

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

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

by

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## TABLE OF CONTENTS

|                                               |              |
|-----------------------------------------------|--------------|
| <b>ACKNOWLEDGEMENTS.....</b>                  | <b>ii</b>    |
| <b>TABLE OF CONTENTS.....</b>                 | <b>iii</b>   |
| <b>LIST OF TABLES .....</b>                   | <b>xii</b>   |
| <b>LIST OF FIGURES .....</b>                  | <b>xv</b>    |
| <b>LIST OF SYMBOLS .....</b>                  | <b>xix</b>   |
| <b>LIST OF ABBREVIATIONS .....</b>            | <b>xx</b>    |
| <b>LIST OF APPENDICES .....</b>               | <b>xxiv</b>  |
| <b>ABSTRAK .....</b>                          | <b>xxv</b>   |
| <b>ABSTRACT .....</b>                         | <b>xxvii</b> |
| <b>CHAPTER 1 INTRODUCTION.....</b>            | <b>1</b>     |
| 1.1 Background of the study .....             | 1            |
| 1.2 Significance of study .....               | 6            |
| 1.3 Research questions .....                  | 7            |
| 1.4 Hypothesis .....                          | 7            |
| 1.5 Research objectives .....                 | 8            |
| 1.5.1 General objectives .....                | 8            |
| 1.5.2 Specific objectives.....                | 8            |
| <b>CHAPTER 2 LITERATURE REVIEW.....</b>       | <b>9</b>     |
| 2.1 Epithelium .....                          | 9            |
| 2.1.1 Physiology of epithelium .....          | 9            |
| 2.1.2 Type of epithelium .....                | 10           |
| 2.1.2(a) Surface epithelium .....             | 13           |
| 2.1.2(a)(i) Keratinised epithelium .....      | 13           |
| 2.1.2(a)(ii) Non-keratinised epithelium ..... | 16           |
| 2.1.2(b) Glandular epithelium .....           | 16           |

|       |                                                                   |    |
|-------|-------------------------------------------------------------------|----|
|       | 2.1.2(b)(i) Endocrine glands .....                                | 16 |
|       | 2.1.2(b)(ii) Exocrine glands .....                                | 17 |
| 2.2   | Epithelial tissue regeneration .....                              | 18 |
| 2.2.1 | Homeostasis .....                                                 | 18 |
| 2.2.2 | Re-epithelialisation.....                                         | 19 |
| 2.3   | Stem cells .....                                                  | 20 |
| 2.3.1 | Type of stem cells .....                                          | 21 |
| 2.3.2 | Dental stem cells .....                                           | 24 |
| 2.3.3 | SHED .....                                                        | 25 |
| 2.3.4 | Stem cell markers .....                                           | 26 |
|       | 2.3.4(a) Nanog.....                                               | 26 |
|       | 2.3.4(b) Nestin.....                                              | 27 |
|       | 2.3.4(c) CD73.....                                                | 27 |
|       | 2.3.4(d) CD90.....                                                | 27 |
|       | 2.3.4(e) CD105.....                                               | 28 |
| 2.4   | Stem cell-epithelial differentiation.....                         | 28 |
| 2.4.1 | Factor influencing stem cell-epithelial differentiation.....      | 29 |
|       | 2.4.1(a) Biological factor .....                                  | 29 |
|       | 2.4.1(b) Chemical factor.....                                     | 31 |
|       | 2.4.1(c) Physical factor .....                                    | 32 |
|       | 2.4.1(d) Temporal factor .....                                    | 34 |
| 2.4.2 | Mechanotransduction of stem cell-epithelial differentiation ..... | 35 |
|       | 2.4.2(a) Monoculture for mechanotransduction.....                 | 37 |
|       | 2.4.2(b) Co-culture for mechanotransduction .....                 | 37 |
|       | 2.4.2(b)(i) Direct co-culture.....                                | 38 |
|       | 2.4.2(b)(ii) Indirect co-culture .....                            | 39 |
|       | 2.4.2(c) 3D biological scaffold.....                              | 40 |
| 2.4.3 | Characterisation of stem cell-epithelial differentiation.....     | 42 |

|                  |                                                                                                            |           |
|------------------|------------------------------------------------------------------------------------------------------------|-----------|
| 2.4.3(a)         | Cell morphology .....                                                                                      | 42        |
| 2.4.3(b)         | Epithelial-specification markers .....                                                                     | 42        |
| 2.4.3(b)(i)      | Commonly used epithelial markers .....                                                                     | 42        |
| 2.4.3(b)(ii)     | Other epithelial markers used in this study .....                                                          | 45        |
| 2.4.4            | Pathways that regulate stem cell-epithelial differentiation .....                                          | 46        |
| 2.4.4(a)         | TGF $\beta$ pathway .....                                                                                  | 48        |
| 2.4.4(a)(i)      | TGF $\beta$ pathway in cell cycle .....                                                                    | 51        |
| 2.4.4(a)(ii)     | TGF $\beta$ pathway in EMT .....                                                                           | 52        |
| 2.4.4(a)(iii)    | TGF $\beta$ pathway in wound healing .....                                                                 | 53        |
| 2.4.4(a)(iv)     | TGF $\beta$ pathway in stem cell-epithelial differentiation .....                                          | 54        |
| 2.4.4(a)(v)      | TGF $\beta$ pathway inhibitor, A83-01 inhibitor .....                                                      | 56        |
| 2.4.4(a)(vi)     | TGF $\beta$ pathway markers .....                                                                          | 58        |
| 2.4.5            | Advanced tools and techniques used in screening and identifying stem cell-epithelial differentiation ..... | 62        |
| <b>CHAPTER 3</b> | <b>METHODOLOGY.....</b>                                                                                    | <b>65</b> |
| 3.1              | Study design .....                                                                                         | 65        |
| 3.2              | Materials .....                                                                                            | 67        |
| 3.2.1            | Cell culture study .....                                                                                   | 67        |
| 3.2.2            | Agarose gel electrophoresis .....                                                                          | 68        |
| 3.2.3            | Microarray study .....                                                                                     | 69        |
| 3.2.4            | Immunocytochemistry study .....                                                                            | 70        |
| 3.2.5            | List of consumable items.....                                                                              | 71        |
| 3.2.6            | Equipment .....                                                                                            | 72        |
| 3.2.7            | List of kits.....                                                                                          | 73        |
| 3.2.8            | Software .....                                                                                             | 74        |
| 3.3              | Methods .....                                                                                              | 75        |
| 3.3.1            | Reagent preparation.....                                                                                   | 75        |
| 3.3.1(a)         | Dilution of A83-01 inhibitor.....                                                                          | 75        |

|          |                                                                    |    |
|----------|--------------------------------------------------------------------|----|
| 3.3.1(b) | Dilution of antibody.....                                          | 75 |
| 3.3.1(c) | Dilution of ethanol.....                                           | 75 |
| 3.3.1(d) | Dilution of primer.....                                            | 76 |
| 3.3.1(e) | Preparation of agarose gel .....                                   | 76 |
| 3.3.1(f) | Preparation of diethyl pyrocarbonate (DEPC)-<br>treated water..... | 80 |
| 3.3.1(g) | Preparation of growth medium for SHED .....                        | 80 |
| 3.3.1(h) | Preparation of growth medium for HEK001 .....                      | 80 |
| 3.3.1(i) | Preparation of lithium borate (LB) buffer .....                    | 80 |
| 3.3.1(j) | Preparation of PBS .....                                           | 80 |
| 3.3.1(k) | Preparation of Thiazolyl Blue Tetrazolium Bromide<br>(MTT).....    | 81 |
| 3.4      | Cell culture .....                                                 | 81 |
| 3.4.1    | SHED .....                                                         | 81 |
| 3.4.1(a) | Cell retrieval from storage.....                                   | 81 |
| 3.4.1(b) | Sub-culturing of SHED.....                                         | 82 |
| 3.4.1(c) | Cell counting.....                                                 | 82 |
| 3.4.1(d) | Cell freezing for storage .....                                    | 83 |
| 3.4.1(e) | Cell pellet.....                                                   | 83 |
| 3.4.2    | HEK001.....                                                        | 84 |
| 3.4.2(a) | Cell retrieval from storage .....                                  | 84 |
| 3.4.2(b) | Sub-culturing of HEK001.....                                       | 84 |
| 3.4.2(c) | Cell counting.....                                                 | 84 |
| 3.4.2(d) | Cell freezing for storage .....                                    | 84 |
| 3.4.2(e) | Cell pellet.....                                                   | 84 |
| 3.4.3    | Co-culture of SHED with HEK001.....                                | 84 |
| 3.4.4    | Cell growth and proliferation .....                                | 89 |
| 3.4.4(a) | PrestoBlue assay to determine cell proliferation .....             | 89 |

|               |                                                                                        |     |
|---------------|----------------------------------------------------------------------------------------|-----|
|               | 3.4.4(b) Determination of cell morphology of SHED.....                                 | 89  |
| 3.5           | Gene expression analysis .....                                                         | 90  |
| 3.5.1         | Standard protocol to validate the integrity of RNA .....                               | 90  |
| 3.5.1(a)      | RNA extraction and quantification.....                                                 | 90  |
| 3.5.1(a)(i)   | innuPREP RNA Mini Kit with DNase treatment.....                                        | 90  |
| 3.5.1(a)(ii)  | GENEzol™ TriRNA Pure Kit.....                                                          | 94  |
| 3.5.1(b)      | Agarose gel electrophoresis for RNA.....                                               | 95  |
| 3.5.2         | Identification of stem cell and epithelial markers of co-cultured SHED by RT-PCR ..... | 95  |
| 3.5.2(a)      | Optimisation of experimental condition for RT-PCR.....                                 | 96  |
| 3.5.2(b)      | Cell culture and harvesting .....                                                      | 102 |
| 3.5.2(c)      | Gene expression analysis by RT-PCR.....                                                | 102 |
| 3.6           | Transcriptomic profiling of SHED-epithelial differentiation by microarray              | 102 |
| 3.6.1         | Cell culture, treatment, and harvesting.....                                           | 102 |
| 3.6.2         | RNA extraction and quantification .....                                                | 103 |
| 3.6.3         | Microarray profiling.....                                                              | 103 |
| 3.6.3(a)      | Filter.....                                                                            | 106 |
| 3.6.3(a)(i)   | Filter by probe sets expression.....                                                   | 106 |
| 3.6.3(a)(ii)  | Filter by volcano plot .....                                                           | 106 |
| 3.6.3(b)      | Identification of DEGs.....                                                            | 107 |
| 3.6.3(c)      | Functional and pathway enrichment analysis.....                                        | 107 |
| 3.6.3(d)      | Cytoscape visualisation and analyses .....                                             | 107 |
| 3.6.3(d)(i)   | PPI network .....                                                                      | 108 |
| 3.6.3(d)(ii)  | Molecular Complex Detection (MCODE)                                                    | 108 |
| 3.6.3(d)(iii) | Biological Networks Gene Ontology (BiNGO).....                                         | 108 |
| 3.6.3(d)(iv)  | Analysis of hub gene .....                                                             | 108 |
| 3.6.3(e)      | Validation of DEGs through RT-qPCR.....                                                | 109 |

|          |                                                                                                                              |            |
|----------|------------------------------------------------------------------------------------------------------------------------------|------------|
|          | 3.6.3(e)(i) Melting curve .....                                                                                              | 109        |
|          | 3.6.3(e)(ii) Standard curve.....                                                                                             | 113        |
|          | 3.6.3(e)(iii) Data quantification of RT-qPCR .....                                                                           | 116        |
| 3.7      | Investigation on TGF $\beta$ pathway modulation in co-cultured SHED after A83-01 inhibition by RT-qPCR .....                 | 117        |
| 3.7.1    | MTT assay to optimise A83-01 inhibitor concentration .....                                                                   | 117        |
| 3.7.2    | Cell culture and harvesting.....                                                                                             | 117        |
| 3.7.3    | Determination of cell morphology of SHED after A83-01 inhibition .....                                                       | 118        |
| 3.7.4    | RNA integrity and concentration .....                                                                                        | 118        |
| 3.7.5    | Quantification of gene expression by RT-qPCR.....                                                                            | 119        |
| 3.8      | Protein expression analysis .....                                                                                            | 122        |
| 3.8.1    | Investigating the role of the TGF $\beta$ signalling pathway in SHED-epithelial differentiation by flow cytometry.....       | 122        |
| 3.8.1(a) | Cell culture and harvesting .....                                                                                            | 122        |
| 3.8.1(b) | Flow cytometry .....                                                                                                         | 123        |
| 3.8.2    | Investigating the role of the TGF $\beta$ signalling pathway in SHED-epithelial differentiation by immunocytochemistry ..... | 125        |
| 3.8.2(a) | Cell culture, treatment, and harvesting .....                                                                                | 125        |
| 3.8.2(b) | Cell division assessment .....                                                                                               | 126        |
| 3.9      | Statistical analysis .....                                                                                                   | 126        |
|          | <b>CHAPTER 4 RESULTS.....</b>                                                                                                | <b>127</b> |
| 4.1      | Cell growth in a co-culture setup in an induction medium .....                                                               | 127        |
| 4.1.1    | Cell proliferation of SHED .....                                                                                             | 127        |
| 4.1.2    | Cell morphology of SHED.....                                                                                                 | 129        |
| 4.2      | Identification of epithelial markers of SHED using RT-PCR.....                                                               | 131        |
| 4.2.1    | RNA integrity of SHED .....                                                                                                  | 131        |
| 4.2.2    | Gene expression analysis .....                                                                                               | 133        |
| 4.3      | Microarray analysis .....                                                                                                    | 137        |
| 4.3.1    | RNA concentration and RIN number.....                                                                                        | 137        |

|               |                                                                                                                                                   |     |
|---------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 4.3.2         | Separation of RNA samples .....                                                                                                                   | 139 |
| 4.3.3         | Bioinformatics analyses .....                                                                                                                     | 141 |
| 4.3.3(a)      | Determination of DEGs .....                                                                                                                       | 141 |
| 4.3.3(b)      | Enrichment analyses .....                                                                                                                         | 148 |
| 4.3.3(b)(i)   | GO enrichment analysis .....                                                                                                                      | 148 |
| 4.3.3(b)(ii)  | KEGG pathway enrichment analysis .....                                                                                                            | 153 |
| 4.3.3(c)      | Construction of PPI network .....                                                                                                                 | 157 |
| 4.3.3(c)(i)   | MCODE analysis.....                                                                                                                               | 157 |
| 4.3.3(c)(ii)  | BiNGO enrichment analysis.....                                                                                                                    | 165 |
| 4.3.3(c)(iii) | CytoHubba analysis.....                                                                                                                           | 173 |
| 4.3.4         | Validation of microarray gene expression using RT-qPCR.....                                                                                       | 179 |
| 4.3.4(a)      | Optimisation of RT-qPCR condition .....                                                                                                           | 179 |
| 4.3.4(a)(i)   | Melt curve .....                                                                                                                                  | 179 |
| 4.3.4(a)(ii)  | Standard curve.....                                                                                                                               | 181 |
| 4.3.4(b)      | Gene expression analysis by RT-qPCR.....                                                                                                          | 184 |
| 4.4           | Investigation of the effect of A83-01 inhibitor on TGF $\beta$ signalling on the epithelial differentiation of co-cultured SHED with HEK001 ..... | 187 |
| 4.4.1         | Proliferation analysis of A83-01 inhibition on SHED by MTT... ..                                                                                  | 187 |
| 4.4.2         | Cell morphology of SHED inhibited by A83-01 .....                                                                                                 | 189 |
| 4.4.3         | Gene expression analysis of co-cultured SHED inhibited by A83-01.....                                                                             | 192 |
| 4.4.3(a)      | RNA integrity of SHED.....                                                                                                                        | 192 |
| 4.4.3(b)      | Optimisation of RT-qPCR primer conditions.....                                                                                                    | 194 |
| 4.4.3(c)      | Gene expression analysis by RT-qPCR.....                                                                                                          | 198 |
| 4.4.3(c)(i)   | Stem cell markers .....                                                                                                                           | 198 |
| 4.4.3(c)(ii)  | TGF $\beta$ pathway markers.....                                                                                                                  | 201 |
| 4.4.3(c)(iii) | Epithelial markers .....                                                                                                                          | 205 |
| 4.4.3(c)(iv)  | EMT markers.....                                                                                                                                  | 208 |
| 4.4.3(c)(v)   | Selected gene markers based on microarray highly regulated DEGs .....                                                                             | 211 |
| 4.5           | Protein expression analysis of co-cultured SHED inhibited by A83-01 .....                                                                         | 214 |

|                  |                                                                                                                                               |            |
|------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 4.5.1            | Analysis of protein expression of co-cultured SHED using flow cytometry .....                                                                 | 214        |
| 4.5.1(a)         | Cell morphology .....                                                                                                                         | 214        |
| 4.5.1(b)         | Gating strategy .....                                                                                                                         | 216        |
| 4.5.1(c)         | Stem cell marker expression of co-cultured SHED on day 14.....                                                                                | 218        |
| 4.5.2            | Analysis of epithelial protein expression of SHED associated with TGF $\beta$ pathway inhibition by immunocytochemistry .....                 | 220        |
| 4.5.2(a)         | Cell differentiation of SHED .....                                                                                                            | 220        |
| 4.5.2(b)         | Cell division of SHED .....                                                                                                                   | 225        |
| <b>CHAPTER 5</b> | <b>DISCUSSION .....</b>                                                                                                                       | <b>226</b> |
| 5.1              | Co-cultured SHED for epithelial differentiation .....                                                                                         | 226        |
| 5.2              | Gene expression analysis of epithelial markers in co-cultured SHED.....                                                                       | 228        |
| 5.3              | Microarray gene expression and bioinformatics analysis to identify pathway that modulates epithelial differentiation of co-cultured SHED..... | 231        |
| 5.3.1            | RNA integrity .....                                                                                                                           | 231        |
| 5.3.2            | Identification of DEGs .....                                                                                                                  | 231        |
| 5.3.3            | Enrichment analyses.....                                                                                                                      | 233        |
| 5.3.3(a)         | GO enrichment analysis.....                                                                                                                   | 233        |
| 5.3.3(b)         | KEGG pathway enrichment analysis.....                                                                                                         | 236        |
| 5.3.4            | Protein-protein interaction (PPI) network.....                                                                                                | 240        |
| 5.3.4(a)         | Cluster identification via MCODE .....                                                                                                        | 242        |
| 5.3.4(b)         | BiNGO visualisation and analysis.....                                                                                                         | 246        |
| 5.3.4(c)         | Identification of hub genes .....                                                                                                             | 247        |
| 5.3.4(d)         | Gene expression analysis for microarray validation....                                                                                        | 249        |
| 5.4              | Investigation on TGF $\beta$ pathway modulation in co-cultured SHED after A83-01 inhibition .....                                             | 251        |
| 5.4.1            | Optimisation of A83-01 concentration on SHED .....                                                                                            | 251        |
| 5.4.2            | Morphological analysis of co-cultured SHED through an inverted microscope after A83-01 inhibition .....                                       | 252        |

|                                                                            |                                                                                             |            |
|----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|------------|
| 5.4.3                                                                      | Gene expression of co-cultured SHED after A83-01 inhibition ..                              | 253        |
| 5.4.3(a)                                                                   | Stem cell gene expression on co-cultured SHED .....                                         | 253        |
| 5.4.3(b)                                                                   | TGF $\beta$ pathway markers expression on co-cultured SHED.....                             | 255        |
| 5.4.3(c)                                                                   | Epithelial markers expression on co-cultured SHED.....                                      | 257        |
| 5.4.3(d)                                                                   | EMT markers expression on co-cultured SHED .....                                            | 258        |
| 5.4.3(e)                                                                   | Selected gene markers expression based on microarray highly regulated DEGs.....             | 259        |
| 5.4.4                                                                      | Protein analysis of co-cultured SHED after A83-01 inhibition ..                             | 262        |
| 5.4.4(a)                                                                   | Stem cell protein expression of co-cultured SHED on day 14 by flow cytometry analysis ..... | 262        |
| 5.4.4(b)                                                                   | Epithelial protein expression of co-cultured SHED by immunocytochemistry analysis .....     | 264        |
| 5.4.4(b)(i)                                                                | Pan-cytokeratin.....                                                                        | 264        |
| 5.4.4(b)(ii)                                                               | Mucin 1 .....                                                                               | 266        |
| 5.4.4(b)(iii)                                                              | Mitosis.....                                                                                | 267        |
| 5.5                                                                        | Overall discussion .....                                                                    | 268        |
| <b>CHAPTER 6 CONCLUSIONS, LIMITATIONS, AND FUTURE RECOMMENDATIONS.....</b> |                                                                                             | <b>271</b> |
| 6.1                                                                        | Conclusions .....                                                                           | 271        |
| 6.2                                                                        | Limitations of the study.....                                                               | 272        |
| 6.3                                                                        | Future directions.....                                                                      | 273        |
| <b>REFERENCES.....</b>                                                     |                                                                                             | <b>274</b> |
| APPENDICES                                                                 |                                                                                             |            |
| LIST OF PUBLICATION                                                        |                                                                                             |            |

## LIST OF TABLES

|            | <b>Page</b>                                                                                                                        |
|------------|------------------------------------------------------------------------------------------------------------------------------------|
| Table 3.1  | Chemicals and reagents used in cell culture .....67                                                                                |
| Table 3.2  | Chemicals and reagents used for gel electrophoresis.....68                                                                         |
| Table 3.3  | Chemicals and reagents used in the microarray study .....69                                                                        |
| Table 3.4  | Chemicals and reagents used in the immunocytochemistry study.....70                                                                |
| Table 3.5  | List of consumable items .....71                                                                                                   |
| Table 3.6  | List of equipment .....72                                                                                                          |
| Table 3.7  | List of kits .....73                                                                                                               |
| Table 3.8  | Software utilised in this study .....74                                                                                            |
| Table 3.9  | List of primers used in this study .....77                                                                                         |
| Table 3.10 | The abbreviations of treatment groups used in this study.....87                                                                    |
| Table 3.11 | The treatment groups and time point(s) used for each experiment<br>in this study .....88                                           |
| Table 3.12 | Components of DNase I, RNase-free Kit.....93                                                                                       |
| Table 3.13 | Components of MyTaq™ One-Step RT-PCR Kit .....97                                                                                   |
| Table 3.14 | General conditions of the RT-PCR as suggested by the<br>manufacturer (MyTaq™ One-Step RT-PCR Kit, Bioline, UK) .....98             |
| Table 3.15 | Optimised conditions of the RT-PCR used in the study .....99                                                                       |
| Table 3.16 | Primer sequences for RT-PCR.....101                                                                                                |
| Table 3.17 | Primer sequences for microarray result validation via RT-qPCR....110                                                               |
| Table 3.18 | Components of RNA-direct™ SYBR® Green Realtime PCR<br>Master Mix.....111                                                           |
| Table 3.19 | General conditions of the RT-qPCR as suggested by the<br>manufacturer (RNA-direct™ SYBR® Green Realtime PCR<br>Master Mix).....112 |

|            |                                                                                                                                                                                                            |     |
|------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 3.20 | RNA and primer concentration used for standard curve.....                                                                                                                                                  | 115 |
| Table 3.21 | Primer sequences used to determine the involvement of the TGF $\beta$ pathway via RT-qPCR .....                                                                                                            | 120 |
| Table 3.22 | RNA and primer concentration used for standard curve.....                                                                                                                                                  | 121 |
| Table 3.23 | Volume of antibodies and cells needed for each flow cytometry tube.....                                                                                                                                    | 124 |
| Table 4.1  | The RNA concentration and RIN number of SHED in the Minimum Essential Medium (MEM) Alpha ( $\alpha$ -MEM) (SA) and co-cultured SHED in keratinocyte serum free medium (KSFM) (CC SK) on days 5 and 7 ..... | 138 |
| Table 4.2  | The most upregulated and downregulated DEGs of CC SK on day 5, compared to SA on day 1 .....                                                                                                               | 146 |
| Table 4.3  | The most upregulated and downregulated DEGs of CC SK on day 7, compared to SA on day 1 .....                                                                                                               | 147 |
| Table 4.4  | List of DEGs found in GO BP related to epithelial cell differentiation on days 5 and 7 based on the DAVID enrichment analysis.....                                                                         | 151 |
| Table 4.5  | List of log fold change and <i>p</i> -value of DEGs involved in the GO BP related to epithelial cell differentiation on days 5 and 7 based on the DAVID enrichment analysis .....                          | 152 |
| Table 4.6  | DEGs in the KEGG pathway enrichment of the TGF $\beta$ signalling pathway on days 5 and 7 .....                                                                                                            | 156 |
| Table 4.7  | Clusters identified using the MCODE in the PPI network of CC SK on day 5.....                                                                                                                              | 161 |
| Table 4.8  | Clusters identified using the MCODE in the PPI network of CC SK on day 7 .....                                                                                                                             | 162 |
| Table 4.9  | Main activities of DEGs based on the MCODE clustering on days 5 and 7 .....                                                                                                                                | 164 |
| Table 4.10 | Top 10 hub genes ranked by the MCC on day 5 network.....                                                                                                                                                   | 174 |
| Table 4.11 | Top 10 hub genes ranked by the MCC on day 7 network.....                                                                                                                                                   | 175 |

|            |                                                                                                       |     |
|------------|-------------------------------------------------------------------------------------------------------|-----|
| Table 4.12 | Top 10 upregulated hub genes ranked by the MCC based on the DEGs of upregulated network on day 5..... | 177 |
| Table 4.13 | Top 10 upregulated hub genes ranked by the MCC based on the DEGs of upregulated network on day 7..... | 178 |
| Table 4.14 | The correlation coefficient ( $R_2$ ) and efficiency (%) values of the primers.....                   | 183 |
| Table 4.15 | Comparison of fold change between microarray and RT-qPCR data .....                                   | 186 |
| Table 4.16 | The correlation coefficient ( $R_2$ ) and efficiency (%) values of the primers.....                   | 197 |

## LIST OF FIGURES

|                                                                                                                                                                          | <b>Page</b> |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| Figure 2.1      Diagram illustrating the physiology of the epithelium, showcasing its distinct polarity: the apical region, basal region, and lateral regions. ....      | 11          |
| Figure 2.2      Classifications of epithelium. Epithelium can be classified as surface epithelium and glandular epithelium. ....                                         | 12          |
| Figure 2.3      The layer of keratinised epithelium consisting of basal, spinous, granular, and cornified layers. ....                                                   | 15          |
| Figure 2.4      Illustration of monoculture, co-culture, and three-dimensional (3D) scaffold .....                                                                       | 36          |
| Figure 2.5      The canonical TGF $\beta$ signalling pathway. ....                                                                                                       | 50          |
| Figure 3.1      Flow chart of experimental design. ....                                                                                                                  | 66          |
| Figure 3.2      Co-culture model of SHED grown in an epithelial microenvironment, co-cultured with HEK001. ....                                                          | 86          |
| Figure 3.3      A comprehensive workflow of microarray profiling. ....                                                                                                   | 104         |
| Figure 3.4      Preparation of RNA serial dilution for standard curve assay. ....                                                                                        | 114         |
| Figure 4.1      Cell proliferation of SHED in SA, SK, and CC SK culture groups (n = 3). ....                                                                             | 128         |
| Figure 4.2      Cell morphology of SA, SK, and HK on day 1 (monoculture) and CC SK on days 1, 3, 5, 7, 10, and 14 (co-culture), respectively at 100x magnification. .... | 130         |
| Figure 4.3      Representative image of 1% agarose gel electrophoresis showing RNA integrity of samples used in the RT-PCR study. ....                                   | 132         |
| Figure 4.4      Image of RT-PCR product of stem cell and epithelial cell markers (n = 1). ....                                                                           | 136         |
| Figure 4.5      RNA sample separation according to nucleotide size using RNA 6000 Nano Assay (n = 3).. ....                                                              | 140         |

|             |                                                                                                                           |     |
|-------------|---------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 4.6  | Hierarchical cluster of differentially expressed genes (DEGs) on days 5 and 7 are illustrated in the heat map (n = 3)..   | 144 |
| Figure 4.7  | Volcano plot of DEGs.....                                                                                                 | 145 |
| Figure 4.8  | The most significant GO based on the DAVID enrichment analysis of CC SK.....                                              | 149 |
| Figure 4.9  | The 15 most significant KEGG pathway based on the DAVID enrichment analysis.....                                          | 154 |
| Figure 4.10 | PPI network of DEGs predicted using the STRING and the clusters identified using the MCODE (Score > 10) on day 5.....     | 159 |
| Figure 4.11 | PPI network of DEGs predicted using the STRING and the clusters identified using the MCODE (Score > 10) on day 7.....     | 160 |
| Figure 4.12 | Visualisation of the selected GO hierarchy of BP on day 5 using the BiNGO..                                               | 166 |
| Figure 4.13 | Visualisation of the selected GO hierarchy of CC based on the critical cell organelles on day 5 using the BiNGO.....      | 167 |
| Figure 4.14 | Visualisation of the GO hierarchy of MF on day 5 using the BiNGO. ....                                                    | 168 |
| Figure 4.15 | Visualisation of the selected GO hierarchy of BP on day 7 using the BiNGO .....                                           | 170 |
| Figure 4.16 | Visualisation of the selected GO hierarchy of CC based on the critical cell organelles on day 7 using the BiNGO.....      | 171 |
| Figure 4.17 | Visualisation of GO hierarchy of MF on day 7 using the BiNGO...                                                           | 172 |
| Figure 4.18 | The melt curve graphs of RT-qPCR primers used for microarray validation.....                                              | 180 |
| Figure 4.19 | The standard curve of RT-qPCR primers used for microarray validation.....                                                 | 182 |
| Figure 4.20 | Regulation of gene expression of CC SK on days 5 and 7. ....                                                              | 185 |
| Figure 4.21 | Effect of A83-01 inhibitor on SHED proliferation using Thiazolyl Blue Tetrazolium Bromide (MTT) cell viability assay..... | 188 |

|             |                                                                                                                                                      |     |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 4.22 | Cell morphology of control groups using an inverted microscope at 100x magnification.....                                                            | 190 |
| Figure 4.23 | Morphology of co-cultured SHED without and with the A83-01 inhibitor using an inverted microscope on days 3, 5, 7, and 14 at 100x magnification..... | 191 |
| Figure 4.24 | Representative image of 1% agarose gel electrophoresis showing RNA integrity of RNA samples used in the inhibition study.....                        | 193 |
| Figure 4.25 | The melt curve graphs of the RT-qPCR primers used for the inhibition study (n = 3).....                                                              | 195 |
| Figure 4.26 | The standard curve of RT-qPCR primers used for the inhibition study (n = 3). .....                                                                   | 196 |
| Figure 4.27 | Gene expression analysis of stem cell markers, <i>CD73</i> , <i>CD90</i> , and <i>CD105</i> (n = 3) .....                                            | 200 |
| Figure 4.28 | Gene expression analysis of TGF $\beta$ pathway markers, <i>TGF<math>\beta</math>3</i> , <i>MYC</i> , and <i>BMP8A</i> (n = 3) .....                 | 204 |
| Figure 4.29 | Gene expression analysis of epithelial markers, <i>BDH2</i> , <i>EHF</i> , and <i>PPARG</i> (n = 3) .....                                            | 207 |
| Figure 4.30 | Gene expression analysis of epithelial-mesenchymal transition (EMT) gene markers, <i>SNAI2</i> and <i>TP63</i> (n = 3). .....                        | 210 |
| Figure 4.31 | Gene expression analysis of highly regulated DEGs in microarray (n = 3).....                                                                         | 213 |
| Figure 4.32 | Morphology of treated SHED on day 14 after 5-days culture in a monolayer environment at 100x magnification.. .....                                   | 215 |
| Figure 4.33 | Images of the forward-scatter (FSC) and side-scatter (SSC) of the SHED population in different groups (n =1).. .....                                 | 217 |
| Figure 4.34 | Dot-plot analysis of stem cell protein markers of SHED on day 14 (n = 1).....                                                                        | 219 |
| Figure 4.35 | Protein expression of Pan-CK in epithelial-like differentiation of treated SHED (n = 1). .....                                                       | 221 |

|             |                                                                                                                |     |
|-------------|----------------------------------------------------------------------------------------------------------------|-----|
| Figure 4.36 | Protein expression of Muc 1 in epithelial-like differentiation of treated SHED (n = 1) .....                   | 223 |
| Figure 4.37 | Formation of nuclear speckles stained with Muc 1 (n = 1).....                                                  | 224 |
| Figure 5.1  | The illustration summarises the key molecular and pathway involved during SHED-epithelial differentiation..... | 270 |

## LIST OF SYMBOLS

|          |                       |
|----------|-----------------------|
| %        | Percentage            |
| <        | Less than             |
| >        | More than             |
| $\leq$   | Less than or equal to |
| $\geq$   | More than or equal to |
| ®        | Registered            |
| °C       | Degree Celsius        |
| h        | Hour                  |
| s        | Second                |
| ™        | Trademark             |
| U        | Enzyme unit           |
| V        | Voltage               |
| $\alpha$ | Alpha                 |
| $\beta$  | Beta                  |
| $\Delta$ | Delta                 |
| $\gamma$ | 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 <sup>2</sup> | Square centimetre                                                |
| CO <sub>2</sub> | 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                  |
| <i>et al.</i> | 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) <sub>2</sub> | Magnesium acetate                       |
| MgCl <sub>2</sub>    | 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 <sup>2</sup>       | 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 $\alpha$ -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        |
| TGF $\beta$   | Transforming growth factor beta                           |
| TGF $\beta$ R | 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                                                   |
| $\alpha$ -MEM | Minimum Essential Medium Alpha                            |

## **LIST OF APPENDICES**

|            |                                               |
|------------|-----------------------------------------------|
| Appendix A | The quality of RNA samples for microarray     |
| Appendix B | List of DEGs                                  |
| Appendix C | List of MCODE                                 |
| Appendix D | Protein-protein interaction (PPI) networks    |
| Appendix E | Immunocytochemistry of Pan-CK and Muc 1       |
| Appendix F | Cluster visualisation based on MCODE analysis |
| Appendix G | Statistical analysis                          |

# **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,  $\alpha$ -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 TGF $\beta$  terlibat dalam pembezaan SHED-epitelium. Oleh itu,

perencat A83-01 (1  $\mu$ M) digunakan untuk menyekat TGF $\beta$ R1 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, *ANP63*, 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, pemerikayaan DEG yang berkaitan dengan tapak jalan TGF $\beta$  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 pengekspresan gen yang tinggi pada penanda yang berkait seperti *TGF $\beta$ 3* dan *BMP8A*. Menariknya, hasil kajian ini turut menunjukkan kesan perencatan tapak jalan TGF $\beta$  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 TGF $\beta$  dalam mengawal pembezaan SHED kepada sel-seperti-epitelium. Walaupun begitu, perencatan tapak jalan TGF $\beta$  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 ( $\alpha$ -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 TGF $\beta$  signalling pathway is involved in SHED-epithelium differentiation. Therefore, a downstream TGF $\beta$  pathway inhibition study was conducted using A83-01 (1  $\mu$ M) to inhibit TGF $\beta$ R1 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-epithelialisation. 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-epithelialisation, 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)/ $\beta$ -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 (TGF $\beta$ )/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 TGF $\beta$  signalling pathway corresponded to regulating epithelial differentiation of the co-cultured SHED. The identified pathway was confirmed based on bioinformatics analyses. Since the TGF $\beta$  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 TGF $\beta$  pathway, which showed a high possibility of involvement. A83-01 inhibits several receptors in the TGF $\beta$  pathway, such as TGF $\beta$ R1, 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 TGF $\beta$  pathway, such as *Transforming Growth Factor Beta 3 (TGF $\beta$ 3)*, *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 TGF $\beta$  pathway was selected based on the preliminary findings from the bioinformatics analysis. The TGF $\beta$  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 TGF $\beta$  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 TGF $\beta$  pathway of SHED after co-cultured with HEK0001 treated with and without TGF $\beta$  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 TGF $\beta$  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 TGF $\beta$  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).

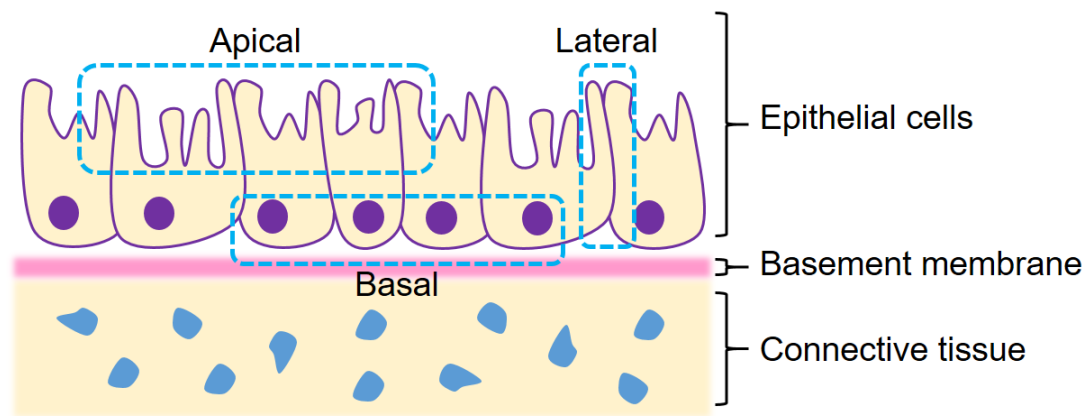


Figure 2.1 Diagram illustrating the physiology of the epithelium, showcasing its distinct polarity: the apical region, basal region, and lateral regions.

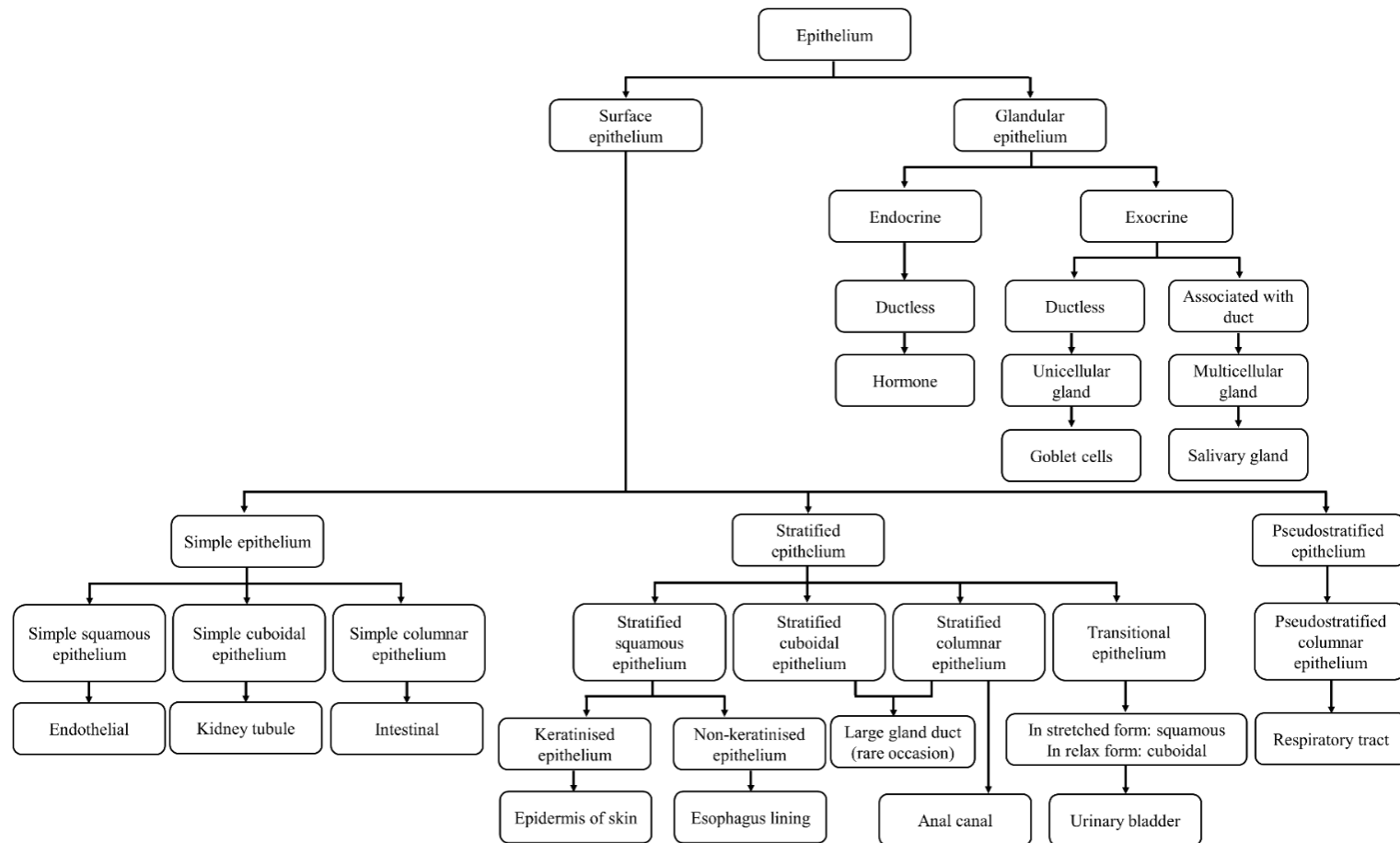


Figure 2.2 Classifications of epithelium. Epithelium can be classified as surface epithelium and glandular epithelium.

### **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.

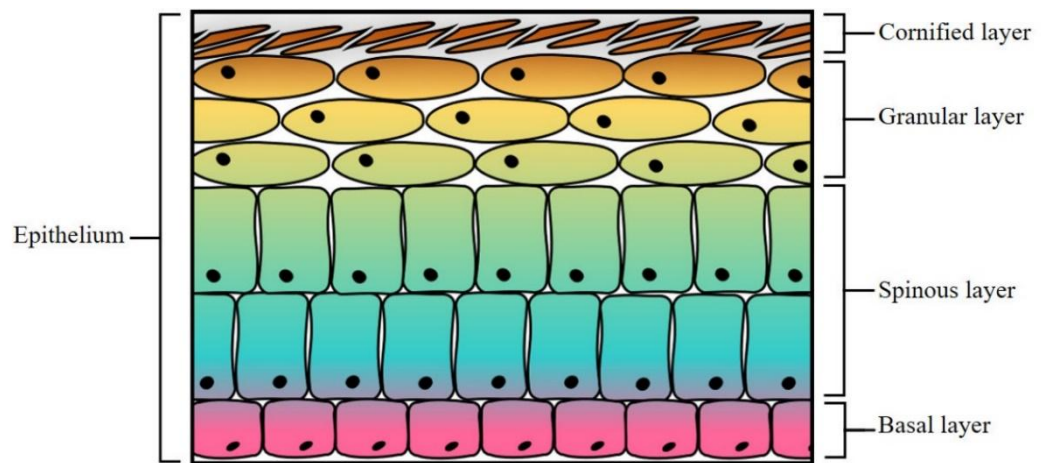


Figure 2.3 The layer of keratinised epithelium consisting of basal, spinous, granular, and cornified layers.

### **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 (Iftekhhar 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