetic ulcers, and skin diseases, both through topical application and injection (Riedl *et al.*, 2021). Nonetheless, the aspiration of stem cells from the bone marrow is painful and results in low yields (Drela *et al.*, 2019).

Considering ethical concerns related to the use of ESCs (Ghimire *et al.*, 2021) and the production costs associated with isolating iPSCs (Moy *et al.*, 2023), the preference leans towards utilising ASCs. Moreover, the ASCs pose fewer ethical concerns during cell isolation, making them more suitable for *in vitro* and *in vivo* studies

(Gopalarethinam *et al.*, 2023). The only disadvantage of using ASCs is that their regulation is still elusive and necessitates more intense research. It is still a challenge to find ASCs that are less painful with promising outcomes and high yields. Previously, ASCs have shown a potential to differentiate into osteoblast-like (Hemming *et al.*, 2016), adipose-like (Marquez *et al.*, 2017), hepatocyte-like (Yu *et al.*, 2018), and neuronal-like cells (He *et al.*, 2021). Dental stem cells, a type of ASC, have gathered attention among researchers due to their minimal ethical concerns, abundant availability, and potential to be used for large-scale applications (Aly, 2020).

2.3.2 Dental stem cells

ASCs can be found in various tissues, including the dental pulp, which serves as a reservoir for dental stem cells (Zakrzewski *et al.*, 2019; Granz and Gorji, 2020). The dental stem cells include stem cells from human exfoliated deciduous teeth (SHED), dental pulp stem cells (DPSCs), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), and dental follicle stem cells (DFSCs) (Granz and Gorji, 2020). Dental stem cells are easily acquired because they are ample with fewer ethical concerns and can be used on a large scale (Granz and Gorji, 2020; Wu *et al.*, 2024). Furthermore, it is relatively easy to obtain dental stem cells since human viable teeth act as stem cell storage (Chen *et al.*, 2022). The dental stem cells also exhibit mesenchyme characteristics, such as showing a fibroblast morphology similar to MSCs and being able to express MSC markers (Marei and El Backly, 2018). Moreover, dental stem cells are known to be able to differentiate into odontogenic, adipogenic, angiogenic, and neurogenic (Huang *et al.*, 2009; Sakai *et al.*, 2010).

Due to their multipotency, dental stem cells have become a strong candidate for epithelial differentiation research. Dental stem cells come from the same ectodermal