Exploring the Effects of Estrogen Receptor Beta Polymorphisms on Wound Repair

A thesis submitted to the University of Manchester for the degree of Doctorate of Philosophy in the Faculty of Biology, Medicine and Health
School of Biological Sciences, Division of Cell Matrix Biology and Regenerative Medicine

2016

Matthew Smith
1. Chapter 1 - Introduction

1.1 Skin and Wounds

1.1.1 Skin Structure and Function

1.1.2 Acute Wound Healing

1.1.2.1 Haemostasis

1.1.2.2 Inflammatory Phase

1.1.2.3 Proliferation Phase

1.1.2.3.1 Granulation Tissue Formation

1.1.2.3.2 Re-epithelialisation

1.1.2.3.3 Angiogenesis

1.1.2.4 Remodelling Phase

1.1.3 Chronic Wounds

1.1.3.1 Venous Ulcers

1.1.3.2 Pressure Ulcers

1.1.3.3 Diabetic Ulcers

1.2 Estrogens

1.2.1 Estrogen Biosynthesis

1.2.2 Estrogen Receptors

1.2.2.1 Estrogen Receptor Protein Structure

1.2.2.2 Estrogen Receptor Genes

1.2.2.2.1 ERα

1.2.2.2.2 ERβ

1.2.2.3 Estrogen receptor Expression and Localisation

1.2.3 Estrogen Signalling

1.2.3.1 Ligand-dependent

1.2.3.2 Ligand-independent

1.2.3.3 ERE-independent

1.2.3.4 Non-genomic

1.2.3.5 Estrogen Receptor Agonists and SERMs

1.3. Estrogen and Wound Healing

1.3.1. Estrogen Effects on The Skin

Contents

List of Figures ........................................................................................................... 6
List of Tables ............................................................................................................. 8
Abbreviations .......................................................................................................... 9
Abstract .................................................................................................................. 12
Declaration .............................................................................................................. 13
Copyright Statement .............................................................................................. 13
Acknowledgments .................................................................................................. 14
2. Chapter 2 - Materials and Methods .................................................. 55
  2.1 Cell culture ................................................................. 55
      2.1.1 Cell culture conditions ........................................... 55
      2.1.2 Scratch-wound activation ....................................... 55
      2.1.3 Migration assay ................................................... 55
      2.1.4 Proliferation assay ............................................... 56
      2.1.5 Mitomycin C treatment ......................................... 57
      2.1.6 Fibroblast and Keratinocyte co-culture with conditioned media ........................................... 57
      2.1.7 Monocyte and fibroblast co-culture ......................... 58
  2.2 DNA and RNA preparation and genomic assays .......................... 58
      2.2.1 DNA extraction .................................................... 58
      2.2.2 DNA quantification .............................................. 59
      2.2.3 Tetra-primer ARMS-PCR genotyping .................... 59
      2.2.4 Gel electrophoresis .............................................. 60
      2.2.5 Sanger sequencing ............................................... 60
      2.2.6 RNA extraction .................................................. 61
      2.2.7 cDNA synthesis (reverse transcription) .................. 61
      2.2.8 Quantitative-PCR ............................................... 62
      2.2.9 Allele specific expression (ASE) ............................. 62
  2.3 Protein expression .......................................................... 63
      2.3.1 Protein extraction .................................................. 63
      2.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) ......................... 63
      2.3.4 Western blotting ................................................... 64
      2.3.4 Immunocytochemistry .......................................... 64

1.3.2 Estrogen Effects on Wound Healing ........................................ 41
  1.3.2.1 Estrogen Effects on the Inflammatory Phase ................... 42
  1.3.2.2 Estrogen Effects on the Proliferation Phase ................... 43
  1.3.2.3 Estrogen Effects on the Remodelling Phase ................... 43
  1.3.2.4 Estrogen Receptors and Wound Healing ..................... 44
  1.3.2.5 Estrogen and Chronic Wounds ................................ 45

1.4 Single Nucleotide Polymorphisms (SNPs) .................................. 46
  1.4.1 ERβ Polymorphisms and Disease Association Studies ............ 46
      1.4.1.1 ERB Polymorphisms and Chronic Wounds - Case-control Association Study ..................... 46
      1.4.1.2 Possible Effects of Polymorphisms/ SNP function in ERβ expression and Chronic wound formation ...................................................... 49
      1.4.1.3 Transcriptional Effects of SNPs ............................. 50
      1.4.1.4 Post-transcriptional Effects of SNPs ........................ 51

1.5 Hypothesis and Aims .......................................................... 53
2.4 Electrophoretic Mobility Shift Assays (EMSA) .......................................................... 64
2.5 RNAseq preparation and analysis ............................................................................ 66
2.6 Primers and antibodies ......................................................................................... 67

3. Chapter 3 - Functional characterisation of cells carrying venous ulcer associated SNPs
................................................................................................................................. 70
3.1 Introduction ........................................................................................................... 70
3.2 Cell acquisition and model system ....................................................................... 70
    3.2.1 Singapore Skin Cell Bank .............................................................................. 70
    3.2.2 Genotyping for venous ulcer SNPs ................................................................. 70
    3.2.3 Model for venous ulcer SNPs ....................................................................... 73
3.3 Specific dependence of ERβ levels on SNP genotype ........................................... 73
    3.3.1 Histological and cytological investigation reveals difference in estrogen receptor
expression .................................................................................................................... 73
    3.3.2 ERβ expression at the transcriptional level ..................................................... 76
    3.3.3 ERβ expression at the translational level........................................................ 76
3.4. The Effects of ERβ SNPs on Proliferation ........................................................... 76
    3.4.1 Keratinocyte proliferation is affected by SNP genotype............................... 77
    3.4.2 Fibroblast proliferation is affected by SNP genotype in the presence of estradiol... 79
    3.4.3 Keratinocyte proliferation with ER selective agonist treatment is comparable to
estradiol ....................................................................................................................... 79
    3.4.4 Fibroblast proliferation with ER selective agonist treatment reveals genotype-
dependent responses ............................................................................................... 79
3.5 The Effects of ERβ SNPs on Migration .................................................................. 79
    3.5.1 Keratinocyte migration is affected by SNP genotype ..................................... 80
    3.5.2 Keratinocyte migration with Mitomycin C treatment .................................... 82
    3.5.3 Fibroblast migration is affected by SNP genotype ....................................... 83
    3.5.4 Fibroblast migration with Mitomycin C treatment ....................................... 85
    3.5.5 Keratinocyte migration with ER selective agonist treatment reveals a beneficial role for
ERα ............................................................................................................................ 86
    3.5.6 Fibroblast migration with ER selective agonist treatment reveals a beneficial role for
ERβ ............................................................................................................................ 86
    3.5.7 Fibroblast and Keratinocyte co-culture reveals genotype-specific interactions .... 89
3.6 Discussion ............................................................................................................ 91

4. Chapter 4 - The effects of venous ulcer SNPs on ERβ transcriptional regulation .... 97
4.1 Introduction ........................................................................................................... 97
4.2 Allele Specific Expression assay (ASE) ............................................................... 98
    4.2.1 Cell selection and isolation .......................................................................... 99
    4.2.2 Assay validation ........................................................................................... 99
    4.2.3 Primer efficiency and assay normalisation ................................................... 103
4.2.4 Allele specific expression assays reveal that venous ulcer-associated SNPs effect ERβ expression .................................................................................................................................107

4.3 Transcription factor binding..............................................................................................................................................................................................................107

4.3.1 Bioinformatic predictions..............................................................................................................................................................................................................107

4.3.2 Electrophoretic mobility shift assays (EMSAs) reveal differential transcription factor binding .........................................................................................................................113

4.4 Discussion..................................................................................................................................................................................................................................................114

5. Chapter 5 - Venous ulcer-associated SNPs affect ERβ signalling during wound progression and alter the inflammatory profile ..............................................................................................119

5.1 Introduction ......................................................................................................................................................................................................................119

5.2 Estrogen receptor beta expression during wounding .................................................................................................................................................................120

5.2.1 Estrogen receptor beta mRNA expression in wounded keratinocytes .................................................................................................................................................................122

5.2.2 Estrogen receptor beta protein expression in wounded keratinocytes .................................................................................................................................................................123

5.3 Venous ulcer SNPs influence keratinocyte cytokine and growth factor expression ...............125

5.4 Venous ulcer SNP effects on cytokine and growth factor expression in fibroblasts ...............127

5.5 Venous ulcer SNP effects on the inflammatory profile of monocyte co-cultures ...............130

5.5.1 Fibroblast inflammatory markers in co-culture with monocytes .................................................................130

5.5.2 Monocyte inflammatory factors in co-culture with fibroblasts .................................................................132

5.5.3 M1/M2-like polarisation is dependent on fibroblast SNP genotype .................................................................135

5.6 Discussion..............................................................................................................................................................................................................138

6. Chapter 6 - RNA-sequencing of wound-activated ESR2 SNP variant keratinocytes .. 142

6.1 Introduction ......................................................................................................................................................................................................................142

6.2 Establishing sample variance ..............................................................................................................................................................................................................143

6.3 Differentially regulated genes by genotype and by estradiol treatment ........................................145

6.4 Gene ontology analysis ..............................................................................................................................................................................................................151

6.4.1 Overrepresented GO groups downregulated in disease cells include ‘cell migration’, ‘negative regulation of inflammatory response’ and ‘steroid hormone biosynthesis’ ......159

6.4.2 Overrepresented GO groups upregulated in disease cells include ‘MHC Class I processing and presentation’ and ‘homophilic cell adhesion molecules’ ........................................166

6.5 Discussion..............................................................................................................................................................................................................170

7. Conclusions and Future Work ......................................................................................................................... 176

8. Supplementary Material ................................................................................................................................. 186

9. References ......................................................................................................................................................................................... 189

Word count: 67778
List of Figures

1.1 The structure of human skin ............................................. 17
1.2 Wound healing profiles of acute and chronic wounds .......... 18
1.3 Types of chronic wound ............................................... 24
1.4 The biosynthesis of estrogen from cholesterol and androgen precursors ............................................ 28
1.5 Schematic representation and comparison of the structure of human ERα and ERβ ............................................. 30
1.6 Genomic structure and SNPs of the ERβ gene ................... 33
1.7 Schematic representation of estrogen signalling pathways ...... 36
1.8 The effects of estrogen on wound healing ......................... 44
2.1 Tetra-primer ARMS-PCR method schematic ...................... 59
3.1 Genotyping venous ulcer-associated SNPs through tetra-primer amplification refractory mutation system-polymerase chain reaction 72
3.2 Estrogen receptor expression in the skin ......................... 74
3.3 RNA expression of the ERβ gene in Wild type and venous ulcer-SNP Disease keratinocytes with estradiol treatment 75
3.4 ERβ protein expression in Wild type and venous ulcer-SNP Disease with estradiol treatment .................................... 75
3.5 The effects of estrogen ligands on T Wild type and Disease keratinocytes and fibroblast doubling times ..................... 78
3.6 Wound closure of Wild type and Disease keratinocytes with estradiol treatment .............................................. 81
3.7 Scratch wound closure of Wild type and Disease keratinocytes with estradiol and Mitomycin C treatment ...................... 82
3.8 Figure 3.8 – Wound closure of Wild type and Disease fibroblasts with estradiol treatment ........................................... 84
3.9 Wound closure of Wild type and Disease fibroblasts with estradiol and Mitomycin C treatment ................................ 85
3.10 Wound closure in Wild type and Disease primary keratinocytes with specific receptor agonist and Mitomycin C treatment .................................................. 87
3.11 Wound closure in Wild type and Disease primary fibroblasts with specific receptor agonist and Mitomycin C ................. 88
3.12 Wound closure of Wild type and Disease keratinocytes treated with conditioned media from wound activated fibroblasts 90
4.1 rs3783736 Allele Specific Expression assay ....................... 104
4.2 rs2987983 Allele Specific Expression assay ....................... 105
4.3 rs2978381 Allele Specific Expression assay ....................... 106
4.4 Transcription factor binding differences revealed by bioinformatic and electrophoretic mobility shift assay analysis of rs3783736 110
4.5 Transcription factor binding differences revealed by bioinformatic and electrophoretic mobility shift assay analysis of rs2987983 111
4.6 Transcription factor binding differences revealed by bioinformatic and electrophoretic mobility shift assay analysis of rs2978381 112
5.1 Estrogen receptor beta and promoter mRNA expression wound time-course in keratinocytes in the presence and absence of estradiol 121
5.2 Estrogen receptor beta protein expression wound time-course in keratinocytes in the presence and absence of estradiol 124
5.3 Wound-related mediator mRNA expression wound time-course in keratinocytes in the presence and absence of estradiol 126
5.4 Wound-related mediator mRNA expression in fibroblasts in the presence and absence of estradiol 2 hours post-wounding 129
5.5 Wound-related mediator mRNA expression in fibroblasts co-cultured with human monocytes at 48 hours post-wounding under low-estrogen conditions 132
5.6 Wound-related gene expression of human monocytes co-cultured with fibroblasts at 48 hours post-wounding under low-estrogen conditions 134
5.7 Macrophage M1/M2 polarisation marker gene expression in human monocytes co-cultured with fibroblasts at 48 hours post-wounding under low-estrogen conditions 137

6.1 Clustering of venous ulcer-associated SNP disease cells and healthy cells 144
6.2 Induced and repressed genes by estrogen and across genotypes 152
6.3 Venous ulcer-associated SNP disease cells display less expression of genes involved in steroid hormone synthesis 161
6.4 Venous ulcer-associated SNP disease cells display less expression of genes involved in the Ras signalling pathway 163
6.5 Venous ulcer-associated SNP disease cells display less expression of genes involved in the Rap1 signalling pathway. 165
6.6 Venous ulcer-associated SNP disease cells display greater expression of genes involved in the MHC Class I antigen processing and presentation 168
6.7 Venous ulcer-associated SNP disease cells display greater expression of genes involved in cell-cell adhesion 169
6.8 Estrogen signalling pathway components are downregulated in disease cells 173

S.1 Supplementary Figure S.1 - Venous ulcer SNP linkage disequilibrium 186
S.2 Supplementary Figure S.2 - Titration of estradiol treatment for scratch wound closure with keratinocytes 187
S.3 Supplementary Figure S.3 - Organotypic skin-equivalent three-dimensional models of wild-type cells 188
List of Tables

Table 1.1  Distribution of ERα and ERβ in adult human tissues  35
Table 1.2  Disease associated estrogen receptor beta single nucleotide polymorphisms  48
Table 2.1  Tetra-ARMS-PCR primers  67
Table 2.2  Allele Specific Expression (ASE) primers  67
Table 2.3  RT-qPCR primers  68
Table 2.4  EMSA probes  69
Table 2.5  Antibodies  69
Table 3.1  Primary cell patient and sample information  71
Table 4.1  rs3783736 Allele Specific Expression assay validation  100
Table 4.2  rs2987983 Allele Specific Expression assay validation  101
Table 4.3  rs2978381 Allele Specific Expression assay validation  102
Table 6.1  Top ten most statistically significant differentially regulated genes by estradiol treatment and genotype  146
Table 6.2  Overrepresented gene ontology (GO) groups in genes downregulated by disease cells (D vs WT)  153
Table 6.3  Overrepresented gene ontology (GO) groups induced in disease cells (Disease v WT)  154
Table 6.4  Overrepresented gene ontology (GO) groups in genes downregulated by disease cells treated with estrogen (D E+ vs WT E+)  155
Table 6.5  Overrepresented gene ontology (GO) groups induced in disease cells treated with estrogen (D E+ vs WT E+)  156
Table 6.6  Overrepresented gene ontology (GO) groups in genes downregulated by disease cells in the absence of estrogen (D E- vs WT E-)  157
Table 6.7  Overrepresented gene ontology (GO) groups induced in disease cells in the absence of estrogen (D E- vs WT E-)  158
Table ST.1  Primary cell patient and sample information  186
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve/-ve</td>
<td>Positive/Negative</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AF</td>
<td>Activation function</td>
</tr>
<tr>
<td>AIB1</td>
<td>Amplified in breast 1/nuclear receptor coactivator 3</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Alu element</td>
<td>DNA region characterized by the action of <em>Arthrobacter luteus</em> restriction endonuclease</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ARMS-PCR</td>
<td>Amplification-refractory mutation system polymerase chain reaction</td>
</tr>
<tr>
<td>ASE</td>
<td>Allele specific expression</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>Chromatin immunoprecipitation sequencing</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>CS-FBS</td>
<td>Charcoal stripped foetal bovine serum</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CXCL11</td>
<td>C-X-C motif chemokine 11</td>
</tr>
<tr>
<td>D</td>
<td>Disease (harbouring venous-ulcer associated ESR2 SNPs)</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DCE-S-III</td>
<td>Downstream core element subelement 3</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DPN</td>
<td>Diarylpropionitrile (2,3-bis(p-hydroxyphenyl)propionitrile)</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol/17β-estradiol</td>
</tr>
<tr>
<td>EAS</td>
<td>5-epiaristolochene synthase</td>
</tr>
<tr>
<td>EBNA</td>
<td>Epstein–Barr virus nuclear antigen 1</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF/R</td>
<td>Epidermal growth factor/receptor</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal–regulated kinases (MAPK)</td>
</tr>
<tr>
<td>ESE</td>
<td>Exonic splicing enhancer</td>
</tr>
<tr>
<td>ESR1</td>
<td>Estrogen receptor 1 (alpha)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>ESR2</td>
<td>Estrogen receptor 2 (beta)</td>
</tr>
<tr>
<td>EUR</td>
<td>European (population code)</td>
</tr>
<tr>
<td>ExoSAP</td>
<td>Exonuclease I - Shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fold change</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GATA</td>
<td>GATA-binding factor</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GIS</td>
<td>Genome Institute of Singapore</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GRIP</td>
<td>Glutamate receptor interacting protein</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HKGS</td>
<td>Human keratinocyte growth supplement</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>HSF</td>
<td>Heat shock factor</td>
</tr>
<tr>
<td>HuR</td>
<td>Human antigen R</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IGF/-R</td>
<td>Insulin-like growth factor/-receptor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMB</td>
<td>Institute of Medical Biology (Singapore)</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's Modified Dulbecco's Medium</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>JUNB</td>
<td>Jun B proto-oncogene</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopaedia of Genes and Genomes</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor (FGF7)</td>
</tr>
<tr>
<td>KRT</td>
<td>Keratin</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>LDS</td>
<td>Lipodermatosclerosis</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>IncRNA</td>
<td>Long non-coding RNA</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDS</td>
<td>Multidimensional scaling</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRC1</td>
<td>Mannose Receptor, C Type 1</td>
</tr>
<tr>
<td>NCOA1</td>
<td>Nuclear Receptor Coactivator</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NHD</td>
<td>N terminal homology domain</td>
</tr>
<tr>
<td>NHEK</td>
<td>Normal human keratinocyte</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>Ovx</td>
<td>Ovariectomised</td>
</tr>
<tr>
<td>p300</td>
<td>E1A binding protein p300 (CBP)</td>
</tr>
<tr>
<td>p38</td>
<td>P38 mitogen-activated protein kinase (MAPK14)</td>
</tr>
<tr>
<td>p68</td>
<td>RNA helicase p68 (DDX5)</td>
</tr>
<tr>
<td>PABP</td>
<td>Polyadenylate binding protein</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease-activated receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PGC</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PPT</td>
<td>Propyl pyrazole triol</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>ref</td>
<td>Reference gene</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAseq</td>
<td>Ribonucleic acid sequencing</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPLP0</td>
<td>Ribosomal Protein Lateral Stalk Subunit P0</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP-1</td>
<td>Specificity protein 1</td>
</tr>
<tr>
<td>SRA</td>
<td>Steroid receptor RNA activator</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIF</td>
<td>Transcriptional mediators/intermediary factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TMM</td>
<td>M-values normalization method</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thyroid hormone receptor-associated protein</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>uORF</td>
<td>Upstream open reading frame</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type (in regard to venous ulcer SNPs)</td>
</tr>
<tr>
<td>xg</td>
<td>Factor of gravity</td>
</tr>
<tr>
<td>Δ</td>
<td>Delta (change)</td>
</tr>
<tr>
<td>ΔCt</td>
<td>Change in cycle threshold</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
</tbody>
</table>
Abstract

Estrogen is an important regulator and promoter of epithelial wound healing. This is facilitated by increased keratinocyte and fibroblast migration and proliferation, as well as promotion of angiogenesis, matrix deposition and inflammatory response dampening. The potential to target this pathway for therapeutics is highlighted by observations that post-menopausal women on hormone replacement therapy have a significantly lower incidence of venous ulcers. Previous work from this laboratory identified four SNPs (single nucleotide polymorphisms) in the 5’UTR of estrogen receptor beta (ERβ) gene that are associated with venous ulcer predisposition. Disease association is further supported by the identification of ERβ as the main conduit of the beneficial effects of estrogen signalling on wound healing.

SNP’s of the 5’UTR can affect transcriptional expression through the modification of transcription factor binding sites, epigenetic modifications and translational efficiency via mRNA localisation and secondary structure alterations. To investigate the possible biological function of these SNPs, we have developed disease relevant cell based assays where primary keratinocyte and fibroblast cells were selected harbouring disease-associated SNPs. We demonstrate that the presence of venous ulcer-associated ERβ SNPs reduced the expression of ERβ in skin cells and reduced their migration and proliferative capabilities. Evidence gathered here suggests that ERβ expression is curtailed by a change in transcription factor binding, likely facilitated by the change in nucleotide sequence brought about by the rs2987983 SNP. Further, we demonstrate that SNP-induced changes in fibroblast expression of growth factors and inflammatory mediators can hinder keratinocyte migration and induce a pro-inflammatory phenotype in human monocytes. Lastly, RNAseq analysis of keratinocytes reveals a SNP-dependant gene expression profile that is detrimental to wound healing.

This work provides the first evidence of a direct functional link between venous ulcer-associated ERβ SNPs and dysfunctional wound healing. Investigating ERβ SNPs has provided insight into novel mechanisms of estrogen signalling that can be applied for therapeutic development to treat venous ulcers.
**Declaration**

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

**Copyright Statement**

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property University IP Policy (see http://documents.manchester.ac.uk/display.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.library.manchester.ac.uk/about/regulations/) and in The University’s policy on Presentation of Theses.
Acknowledgments

I would like to begin by thanking my Supervisors Doctor Matthew Hardman and Professor Birgit Lane for all their support and guidance and for providing this opportunity. I’d like to thank my Advisor, Professor Tony Day, for all his help throughout my PhD and especially during the trickier times. Special thanks also to Doctor John Common, who acted as my Mentor in Singapore, for all his advice and guidance. I would like to thank The University of Manchester and The Agency for Science, Technology and Research (A*Star), Singapore for their funding of this project. I’d also like to thank Doctor Christoph Ballestrem and Jessica Bowler for their tireless work in organising this PhD programme.

I would like to express my huge appreciation to members of both the Hardman and Lane Lab’s for all their scientific help and advice and for making the last four years such a great experience. There are too many persons to thank individually, but thanks to Clare - particularly for her advice when writing up, Anna, Ben, Charis, Chris, Rachel, Elaine, Helen T, Helen W, Jeya, Laura, Nic, Nurhuda and Pishya from the Manchester side. From the Singapore side, special thanks to Colin (‘cheers bro’) for helping me to get started in a new lab and a new country, to Simon for his expertise and guidance in bioinformatic analysis and to Dec for his histology omniscience and constant merriment. Thanks are also due to Belle, Hideki, Henri, Ildiko, Jasrie, Kim, Lukas, Rosita, Unna, Vincent, Vivien and Yi Zhen for their support, comradery and for introducing me to all the amazing food in Singapore - it’s been damn shiok! Thanks also to Jason Ashworth for his insights and specific knowledge of this research area.

A big thank you to my fellow pioneering students of the Manchester-Singapore research attachment programme; Aleks, Anna, Beth, Ruth and Vassilis. Having your fellowship throughout this experience has been a constant source of support and amusement. I’d like to express my gratitude to all the friends I’ve made both in Manchester and Singapore who have helped me along the way, especially Emily for her unquestioning support. Finally, I’d like to thanks my parents for their endless encouragement and support in every way.
1. Chapter 1 - Introduction

Wound healing is the complex process of tissue repair after an injury to the skin. It loosely follows four overlapping phases; haemostasis, inflammation, proliferation and resolution. Here blood loss is minimised, the wound is cleared of infection and the tissue is regenerated. This process occurs largely without problem in the young, however the aged heal at a slower rate and are more prone to developing chronic wounds. Chronic wounds are slow to heal and often present with excessive inflammation and delayed reepithelialisation. Many factors associated with intrinsic aging contribute to this lack of healing, one of which is hormonal deficiency, particularly estrogen. There is an emerging body of publications and association studies addressing the impact of estrogen on human wound healing and the functional effects of this on a cellular level in murine models. Estrogen treatment can protect against the onset of chronic wounds in humans (Margolis et al., 2002) and signalling through estrogen receptor beta (ERβ) has been shown to be pro-healing in mouse models (Campbell et al., 2010). Furthermore, certain single nucleotide polymorphisms of ERβ have been found to be associated with a predisposition to chronic wounds in a case-control study (Ashworth et al., 2008). However, it remains unclear whether estrogen signalling and the regulation of receptors involved in this have a key role to play in wound healing and the development of chronic wounds in humans. The work presented in this thesis builds upon preliminary research implicating ERβ SNPs in wound chronicity to further characterise and explore the effects of these SNPs on cellular function.

1.1 Skin and Wounds

1.1.1 Skin Structure and Function

The skin is a multifunctional organ accounting for approximately one sixth of the human total body weight. It is responsible for physical and chemical protection by absorbing UV radiation, preventing microorganism invasion, stopping chemical penetration and controlling the passage of water and ions. It also has roles in thermoregulation as well as immunological and sensory functions (Firooz et al., 2012). The skin is formed from three distinct layers (Figure 1.1); the subcutis, dermis and epidermis.

The subcutaneous layer connects the outer layers of the skin to the underlying tissue providing a protective cushion and energy reservoir. It is a loose layer of connective and adipose tissue containing hair roots, nerves and blood vessels (Sherwood, 2010). The dermis sits atop the subcutaneous layer and is a connective tissue containing blood vessels, nerve endings, lymphatics, and skin appendages. It is comprised of collagen fibres which provide strength and elastin fibres
which provide stretch. There is a relatively low density of cells in the dermal layer, with the majority of these being fibroblasts alongside melanocytes, mast cells, macrophages and lymphocytes (Firooz et al., 2012). The basal lamina joins the dermis to the epidermis, formed of collagen and laminin polymers, it allows the passage of chemical signals between the layers (Yurchenco and O’Rear, 1994).

The epidermis is a self-renewing multi-layered sheet of cells (Braun-Falco, 2000). It is made up of five layers; the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and the stratum corneum. Proliferation of keratinocytes takes place in the basal layer, where they are attached to the underlying basement membrane through hemidesmosomes. Cells undergo terminal differentiation as they migrate upwards through the layers until they form corneocytes at the stratum corneum and are then shed at the skin surface (Jones et al., 1995). The stratum spinosum lies above the stratum basale and is made up of spinous cells that are joined together by desmosomes and other intercellular junctions. Above this lies the stratum granulosum which contains diamond shaped keratinocytes which have granular cytoplasm and which are beginning to lose their nuclei. The stratum lucidum and the stratum corneum form the upper layers and are populated by flat, dead, cornified cells. The stratum corneum is important in that it serves as the principle barrier against the penetration of chemicals and microbes (Proksch et al., 2008). These cells contain keratin filaments and are surrounded by a protein/lipid polymer envelope (Proksch et al., 2008). They are joined by corneodesmosomes which have the additional corneodesmosine protein (Jonca et al., 2002). This layer is several cells thick and these cells slough off over time in response to physical and mechanical interactions. Appropriate desquamation requires the action of proteolytic enzymes such as kallikreins (Zeeuwen, 2004). Melanocytes are pigmented cells found in the basal layer that form granules of pigment (melanosomes) which they transfer to keratinocytes to provide protection from ultraviolet light. Skin also contains Langerhans cells which are dendritic antigen presenting cells that can sample the skin micro flora and fauna and participate in the immune barrier of the skin (Hogan and Burks, 1995).
Figure 1.1 – The structure of human skin. (A) Idealised haematoxylin and eosin stained skin structure illustration (Allen, 2008) and (B) a haematoxylin and eosin stained human scalp section.
1.1.2 Acute Wound Healing

Upon insult, the skin undergoes a series of complex, overlapping physiological processes in order to restore the functionality of the skin barrier. Initial blood loss is halted by haemostasis, followed by an inflammatory phase, which progresses to the proliferative phase and finally the remodelling phase (Figure 1.2).

**Figure 1.2 – Wound healing profiles of acute and chronic wounds.** The main overlapping stages of wound healing (haemostasis, inflammation, tissue formation and remodelling), alongside average timescale of acute and chronic wounds in humans. Inflammation is sustained in chronic wounds to the detriment of all other phases.

1.1.2.1 Haemostasis

Haemostasis is the process of limiting blood loss and infection achieved by vasoconstriction and the formation of a platelet plug. Damage to the skin and blood vessels causes an immediate response whereby the vessels contract to limit the outflow of blood (Monaco and Lawrence, 2003). Contact with exposed collagen causes platelets to become activated and adherent, forming a platelet plug at the wound site, activated by thrombin and glycoprotein Ib and protease-activated receptor (PAR)-1 and -4. This causes an increased display of surface receptors (i.e. the \( \alpha_{\text{IIb}}\beta_3 \) receptors, von Willebrands factor, fibrinogen and thrombin), granule release and aggregation. Simultaneously, the coagulation cascade is initiated whereby fibrinogen is converted to fibrin by thrombin, which forms a mesh of insoluble fibres bound to platelets, creating a provisional wound matrix (Laurens et al., 2006). Damaged cells leak cytoplasmic material
containing endogenous molecules such as damage-associated pattern molecules (DAMPs), which are thought to activate other cells in the area. Platelets contain a store of growth factors and pro-inflammatory cytokines that can participate in various processes of wound healing, such as, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) (Guo and Dipietro, 2010). These factors signal to fibroblasts and keratinocytes as well as recruiting inflammatory cells from the circulating blood, paving the way for the inflammatory response (Guo and Dipietro, 2010).

1.1.2.2 Inflammatory Phase

The inflammatory phase initiates almost immediately after wounding. Cytokines and chemokines, released by resident immune cells and as a result of haemostasis, attract inflammatory cells to the wound site in order to clear the site of infection and debris (Reinke and Sorg, 2012). Circulating macrophages and neutrophils are encouraged to marginate and extravasate into the wound tissue by chemokine and cytokine induced expression of endothelial surface cellular adhesion molecules (CAM’s), such as CD11 and CD18 (Walzog et al., 1999). Neutrophils are the first immune cells to infiltrate the wound site and kill invading microbes through the release of reactive oxygen species (ROS) and lysosomal enzymes. They also degrade damaged matrix structures with the use of collagenases and proteinases (Sylvia, 2003). Monocytes follow neutrophils in infiltrating the wound where they differentiate into macrophages, with their numbers peaking at around day 5 post-wound. They are attracted by chemoattractants associated with damage, such as TGF-β (Wahl et al., 1987). Monocytes are provoked to differentiate into macrophages through contact with the extracellular matrix and through interaction with inflammatory signalling molecules (Jendraschak et al., 1998). In the early stages of healing they act to clear the wound of microbes, apoptotic cells (including neutrophils) and the remaining extracellular or fibrin matrices.

Existing damaged extracellular matrix is degraded by proteolytic enzymes such as metalloproteinases. Excessive macrophages have been proposed to drive delayed healing via excessive matrix degradation, producing large amounts of nitric oxide (NO) and ROS which can further damage the site (Mauch et al., 1994) (Schafer and Werner, 2008b). Macrophages can stimulate: reepithelialisation by the release of insulin-like growth factor-1 (IGF-1); keratinocyte proliferation and migration via epidermal growth factor (EGF) and keratinocyte growth factor (KGF) (Ando and Jensen, 1993); angiogenesis facilitated by the release of vascular endothelial growth factor (VEGF) and; wound contraction and the production of dermal collagen implemented by fibroblasts in response to PDGF and FGF. As wound clearing continues, the macrophages undergo a transition from a pro-inflammatory phenotype (M1) to a pro-healing
phenotype (M2) (Mosser and Edwards, 2008). Macrophages stimulate the resolution of the inflammatory phase, overlapping with the proliferative stage. Lymphocytes, such as T cells can also appear towards the end of the inflammatory phase, although their primary wound function is not fully understood (Shah et al., 2012).

The adaptive immune response can also have an important role in regulating inflammation, wound healing and the development of chronic wounds. In contrast to the innate immune response, the adaptive immune response is delayed but specific. It has the ability to retain memory and initiate rapid responses to subsequent immunological challenges. The main classes of cells associated with adaptive response are B cells and T cells which can become activated in response to free antigen or antigen presenting cells. Cell numbers peak at 5-10 days post-wounding (Lin Chen, 2015). Activated B cells produce specific antibodies that can have numerous effects including marking of pathogens for destruction, complement activation, bacterial opsonisation and the inactivation of toxins. T cell response is dependent on the major histocompatibility complex (MHC) molecule with which the antigen is presented. MHC class I presented antigen is recognised by CD8+ cytotoxic T cells, whilst MHC class II presented antigen is recognised by CD4+ helper T lymphocytes. CD8+ cells are much more specific in cell targeting whilst CD4+ cell activation enables cytokine production and paracrine signalling to neighbouring cells. There is strong evidence that the adaptive immune system can have an influence on wound repair. Venous ulcers and diabetic foot ulcers have a significantly lower CD4+/CD8+ ratio compared to acute wounds due to particularly low levels of CD4+ cells and also have an increase in B cells (Loots, 1998). T cells can secrete growth factors that are beneficial to wound healing (Gillitzer, 2001). A particular subset of T cells, gamma delta (γδ) T cells also known as dendritic epidermal T cells, can contribute to reepithelialisation through the production of growth factors, with γδT cell deficient animal models demonstrating a delay in wound healing (Jameson 2002). The presence or absence of CD4+ and CD8+ cells have been shown to alter the infiltration and wound area numbers of neutrophils and macrophages. This has an inevitable effect on the inflammatory regulation of the wound site. Furthermore, it has been demonstrated that an induced deficiency in CD4+ cells can increase the wound levels of IL-1β, IL-6, IL-17, IFNγ and a decrease in IL-4, whilst induced CD8+ deficiency has the inverse effect on the same factors (Lin Chen, 2015). A balanced orchestration of immune reaction and inflammation is necessary for timely wound healing and any dysfunction, whether at the innate or adaptive level, can have an effect on wound healing and the development of chronic wounds.
1.1.2.3 Proliferation Phase

During the proliferative phase, contractile forces reduce the wound area, granulation tissue is formed in the dermis from proliferating and migrating fibroblasts, whilst reepithelialisation is achieved by the proliferation and migration of keratinocytes across the wound surface (Guo and Dipietro, 2010).

1.1.2.3.1 Granulation Tissue Formation

The formation of granulation tissue overlaps with the inflammatory phase and develops approximately between 3 and 10 days after injury. The provisional matrix of the clot in the dermis is replaced with the more sophisticated granulation tissue which provides a framework for successful healing. Granulation tissue, predominantly made up of fibroblasts but also containing granulocytes, macrophages, capillaries and collagen bundles, forms in the damaged dermis to replace the provisional matrix of the blood clot. These fibroblasts are sourced from the surrounding healthy dermis, circulating fibroblasts and bone marrow progenitor cells. Fibroblasts have a number of roles that are beneficial to the healing process including wound contraction and extra cellular matrix (ECM) deposition. They have been shown to be activated by PDGF and FGF-2, and stimulated to proliferate by insulin-like growth factor 1 (IGF-1) and TGF-β1 (Singer and Clark, 1999). TGFβ1, produced by macrophages in the inflammatory response, can drive fibroblasts to differentiate into myofibroblasts, which express α-smooth muscle actin and have contractile properties (Gabbiani, 2003). It has been suggested that differentiation can be influenced by mechanical stress and tension in the ECM (Hinz and Gabbiani, 2003). They connect with each other via gap junctions and to the extracellular matrix by fibronexus; a transmembrane structure which joins the intracellular microfilaments in continuity with fibronectin fibres in the developing ECM (Eyden, 1993). Wound edge fibroblasts can also form stress fibres of weak contractile actin bundles (Shaw and Martin, 2009). Together these cells create contractile forces to draw the wound closed. Fibroblasts also contribute to the formation of the new ECM by producing collagen, fibronectin, elastin, glycosaminoglycans, proteoglycans and hyaluronic acid (Reinke and Sorg, 2012).

1.1.2.3.2 Re-epithelialisation

Re-epithelialisation is the process of re-establishing a cutaneous covering through the migration and proliferation of keratinocytes. The epidermis is repopulated by resident cells from the wound margin and by stem cells from the bulge region of hair follicles (Ito et al., 2005). The three main events in re-epithelialisation are keratinocyte detachment, migration and proliferation.
Migrating keratinocytes do not proliferate, so it is the cells at the wound margin that must proliferate to replace these migrating cells (Laplante et al., 2001). Keratinocytes are activated to migrate by KGF, IGF-1, nerve-growth factor (NGF) and particularly epidermal growth factor (EGF) (Werner and Grose, 2003). Activated platelets release growth factors immediately after the initial wounding event, including EGF. These signals are reinforced by macrophages and other immune cells during the inflammatory phase. This provides the keratinocytes with an immediate and lasting signal, stimulating re-epithelialisation (Ando and Jensen, 1993). Migrating keratinocytes at the wound edge downregulate desmosomal adhesion, switching from the hyper-adhesive Ca2+ dependent form, to the less adhesive Ca2+ dependent form (Thomason et al., 2012). This alteration in adhesion enables the cells to migrate from the basement membrane at the wound edge and onto the provisional matrix that forms the clot. The removal of tension at the desmosome junctions results in the production of lipid mediators which activate associated kinases resulting in increased permeability to ions. This provides an initiating signal to the cells at the wound margin and polarises them, through a reorganisation of their intracellular tonofilaments, in the direction of migration (Reinke and Sorg, 2012). The act of migration itself is achieved through lamellipodial crawling via the polymerisation of cytoskeletal actin fibres (Mitchison and Cramer, 1996). Focal adhesions are made with the ECM and fibrin clot constituents, mediated by integrins. RhoGTPases coordinate the intracellular organisation of the cytoskeleton and fibre production, and are essential for the control of migration. Migrating keratinocytes also upregulate production of proteolytic enzymes, such as matrix metalloproteinase (MMPs) (Pilcher et al., 1999), which allows them to carve a path between the scab and tissue below. Once the migrating cell fronts intercept, contact inhibition cause migration to cease and the GTPases are switched off leading to a reorganisation of the cytoskeleton (Mayor and Carmona-Fontaine, 2010). The keratinocytes can then differentiate and stratify to form a new epidermis.

Intact, healthy epidermal basal keratinocytes express the keratin pair K5/K14 and suprabasal keratinocytes express K1/K10 (Stoler et al., 1988). However, when wounded, expression of K6, K16 and K17 is induced. This change is thought to be caused by cytokines and growth factors in the wound milieu, but functional significance is less clear. One theory, although lacking supporting evidence, is that the upregulated wound keratins have advantageous viscoelastic properties for migration (Wong and Coulombe, 2003).

1.1.2.3.3 Angiogenesis

Angiogenesis is the process of revascularisation, necessary to restore a blood supply to the highly metabolically active wound tissue. Angiogenesis is initiated by growth factors, such as VEGF, FGF-2, PDGF and members of the TGF-β family, which are released in response to damage by
inflammatory cells (Pardali et al., 2010). Endothelial cells secrete proteolytic enzymes that enable them to break through the basal lamina and gain access to the wound site where they proliferate and arrange themselves into tubular canals, connected by the superficial integrin adhesion molecules αvβ3, αvβ5 and α5β1 (Reinke and Sorg, 2012), and release metalloproteinases to lyse the surrounding tissue. They differentiate into venules and arterioles and strengthen their vessel walls with the recruitment of smooth muscle cells and pericytes, restoring blood flow to the damaged tissue.

1.1.2.4 Remodelling Phase

The remodelling phase is the last phase of wound healing that can continue for several years post-wounding. In this time, the inflammatory response is terminated, the vascular network matures and the extra-cellular matrix (ECM) is remodelled.

There is a cessation of inflammatory cytokine production and clearing of inflammatory and immune cells from the wound site. Neutrophils are removed from the site by a combination of apoptosis and phagocytosis by macrophages or can exit the tissue through the lymphatics. Macrophages can be deactivated by anti-inflammatory cytokines and glucocorticosteroids, such as resolvin-E, or by phagocytosis. Resolvin and protectin families, as well as the lipoxin class of eicosanoids, have been identified as anti-inflammatory and pro-resolution mediators that restore homeostasis to the tissue (Serhan et al., 2008). The production of functionally inactive, competitor receptors on inflammatory cells are also thought to dampen the inflammatory response (D’Amico et al., 2000).

The architecture of the skin is never fully restored as appendages (hair follicles and sebaceous glands) do not readily develop in scar tissue. However, it has been suggested that inflammatory elements can promote appendage re-growth (Osaka et al., 2007), involving the action of Wnt signalling to bring about the re-enactment of epidermal development (Ito et al., 2007). The dense ECM is remodelled to form a stronger structure, but not to the extent of unwounded skin. Collagen III of the scar tissue, deposited during the proliferative stage, is replaced by the more adept regimented bundles of collagen I. Blood vessels also regress and refine to form a more functionally competent blood supply (Wietecha et al., 2013).

1.1.3 Chronic Wounds

Chronic wounds develop as a result of the disruption of normal wound healing and specifically a failure to progress through one or more phases of healing (see Figure 1.2). They can take many
months or even years to fully heal. Chronic wounds are predominantly a disease of the elderly, with an average age of a patient being over 65, and represent a major clinical problem and demand a large proportion of healthcare resources (Mustoe, 2004). It is estimated that the National Health Service spends approximately £5 billion per annum on their care and treatment (Guest et al., 2016), not to mention the impaired quality of life, suffering and economic loss experienced by the patients themselves. There are currently no effective treatments for chronic wounds and research is hindered by the lack of suitable and accurate animal models, making this an area of research that needs to be addressed. Factors linked to the development of chronic wounds include reduction in tissue growth factors and cytokine levels, decreased matrix synthesis, imbalance in proteolytic enzymes and their inhibitors, prolonged inflammation, impaired angiogenesis, delayed reepithelialisation, hypoxia, bacterial infection and defective macrophage function (Harding et al., 2002), (Mustoe et al., 2006), (Mustoe, 2004), (Tarnuzzer and Schultz, 1996). Three main classes account for over ninety percent of all chronic wounds; namely venous, diabetic and pressure ulcers (Mustoe et al., 2006). Despite very different aetiology there are a number of commonalities between these wound types.

![Figure 1.3 – Types of chronic wound. Examples of a typical; (A) venous ulcer (Grey et al., 2006b), (B) diabetic foot ulcer (Amin and Doupis, 2016) and (C) pressure ulcer (Grey et al., 2006a).](image)

1.1.3.1 Venous Ulcers

Venous ulcers (Figure 1.3a) are most commonly found in the elderly and are responsible for around 70% of chronic wounds of the lower leg (Bergqvist et al., 1999). Approximately 0.2% of the European population will develop a venous ulcer in the course of their life (Grey et al., 2006b). Most venous ulcers occur in the leg gaiter area and can range in size from small and discrete to circumferential. They are usually well vascularised shallow wounds which can be covered with a
fibrinous layer and have unhealthy granulation tissue. Risk factors include varicose veins, deep vein thrombosis, chronic venous insufficiency and poor calf muscle function (Grey et al., 2006b). Venous ulcers are a clinical manifestation of sustained venous hypertension. Normal venous return of the leg is facilitated by the muscular pump of the calf and valvular function which prevents reflux (Abbade and Lastoria, 2005). Dysfunction of the lower limb valves allows blood to flow back into the superficial veins, creating a state of hypertension. This pressure is transferred to the cutaneous capillary beds with the consequence of the formation of an ulcer. If this hypertension is prolonged, lipodermatosclerosis (LDS) can occur, which is characterised by an induration and darkening of the skin around the lower leg (Kirsner et al., 1993). This high pressure can result in erythrocyte extravasation to skin tissue, stimulating melanin production and giving rise to the characteristic brown skin seen in patients with venous insufficiency and venous ulcers (Grey et al., 2006b).

Various pathophysiological changes have been observed in LDS skin and venous ulcers. Endothelial hypertrophy and the widening of interendothelial pores can be seen (Chatterjee, 2012). This increases the permeability of the vessels and allows for the extravasation of macromolecules into the dermis and subcutaneous tissue. This can lead to ischemia, hypoxia and a lack of nutrients to the skin tissue, resulting in necrosis (Browse and Burnand, 1982). Fibrinogen extravasation and deposition creates what are known as ‘fibrin-rich cuffs’ (Burnand et al., 1982). Some studies have suggested that the development and delayed healing of venous ulcers are associated with an inappropriate and prolonged inflammatory response (Smith, 2001). Leukocytes can become trapped in the microcirculation of the lower limb and cutaneous capillary beds (Thomas et al., 1988). Trapped leukocytes are thought to secrete inflammatory mediators which recruit more leukocytes to the area, where they accumulate and release free radicals and proteolytic enzymes that irritate the skin architecture, expediting the formation of an ulcer. An imbalance in MMPs and their inhibitors are also thought to create a destructive environment in the venous ulcer (Bullen et al., 1995).

Considering that the systemic level of estrogen decreases with age, particularly in the post-menopause female, it has been proposed that loss of estrogen may be a causative factor in pathological wound healing. Indeed a recent case-cohort study demonstrated the protective effects of estrogen. Patients of 65 years and over who received HRT, were 30-40% less likely to develop a venous leg ulcer than those who did not use HRT (Margolis et al., 2002).

1.1.3.2 Pressure Ulcers

A pressure ulcer (Figure 1.3c) has been defined as ‘a localized injury to the skin and/or underlying tissue usually over a bony prominence, as a result of pressure, or pressure in combination
with shear’ by the National Pressure Ulcer Advisory Panel (NPUAP) (Black et al., 2007). Most pressure ulcers occur in hospitalised patients, mainly in the elderly and paralysed or intensive care patients who have limited or no mobility. Pressure ulcers usually occur over a bony joint and are caused by the disruption of blood flow to the tissue by sustained pressure. The lack of oxygen and nutrients to the site causes necrosis of the tissue and the formation of a pressure sore. At the most advanced stage, ulcers can progress to extensive full thickness tissue loss with exposed bone or muscle (Black et al., 2007).

1.1.3.3 Diabetic Ulcers

Around 15% of the 2.9 million diabetes patients in the UK are estimated to develop a chronic non-healing diabetic foot ulcer (Guo and Dipietro, 2010), of which 84% will go on to require a lower leg amputation (Brem and Tomic-Canic, 2007). Diabetic ulcers (Figure 1.3b) often occur on the feet and ankles which are constantly exposed to pressure and mechanical stresses. Diabetes creates a pathology which contributes to the development of a chronic wound. Altered blood sugar and metabolic activities can cause nerve damage resulting in autonomic neuropathy and a dysfunction in the microcirculation (Jeffcoate and Harding, 2003). This can create local hypertension and hypoxia, resulting in an ulcer.

1.2 Estrogens

Estrogens are a group of steroid hormones which were first described in the 1920’s by Edward Doisy (Tata, 2005). The three main forms found in humans are estrone (E₁), estradiol (E₂), and estriol (E₃), of which 17-β-estradiol is the most biologically active. Estrogen is the primary female sex hormone and as such, is highest in females of reproductive age. Estrogen is involved in many physiological functions including the development of secondary female sexual characteristics, regulation of fertility and ovulation, bone mass maintenance, lipoprotein synthesis, regulation of insulin responsiveness and maintenance of cognitive function and cell cycle and growth, amongst others (Nelson and Bulun, 2001). In males estrogens are implicated in spermatogenesis, the maintenance of bone density and cardiovascular health (de Ronde et al., 2003).

Estrogen deficiency has been mooted as the main regulator of delayed healing in the elderly (Hardman and Ashcroft, 2008) and shown to have protective anti-inflammatory action in various tissues, such as the brain (Bruce-Keller et al., 2000). Although estrogen has an anti-inflammatory affect in the skin, regulated through macrophage migration inhibitory factor (MIF) (Hardman et al., 2005), it has recently been shown that estrogen can promote wound healing independently from its anti-inflammatory effects (Campbell et al., 2010). Systemic levels of estrogen decrease
with age in both genders (this decrease is highly pronounced in post-menopausal females). Post-menopausal females taking systemic hormone replacement therapy heal standardised acute wounds more quickly than their control counterparts, who have no hormonal supplementation (Ashcroft et al., 1997a). A further study showed that topical estrogen treatment accelerated healing in elderly subjects, associated with the inflammatory response (Ashcroft et al., 1999). Thus estrogen has clear beneficial effects on healing.

1.2.1 Estrogen Biosynthesis

Estrogens are steroidal hormones, synthesised fundamentally from cholesterol (see Figure 1.4). Cholesterol is metabolised by various enzymes to form androgens, such as androstenedione and testosterone. These androgens can then be converted to estrogen molecules through the action of the P450 enzyme aromatase, in the endoplasmic reticulum of estrogen producing cells (Simpson et al., 1994). Testosterone is converted to estradiol in ovarian granulosa cells and androstendione to estrone in the adipose tissue. Aromatase is expressed in various tissues other than the ovary, such as the stromal mesenchymal cells of adipose tissue (Simpson et al., 2002), in bone and bone derived cells (Sasano et al., 1997), in the brain of rats (Lauber and Lichtensteiger, 1994) and in vascular smooth muscle cells (Harada et al., 1999).

In females of reproductive age, the ovary is the main source of systemic estrogen. The granulose cells of the ovarian follicles and the corpora lutea are the main sites of biosynthesis. In males, it is the gonad that provides a large proportion of systemic estrogen. However, there is also the production at the local level in the peripheral tissue, acting in an autocrine and paracrine fashion (reviewed (Zouboulis, 2009)). The adrenals secrete large amounts of steroid precursors, which can be converted into estrogens and androgens at the peripheral tissues. Local synthesis of estrogen is also present in both young and elderly females and becomes increasingly important post-menopause, once the systemic source is diminished (Labrie et al., 2000).

The menopause marks the point where female estrogen biosynthesis changes from the ovaries as a systemic source to local synthesis in the peripheral tissues. Although local estrogen synthesis occurs in the young, it is not until the systemic source is lost that it becomes functionally important. However, the local generation of estrogen is inadequate to compensate the shortfall created by the loss in systemic levels. Aromatase can be found in the adipose tissue of the skin and it is thought that this provides the supply of estrogen that plays a role in wound healing in males and the elderly of both genders (Purohit and Reed, 2002).
Figure 1.4 – The biosynthesis of estrogen from cholesterol and androgen precursors (Blair, 2010). Members of the enzyme families Cytochrome P450 (CYP), Aldo-keto reductase (AKR) and Hydroxysteroid dehydrogenase (HSD) facilitate hormone synthesis.

1.2.2 Estrogen Receptors

Estrogen has a multitude of functions including the regulation of cell growth, development, differentiation, inflammatory regulation and homeostasis. Estrogen signals through the nuclear estrogen receptors (ER) of which there are two forms; ERα and ERβ. ERα was first characterised in 1958 (Jensen, 1960), but it was not until 1996 that ERβ was discovered in rat prostate and ovary (Kuiper et al., 1996). The effects of estrogen can be directed through a number of signal transduction pathways, including ligand-dependent, ligand-independent, ERE-independent and non-genomic pathways. The main pathway is the ligand-dependent pathway, which involves the activation of an ER by ligand followed by dimerisation and nuclear translocation and transcriptional activity of target genes containing an estrogen response element (ERE). Interestingly, estrogen signalling can target estrogen receptor genes themselves and upregulate mRNA expression, especially in the case of ERβ (Tessier et al., 2000).
1.2.2.1 Estrogen Receptor Protein Structure

The biological effects of estrogen, particularly 17-β-estradiol, are mediated by the two estrogen receptors, ERα and ERβ (Figure 1.5). These are ligand-inducible transcription factors that, despite having similar structures and functionality, are transcribed from separate genes on different chromosomes. In the classical, ligand-dependent pathway, estrogen diffuses into the cell where it binds and activates ER’s allowing them to dimerise and translocate to the nucleus. Here the dimer can bind directly to estrogen response element (ERE) sequences of target genes or indirectly through protein to protein interactions with AP1 or Sp1. This results in the recruitment of coregulatory proteins to the estrogen-responsive gene promoter site and the promotion or repression of gene transcription (Klinge, 2000).

The ERα is constructed of 595 amino acids with a molecular weight of 66kDa, whilst the ERβ is 530 amino acids with a molecular weight of 59kDa. ERα and ERβ share a similar 5 domain structure (Figure 1.5). The DNA-binding domain (C domain) is the most homologous, sharing 97% amino acid sequence, accounting for common interaction with the ERE motif. The domain contains two functionally distinct zinc finger motifs, with each composed of 8 cysteines that create a tetrahedral orientation of two zinc ions, to enable interaction directly with the DNA helix (Ruff et al., 2000).

There are significant differences in their primary structure however, with the ligand binding domains sharing 55% homology and as little as 17% homology in the transcriptionally active A/B domain. The A/B domain is located at the N’ terminus of the ER protein and provides transcriptional activity through activation function-1 (AF-1), which recruits a range of co-regulatory protein complexes to the DNA-bound receptor. AF1 can regulate transcription ligand-independently. The ERα and ERβ proteins share a low homology in this region, which is thought to account for the difference in gene transcription and signal transduction.

The central D domain is a hinge region and serves as a flexible section, connecting the DBD and ligand binding domains (LBD). This region is also a site of ER dimerisation and is involved in the association with the chaperone heat shock protein 90 (hsp90).

The E domain or ligand binding domain (LBD) is the site of ligand interaction. The two receptors have 55% conformity in this region, but due to stretches of conserved sequence have remarkably similar tertiary structures. Activation function-2 (AF2), which can recruit co-activators, is in this region and responsible for ligand-dependent transcriptional activity. The region is composed of 11 anti-parallel α-helices (steroid receptor family LBD’s H1-H12, excluding H2 which is not present in ER), which form a ligand binding pocket, with an anti-parallel β-sheet at the base (Tanenbaum et al., 1998). Ligand binding induces a conformational change where helix 12, which is part of the AF-
2, closes the pocket and encapsulates the ligand in a highly hydrophobic environment (Brzozowski et al., 1997) (Shiau et al., 2002). This ligand-binding pocket of ERβ is smaller than that of ERα and the two have different amino acids lining the cavity. This enables the two receptors to interact with different affinity to ligands and to cause different levels of transcriptional activity of target genes.

Following the LBD domain is the F domain of the C terminus. This region shares an 18% between receptors and is not well conserved between vertebrate species (Montano et al., 1995). The F domain has been found to modulate gene expression in a ligand, promoter and cell specific manner and has a role to play in receptor dimerization along with the E domain (Koide et al., 2007; Peters and Khan, 1999; Yang et al., 2008). It is the differences in the F domain that is thought to lead to the differences in agonist and antagonist signalling outputs (Skafar and Zhao, 2008).

**Figure 1.5 – Schematic representation and comparison of the structure of human ERα and ERβ.** Percentage homology between each domain is denoted in grey. Numbers above each box signify amino acid number. NHD= N terminal homology domain, DBD= DNA binding domain, LBD= ligand binding domain. (Adapted from (Klinge, 2000))

1.2.2.2 Estrogen Receptor Genes

1.2.2.2.1 ERα

The estrogen receptor α gene (ESR1) is located on chromosome 6, at position 6q25.1 and is approximately 473kb in length. The transcription of the gene is regulated by a complex system of multiple promoters and untranslated exons at the 5’ end. The upstream region contains 9 untranslated exons, transcribed from 7 independent promoters (at E1, F, T1, D, C, B and A). There are 8 coding exons that code for ER protein domains. The complex promoter and exon structure result in the possibility of numerous splice variants (Donaghue et al., 1999).
1.2.2.2 ERβ

ERβ is transcribed from the 255kb ESR2 gene located on chromosome 14 at position 14q23.2. It also has a complex system of multiple non-coding exons (Figure 1.6), which are different to those found in ESR1. The ERα and ERβ coding exons are similar, but the 5’ untranslated regions are distinct. The estrogen receptor genes are complex and although coding for similar proteins, they have very different gene structures, regulatory mechanisms and splice variants.

Depending on which ESR2 promoter is engaged, a different 5’UTR on the primary mRNA transcript will be produced. Mature mRNA, where transcription has been initiated at the OK promoter (located 55111bp upstream of the translation start site), will include the non-coding OK exon which can include the 5 cassette exons 0X1 to 0X5, or can simply splice straight to the first coding exon. Transcriptional initiation from the ON promoter (located 11374bp upstream of the translation start site) will result in mature mRNA containing the ON non-coding exon, before exon 1. It has more recently been proposed that a third promoter region is located immediately upstream of exon 1, identified from a novel ERβ mRNA 5’UTR which contained neither ON nor OK exons, but which did contain sequence from exon 1 and from the region immediately upstream of the 5’ splice site, however this has not been extensively studied (Smith et al., 2010b).

There is little understood on the regulatory significance of these alternative 5’UTR sequences and how they affect ERβ expression, but investigation into which cells and tissues make use of which promoter have been revealing. Springwald et al saw an increase in ON promoter usage in the endometrium of post-menopausal women compared to pre-menopausal, whilst OK promoter usage decrease to negligible levels (Springwald et al., 2010). This suggests that promoter usage could change with age, disease state or, rather pertinently with changes in estrogen level. Furthermore, it has been shown that alternative promoter usage can be cell specific (Hirata et al., 2001). The use of the OK promoter has been found in spermatozoa, liver, endometrium and myometrium. The ON promoter has also been found in spermatozoa, endometrium and myometrium, but in addition is reportedly favoured in peripheral leukocytes. This is relevant since excessive, persistent inflammation is a major causative factor in the development of chronic wounds. This suggests that they may play a role in the regulation of expression of the receptor, favour a certain isoform or variant, or may alter the function of the ER in a cell specific environment.

Other variants of ERβ can be formed from the splicing of alternative exon 8s. Wild-type ERβ mRNA is composed of exons 1 to 7 and exon 8b. The β2 variant (also known as βcx) mRNA uses exon 8b, the β3 variant uses exon 8e, the β4 variant uses exons 8c and 8d, whilst the β5 variant uses exon 8a. As exon 8 codes for the protein F domain, these variants produce alternative ERβ proteins (Moore et al., 1998). In all but the wild type ERβ, the AF2 is deleted and the structure distorted,
meaning that the isoforms do not bind ligand. The function of these variants has not been extensively explored, although they appear to be expressed in a cell specific manner (Moore et al., 1998). The ERβcx has been characterized and appears to be able to form a heterodimer with wild-type ERβ resulting in a suppression of function (Moore et al., 1998), (Ogawa et al., 1998). A further intronic ‘M’ exon has been reported between exons 4 and 5 (Shoda et al., 2002). The final form of variant found in the mRNA transcripts of ERβ is exon skipping/deletion (denoted by Δ). The reported exon deleted mRNA variants include Δ2, Δ2+5+6, Δ3, Δ4, Δ5, Δ5+2, Δ6, Δ6+2, Δ6+2+3, Δ5+6 (Poola et al., 2002). The first coding exons can also be deleted giving rise to the mRNA transcripts Δ1 and Δ1+2+3, where the 5’ UTR exons 0K or 0N splice directly to coding exons 2 or 4 (Springwald et al., 2010).

Since most functional studies use the mouse model, it is important to bear in mind the differences between mouse and human ERs and any limitations that this may create. The protein structure of the human and mouse ER’s are very similar sharing a structural homology of 88% (White et al., 1987), giving a similar functional profile. However, the gene structure of each receptor is less homologous, especially in the 5’ untranslated regulatory region (5’UTR). This suggests that the receptor expression may be controlled in a different manner between species.
Figure 1.6 – Genomic structure and SNPs of the ERβ gene. The ERβ gene consists of 8 coding exons (E1-8) (blue) with multiple features for exon 8 and 7 untranslated exons (0k, 0X1-5 and ON) transcribed from 3 distinct promoter sites (indicated by black arrows) in the 5’ untranslated region (red). Splicing events are highlighted by the blue connecting lines (/\). Venous ulcer associated SNP loci are indicated (A), as are all other disease linked SNP loci (B) (see Table 1.2). Based on mRNA transcripts presented by Horata et al. (Hirata et al., 2001), promoters identified by Smith et al. (Smith et al., 2010a) and nomenclature proposed by Shoda et al. (Shoda et al., 2002).
1.2.2.3 Estrogen receptor Expression and Localisation

Estrogen has functional effects throughout the body and as such ERs are distributed in a variety of tissues and cell types. These receptors have both unique and overlapping tissue distributions which suggests that they may have distinct and interacting roles (Mosselman et al., 1996). Immunocytochemistry and mRNA expression studies have revealed distribution patterns of estrogen receptors in a wide range of human tissues (Table 1.1). Both receptors are enriched in the tissues of the female reproductive system, but are also found in the endocrine, skeletal, cardiovascular and the central nervous systems, amongst others (reviewed (Dahlman-Wright et al., 2006)).

The distribution of ER’s in the skin is unclear despite a number of publications addressing the issue. Immunocytochemistry study showed that although ERα has been identified in all of the skin associated cells types, strong presence was not seen anywhere except for the sebaceous gland (Pelletier, 2000). By contrast, ERβ was highly expressed in the epidermis, blood vessels, and dermal fibroblasts (Thornton et al., 2003). These results were affirmed by mRNA studies showing a higher mean level of ERβ in skin associated cells (Haczynski et al., 2002). Most immune cells, which are important in facilitating the positive effects of estrogen during wound healing, express both ERs (Nalbandian et al., 2005). The next layer of complexity involves ER isoform expression in different cell types, which to date has not been addressed in the skin. Mice are the main research model in wound healing, but knowledge of ER skin distribution is lacking. However, work from our own lab reveals equal ERα and ERβ expression (Campbell et al., 2010).

Estrogen signalling can have different actions depending on ER composition within a tissue or cell. This is important because ERs form dimers in order to become active transcription complexes. The ratio of ERα to ERβ within a cell will determine the extent of heterodimer or homodimer formation. Different dimer variations have different stabilities, different levels of affinity to chromatin and DNA ERE’s and different transcription regulatory effects (i.e. promotive or inhibitory) (Charn et al., 2010). ERα homodimers are more stable than ERα/ERβ heterodimers and ERβ homodimers less stable. However ERα homodimers and ERα/β heterodimers bind to DNA with similar affinities, whilst ERβ dimers bind with less affinity (Cowley et al., 1997). This provides a number of variable outcomes, depending on the ER composition. In general ERβ represses ERα transcriptional activity and is suggested to prevent excessive ERα mediated gene transcription (Weihua et al., 2003).
Table 1.1 - Distribution of ERα and ERβ in adult human tissues (Taylor and Al-Azzawi, 2000), (Bord et al., 2001), (Pedersen et al., 2001), (Phiel et al., 2005), (Thornton et al., 2003), (Pelletier, 2000), (Ashcroft et al., 2003), (Haczynski et al., 2002).

<table>
<thead>
<tr>
<th>Tissue/Cell</th>
<th>ERα</th>
<th>ERβ</th>
<th>Tissue/Cell</th>
<th>ERα</th>
<th>ERβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female Reproductive System</td>
<td></td>
<td></td>
<td>Cardiovascular System</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vagina</td>
<td>+</td>
<td>+</td>
<td>Myocardium</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Uterus</td>
<td>+</td>
<td>+</td>
<td>Purkinje fibres</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cervix</td>
<td>+</td>
<td>+</td>
<td>Aorta</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fallopian tubes</td>
<td>+</td>
<td>+</td>
<td>Coronary artery</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Breast</td>
<td>+</td>
<td>+</td>
<td>Inferior vena cava</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Male Reproductive System</td>
<td></td>
<td></td>
<td>Carotid artery</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Testis</td>
<td>+</td>
<td>+</td>
<td>Central Nervous System</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epididymis</td>
<td>+</td>
<td>+</td>
<td>Cerebral cortex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>-</td>
<td>+</td>
<td>Cerebellum</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Prostate</td>
<td>+</td>
<td>+</td>
<td>Hippocampus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Endocrine System</td>
<td></td>
<td></td>
<td>Medulla oblongata</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pituitary</td>
<td>+</td>
<td>+</td>
<td>Pons</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thyroid</td>
<td>-</td>
<td>+</td>
<td>Skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>+</td>
<td>+</td>
<td>Fibroblasts</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ovary</td>
<td>+</td>
<td>+</td>
<td>Keratinocytes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urinary System</td>
<td></td>
<td></td>
<td>Adipose tissue</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>+</td>
<td>+</td>
<td>Hair follicles</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bladder</td>
<td>+</td>
<td>+</td>
<td>Sebaceous glands</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Skeletal System</td>
<td></td>
<td></td>
<td>Sweat glands</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cortical bone</td>
<td>+</td>
<td>-</td>
<td>Endothelial cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cancellous bone</td>
<td>-</td>
<td>+</td>
<td>Immune Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Macrophages</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymphocytes</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1.2.3 Estrogen Signalling

The biological effects of estrogen (17-β-estradiol) can be directed through a number of signal transduction pathways converging at the transcriptional level (as seen in Figure 1.7). In the classical, ligand-dependent pathway, estrogen diffuses into the cell where it binds and activates estrogen receptors. These are ligand-inducible transcription factors that, despite having similar structures and functionality, are transcribed from separate genes. Under certain conditions estrogen receptors can also be activated through phosphorylation (Kato et al., 1995) (Bhatt et al., 2012). Activated receptors can then dimerise and translocate to the nucleus. Here the dimer can bind directly to estrogen response element (ERE) sequences of target genes or indirectly through protein to protein interactions with AP1, Sp1 or other transcription factors (O’Lone et al., 2004). This results in the recruitment of coregulatory proteins to the estrogen-responsive gene promoter site and the promotion (or repression) of gene transcription (Klinge, 2000). Estrogen can also signal through a rapid non-genomic pathway through the interaction with membrane receptors (Simoncini et al., 2003).
Different cells and tissues can respond differently to the same signalling hormone via preferential use of these different pathways and the cellular co-factor profile. ER behaviour depends on the formation of homo- or hetero- dimers, which have differing levels of inhibition or promotion on target gene transcription. The ratio of ERα and ERβ within a certain cell influences the dimer composition and therefore plays a role in transcription of target genes. ERβ is the dominant partner in heterodimeric formation, i.e. hetero-dimers will form preferentially over homo-dimers. ERβ represses ERα transcriptional activity and is thought to modulate ERα mediated gene transcription and prevent excessive action (Pettersson and Gustafsson, 2001). Another layer of complexity is added by the existence of ER splice variants which can form preferential dimers with wild type receptors, influencing transcription. Furthermore, the transcriptional activities of the receptors are influenced by their interactions with transcriptional co-factors. These co-factors are expressed to varying degrees in different cells and have differing functional activities within these cells, leading to diverse transcriptional outcomes. Lastly, there are a number of molecules and other receptors, such as human progesterone A (hPRA) and nuclear receptor co-repressor (NCoR), that can negatively regulate ERα- and ERβ-mediated transcriptional activity (McDonnell and Norris, 2002).

**Figure 1.7 – Schematic representation of estrogen signalling pathways.** Estrogen can signal via multiple pathways including (1.) ligand dependent through estradiol (E2) activation of nuclear estrogen receptors (ER) which bind to estrogen response elements (ERE) and recruit coactivators (CoA) to bring about gene expression, (2.) ligand independent signalling through growth factor (GF) signalling and activation of kinase cascades which can activate receptors, (3.) ERE independent signalling where activated ER’s tether to transcription factors (TF) to bring about gene expression in the absence of an ERE and (4.) non-genomic signalling whereby membrane-bound estrogen receptors can cause rapid non-genomic effects, or converge with kinase pathways to activate transcription factors. E2= estradiol, P= phosphorylation, black arrow represents gene expression.
1.2.3.1 Ligand-dependent

The classical understanding of estrogen signalling is that estrogen interacts with an estrogen receptor, which dimerises and binds to an ERE and transcribes the target gene. In the absence of ligand, estrogen receptors are dormant, bound in a complex with inhibitory proteins such as Hsp90 in the cytoplasm or nucleus. Ligand binds to the groove in the LBD region causing a conformational change. Helix 12 (H12), which is part of the AF-2 element, moves to cover the pocket and sequester the ligand in the binding groove. Depending on the ligand that binds, H12 can move into either an agonist or antagonist position (Nilsson and Gustafsson, 2000). The ER can then form a hetero- or homo-dimer and bind to DNA via the zinc fingers of the DBD. This dimer has a high affinity for estrogen response elements, which are cis-acting enhancers conforming to the general palindromic sequence 5′-GGTCAnnnTGACC-3 (where n is any nucleotide), found upstream in the promoter regions of estrogen target genes. Ligand dependent transcription is facilitated by AF-1 in the A/B domain and the ligand dependent AF-2 in the LBD. Transcriptional activity is dependent on the co-activators or co-repressors that the activation functions recruit. The steric nature of H12 is essential for activity in this pathway and interacts with the SRC family of co-activators (Heery et al., 1997). When ligand binds, the conformational change creates a hydrophobic groove formed by residues of H3, H4, H5 and H12, which acts as a binding site for nuclear co-activator or inhibitory molecules (Ruff et al., 2000). The AF-1 is not dependent on ligand, but will also recruit co-activators and transcription factors, both similar and unique from those recruited by AF-2. The AF-1 of ERα and ERβ are highly disparate and as such ERβ AF-1 shows limited function. The ER dimer, when located at the ERE, is thought to interact with the transcriptional machinery through the co-activators recruited by the activation functions. These co-activators include SRC-1/NCOA1, TIF2/GRIP1, CBP/p300, TRAP220, PGC-1, p68 RNA helicase AIB1, and SRA (Klinge, 2000). The co-activators facilitate ER activity by disrupting the chromatin structure and forming a transcriptional pre-initiation complex. Co-activators, such as CBP/p300 have histone acetyltransferase activity which allows them to reorganise the chromatin structure (Rosenfeld and Glass, 2001). The cofactors are also thought to be responsible for the recruitment of RNA polymerase II to enable transcription of the downstream target gene. Depending on the bound ligand (and the specificity of co-factors that it creates) and the nuclear co-factors available in the cell, the DNA bound ER can exert either a positive or negative effect on target genes. A recent study indicated that wound healing is delayed in the absence of ligand, indicating that it is this pathway that is relevant to cutaneous wound healing (Emmerson et al., 2009).
1.2.3.2 Ligand-independent

The ligand independent pathway involves the cross-talk of estrogen receptors with other signalling pathways. The ability of estrogen dependent genes to be upregulated in the absence of ligand suggests that another signalling pathway can cause ER specific action (reviewed (Marino et al., 2006)). Polypeptide growth factors, such as epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), have been shown to stimulate ER transcriptional activity in the absence of estrogen ligand (Smith, 1998). This interaction has been further confirmed by the use of antibodies to growth factors which causes an inhibition of estrogen induced activity. This transcriptional activity requires the function of the AF-1. The EGF receptor is membrane bound and its activation leads to multiple downstream actions, such as activation of phospholipase C (PLC), phosphatidylinositol 3-kinases (PI3Ks) and the mitogen-activated protein kinase (MAPK) signalling cascade (Kato et al., 1995). It is thought that the phosphorylation of a serine residue at the AF-1 (ser118) by the MAPK signalling cascade can activate ER’s and facilitate interaction with SRC-1 and other co-activators (Kato et al., 1995). This enables interaction with estrogen target genes and the recruitment of transcription machinery.

1.2.3.3 ERE-independent

Estrogen can also have effects on the transcription of genes which do not have an ERE. This involves the translocation of estrogen activated ER dimers to the nucleus, interaction with transcription factors at alternative response elements and the up-regulation of transcription of alternative genes (Hall et al., 2001). ERE-independent transcriptional activity has been identified in the expression of collagenase and IGF-1 genes, through the Fos/Jun family of transcription factors, at the AP-1 binding site (Umayahara et al., 1994). Studies have shown that ER activation of AP-1 response elements requires the action of both the AF-1 and AF-2 regions (Webb et al., 1999). These factors enhance the activity of the cofactors recruited by the Fos/Jun transcription factors, enhancing the expression of the downstream gene. ERβ lacks a functional AF-1 region and accordingly has been shown to be unable to activate transcription of AP-1 regulated genes (Paech et al., 1997). This differential activation between ER isoforms gives rise to the possibility of distinct physiological actions of the two receptors through different gene subsets and signalling pathways.
1.2.3.4 Non-genomic

It has long been observed that estrogen stimulation can induce rapid cellular responses, such as intracellular calcium release, that occur in acute timeframes which cannot be accounted for by the classical genomic pathway (Morley et al., 1992). Furthermore, it has been illustrated that endothelial cells (amongst other cell types) contain plasma membrane binding sites for estrogens, of which presence correlates with rapid non-genomic estrogen signalling (Russell et al., 2000). Membrane associated estrogen receptors have also been identified in mice, but double knockout mice (ERα−/−/ERβ−/−) do not present with membrane associated estrogen receptors (Razandi et al., 2004). This indicates that they are formed from the same genes as the nuclear estrogen receptors, giving rise to the possibility that they are splice variants. The hERα isoforms ER66 and ER46 have been put forward as possible candidates for these membrane receptors (Li et al., 2003). Estrogen was shown to stimulate the acute production of nitric oxide in endothelial cells by activating nitric oxide synthase (eNOS) (Chen et al., 1999). This process was repressed when tyrosine kinases or mitogen activated protein (MAP) kinases were inhibited. This leads to the impression that the rapid production of nitric oxide is mediated by the non-genomic action of estrogen receptors through MAPK pathways. These kinase pathways create second messengers that can phosphorylate genome bound transcription factors, enhancing transcriptional activity, affect ion channels and activate enzymes that influence cell cycle and cell growth.

Another receptor has also been put forth as a potential participant in the rapid cellular effects of estrogen. GPR30 is a member of the seven-transmembrane G protein coupled receptor (GPCR) family that has been shown to be activated by estrogen (Revankar et al., 2005). This receptor, which is found at the endoplasmic reticulum and the plasma membrane (Thomas et al., 2005), can mediate both the rapid non-genomic and the genomic transcriptional effects of estrogen (Prossnitz et al., 2008). The rapid estrogen related effects of GPR30 are achieved through the activation of heterotrimeric G proteins, which activate adenyl cyclase, Src, phospholipase C and sphingosine kinase (SphK). These create second messengers such as cAMP, inositol triphosphate and calcium. Downstream targets of these pathways are matrix metalloproteinases, which release heparin-binding epidermal growth factors and allow for EGFR activation (Filardo and Thomas, 2005). EGFR activation can initiate rapid cellular responses through kinase pathways, but can also have transcriptional effects through the phosphorylation of ERs (the ligand-independent estrogen signalling pathway) (Kato et al., 1995) (Bhatt et al., 2012).
1.2.3.5 Estrogen Receptor Agonists and SERMs

ERs can also bind ligands other than estrogen, such as the structurally similar naturally occurring phytoestrogen genistein (Wang et al., 1996). ERα and ERβ selective agonists have been used to analyse the distinctive roles of estrogen receptors in different processes. Propylpyrazole-triol (PPT) is a selective agonist which has a 410-fold selectivity for ERα over ERβ (Stauffer et al., 2000). Diarylproponitrile (DPN) is an ERβ-selective agonist demonstrating a 70-fold specificity over ERα (Meyers et al., 2001). Selective estrogen receptor molecules (SERMs) are ER interacting molecules which can function as agonists or antagonists depending on the ER subtype, cell and tissue in which they operate (McDonnell, 1999). SERMs were developed to promote the beneficial effects of estrogen signalling in some tissues, whilst reducing estrogen-associated disease risk. Tamoxifen and raloxifene are two common SERMs. They both function as antagonists in breast tissue, but tamoxifen acts as an agonist in the uterus, bone and cardiovascular system, whilst raloxifene acts as a antagonist in the uterus and an agonist in bone (Nott et al., 2008). Estrogen binding molecules have structural differences and it is these differences, and the interaction with the ligand binding pocket of ERs, that influences their agonistic quality. The ligand binding pockets of the two ERs have differences in amino acid sequence, which appear to make contributions to the positioning of ligand. It appears that the ligand structure and its interaction with the secondary structure of the ligand-binding pocket are important in ligand affinity and ER transcriptional output (Nettles et al., 2007).

A recent study identified ERβ as the conduit for the beneficial effects of estrogen signalling during wound healing (Campbell et al., 2010). Due to the detrimental side-effects of estrogen treatment (i.e. cancer risk), any therapeutic wound treatment would need to be selective for ERβ in the skin, with SERMs being the likely option. There is interest in the development of new SERMs and development of new SERMs for different diseases is a very active area of research. Recently, 3rd and 4th generation SERMs have been developed with enhanced efficacy, specificity and anti-neoplastic action. These provide the potential to treat menopause-related symptoms in a more effective way. Two such SERMs are bazedoxifene and arzoxifene (Zhao et al., 2005).
1.3. Estrogen and Wound Healing

1.3.1. Estrogen Effects on The Skin

It is now widely accepted that the reduction in estrogen that accompanies ageing is a causative factor in poor wound healing and skin degeneration. Estrogen deficient post-menopausal women complain of dry, fragile and flaky skin that bruises easily, with reduced thickness and decreased collagen content (Brincat et al., 1985) (Brincat et al., 1987). Estrogen has been found to reverse this, improving collagen content and quality, increasing skin thickness and enhancing vascularisation (Brincat, 2000). Systemic estrogen treatment shows an increase in collagen deposition in post-menopausal women (Ashcroft et al., 1997a), whilst topical treatment increases collagen I and III within the dermis of postmenopausal women, preserving skin thickness (Varila et al., 1995). Furthermore, topical estrogen application can increase the quantity and quality of elastin fibres in the dermis (Punnonen et al., 1987). Other effects include increased deposition of glycosaminoglycans in the ECM, enhancing skin turgor and water retention accompanied by stimulation of keratinocytes and inhibition of Matrix metalloproteinases (MMP) production to reverse skin atrophy (Brincat, 2000).

1.3.2 Estrogen Effects on Wound Healing

With increasing age the rate of cutaneous wound healing decreases (Ashcroft et al., 1997b), being particularly pronounced in estrogen-deficient post-menopausal women. There is a strong indication that there is a direct link between estrogen signalling and wound healing in humans. In vivo and in vitro studies have shown that the rate of cutaneous wound healing in elderly humans can be accelerated by systemic (Ashcroft et al., 1997a) and topical (Ashcroft et al., 1999) estrogen treatments. Micro-array investigations suggest that estrogen may achieve this by modulating the inflammatory response, cytokine expression, matrix deposition, accelerating re-epithelisation, stimulating angiogenesis and wound contraction, and regulating proteolysis (Hardman and Ashcroft, 2008) (Ashcroft and Ashworth, 2003).

Estrogen supplementation, in the form of Hormone Replacement Therapy (HRT) has also been seen to offer protection from the development of chronic wounds (Margolis et al., 2002), with ERβ single nucleotide polymorphisms (SNPs) associated with a predisposition to venous ulcers (Ashworth et al., 2008) (Ashworth et al., 2005). Further evidence that estrogen signalling is important in wound healings in human has been demonstrated by the association of dehydroepiandrosterone (DHEA), a ubiquitous adrenal hormone which can be converted downstream steroid hormones and lead to estrogen signalling, with protection against venous
ulceration in humans (Mills et al., 2005) (Margolis et al., 2002). Patients with venous ulcers presented with significantly decreased serum DHEA levels compared to healthy control patients. This was examined in an impaired healing mouse model (hypogonadal) which showed accelerated healing when treated with DHEA, mediated by the local conversion of DHEA to estrogen and by dampening inflammation (Mills et al., 2005). This study links association findings in humans to functional characterisation of estrogen signalling in the wound environment in mouse models.

1.3.2.1 Estrogen Effects on the Inflammatory Phase

It is widely recognised that age-related impaired cutaneous wound healing is related to an excessive and prolonged inflammatory response, that is associated with cell adhesion and migration and is affected by the increase in pro-inflammatory cytokines (e.g. TNFα) (Ashcroft and Ashworth, 2003). Chronic wounds have been found to contain increased levels of elastase and MMPs, which are secreted by neutrophils and are associated with excessive tissue damage (Wysocki et al., 1993), (Herrick et al., 1997). Estrogen regulates and dampens the inflammatory response by inhibiting neutrophil extravasation and chemotaxis to the wound site through alterations of the expression of neutrophil adhesion molecules (Ashcroft et al., 1999). In reducing the infiltration of neutrophils, estrogen reduces the levels of tissue damaging proteases and increases collagen and fibronectin content. Estrogen is thought to increase the oxidative metabolism of neutrophils through myeloperoxidase and consequentially increase phagocytosis (Jansson, 1991). A deficiency in estrogen would therefore decrease the phagocytic ability of neutrophils and increase the risk of infection and a delay in healing (Ashcroft and Ashworth, 2003). Estrogen is also thought to have an effect on monocytes and macrophages, inferred by their possession of estrogen receptors (Gulshan et al., 1990). Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that has been demonstrated to be a master regulator of the effects of estrogen on wound repair (Hardman et al., 2005), (Emmerson et al., 2009). It is produced by monocytes, macrophages, neutrophils, keratinocytes and endothelial cells (Baumann et al., 2003), (Calandra and Roger, 2003). Estrogen deficient mice show increased MIF combined with enhanced inflammation and a delay in healing (Ashcroft et al., 2003), whilst MIF null-mice show accelerated wound healing, with reduced inflammation and enhanced matrix formation (Ashcroft et al., 2003), (Emmerson et al., 2009). Estrogen down-regulates MIF expression which leads to a reduction in inflammation, enhanced matrix deposition, increased reepithelialisation and an overall accelerated wound repair (Ashcroft et al., 2003).
1.3.2.2 Estrogen Effects on the Proliferation Phase

Estrogen has a positive effect on the proliferative phase by improving re-epithelisation, wound contraction, granulation tissue formation and angiogenesis. Estrogen has a mitogenic effect on keratinocytes and has been shown to increase the rate of re-epithelialisation and wound contraction in post-menopausal women (Ashcroft et al., 1997a). The level of re-epithelialisation of wounds of post-menopausal females who had never taken hormone replacement therapy (HRT) was compared to post-menopausal women who had taken HRT for longer than 3 months. The non-HRT group showed stunted reepithelialisation, whilst the HRT group showed similar levels to premenopausal females. One facet of this increased reepithelialisation is thought to be accounted for by the increase of epidermal keratinocyte proliferation, which has been demonstrated to act through the Erk/Akt signalling pathway (Zhou et al., 2016). Estrogen can modulate the expression of PDGF by monocytes/macrophages (Shanker et al., 1995) which is mitogenic and chemotactic to fibroblasts (Seppa et al., 1982). By stimulating fibroblasts and myofibroblasts wound contraction can occur and lead to an increased rate of healing. It has been demonstrated that estrogen can accelerate murine fibroblast migration, which is beneficial in that it increases recruitment to the wound site (Emmerson et al., 2009). Estrogen has also been shown to promote fibrosis and angiogenesis (Roberts et al., 1986). Estrogen causes an increase in TGF-β1 in the wound site by stimulating expression through fibroblasts (Ashcroft et al., 1999), which promotes collagen deposition, the formation of ECM and stimulates granulation tissue formation (Ashcroft and Ashworth, 2003). There is evidence to suggest that estrogen promotes angiogenesis by directly stimulating endothelial cells. Estrogen was demonstrated to increase the attachment of endothelial cells to laminin, collagen I and IV, and fibronectin (Morales et al., 1995). Furthermore, it appears to influence angiogenesis by improving the formation of capillary-like structures by endothelial cells, when placed on reconstituted basement membrane (Morales et al., 1995).

1.3.2.3 Estrogen Effects on the Remodelling Phase

Estrogen appears to influence wound remodelling by modulating collagen degradation through wound proteases. Topical estrogen treatment increases collagen deposition in wound tissue at 7 and 10 days post-wounding in elderly individuals (Ashcroft et al., 1999). Estrogen can also act through TGF-β1, which it stimulates expression of in fibroblasts, to increase collagen deposition. Matrix collagen deposition at 7 and 84 days post-wounding was reduced in post-menopausal females who had never had HRT. Conversely, post-menopause females who had received HRT for longer than 3 months showed similar levels of matrix collagen deposition as healthy pre-menopause females (Ashcroft et al., 1997a).
Figure 1.8 – The effects of estrogen on wound healing. Estrogen has been suggested to accelerate healing by promoting the migration and proliferation of keratinocytes and fibroblasts (both directly and indirectly through increasing growth factors such as macrophage derived PDGF), dampen the inflammatory response (reducing pro-inflammatory cytokines such as TNFα, MIF and IL-6) and to increase angiogenesis (both directly and again through PDGF). Adapted from (Emmerson and Hardman, 2012).

1.3.2.4 Estrogen Receptors and Wound Healing

A recent study by Campbell et al. has demonstrated different roles for the ERα and ERβ during acute cutaneous wound repair in female murine models (Campbell et al., 2010). Ovariectomized (Ovx) (estrogen deficient) mice were treated with 17β-estradiol, PPT (ERα agonist) or DPN (ERβ specific agonist). Wounds of mice treated with DPN presented with promoted reepithelialisation and reduced wound area, comparable to that seen with 17β-estradiol treatment. In contrast, PPT treatment (i.e. with ERα signalling alone), showed no effect on healing, with wound closure significantly delayed compared to 17β-estradiol. This suggests that the positive effects of estrogen on wound healing are directed through ERβ and not ERα. It was noted that both specific agonists were equally anti-inflammatory, suggesting that whilst ERβ is the main instigator of estrogen-related wound repair, this is unlikely to be in an exclusively anti-inflammatory manner. Epidermal-specific null mice also showed ERβ to promote reepithelialisation rather than ERα and that the signalling of estrogen through ERα in the absence of ERβ delayed healing and showed pro-inflammatory activity. These findings suggest that ERβ might inhibit ERα, as suggested previously (Pettersson and Gustafsson, 2001), or that hetero-dimer formation may play a physiological role.
Insulin-like growth factor-1 (IGF-1) is involved in wound healing and has signalling links between IGF-1 receptor (IGF-1R) and ER’s (Emmerson et al., 2012). It was been shown that local IGF-1 treatment in Ovx mice promotes wound healing by promoting reepithelialisation and dampening inflammation. It was found that IGF-1-mediated effects on reepithelialisation are directly mediated by IGF-1R, but that the anti-inflammatory effects of IGF-1 are predominantly via the ERs, in particular ERα, with the ERα-null mouse failing to promote healing and presenting with excess inflammation. This highlights the complex interactions between IGF-1 and estrogen in the skin and suggests that it may compensate for estrogen deficiency during wound healing. This study also identifies an important role for ERα in wound healing, although global null studies suggest that ERβ is the predominant receptor during wound healing.

The differing actions of the two ER receptors during wound healing has been further investigated using an ERE-luciferase transgenic mouse model, which can assess in real-time in vivo, the level of estrogen receptor activation (Emmerson et al., 2013). This study identified the activation of estrogen signalling in female mice localised to keratinocytes of the neoepidermis and wound margin dermal cells. Receptor specific agonist treatment revealed ERβ induces ERE-mediated signal in epidermal and dermal cells while ERα only induces ERE-mediated signal in dermal cells. This further highlights the differential signalling of the two ER’s during wound healing and strongly suggests that ERβ is important in keratinocytes and fibroblasts during healing, whilst ERα activity may be limited to dermal inflammatory cells.

1.3.2.5 Estrogen and Chronic Wounds

Considering that the systemic level of estrogen decreases with age, particularly in the post-menopause female, it has been proposed that loss of estrogen may be a causative factor in pathological wound healing. Indeed a recent case-cohort study demonstrated the protective effects of estrogen. Patients of 65 years and over who received HRT, were 30-40% less likely to develop a venous leg ulcer than those who did not use HRT (Margolis et al., 2002). Furthermore, association studies have highlighted polymorphisms of the estrogen receptor beta gene to be implicated in a predisposition to the formation of venous ulcers (Ashworth et al., 2005; Ashworth et al., 2008). Importantly, it has been suggested that ERβ expression in the keratinocytes and fibroblasts of human chronic wound margin is reduced, whilst ERα expression is unchanged (Strudwick, 2006). Chronic wounds are characterised by an excessive and prolonged inflammatory state. Chronic wound exudate is high in inflammatory factors such as IL-1β, IL-6, TNFα, TGFβ, IGF-1 and MMP’s (Tarnuzzer and Schultz, 1996), which can be downregulated through the action of estrogen (Ito et al., 2001; Schaefer et al., 2005; Son et al., 2005). Pro-healing factors such as EGF
can be degraded by the action of the high levels of proteases, further reducing the ability of the wound to heal (Tarnuzzer and Schultz, 1996).

1.4 Single Nucleotide Polymorphisms (SNPs)

Polymorphisms are natural DNA sequence variants, or alleles, which are largely defined to be occurring in greater than 1% of the population (Chakravarti, 1999). Polymorphisms can be in the form of microsatellite repeats, which are regions of the genome where short nucleotide sequences are repeated in tandem arrays, or the more common single nucleotide polymorphisms (SNPs). A SNP is a single base pair change in a DNA sequence, which results from errors in DNA replication or repair. Frequency of SNPs varies widely between genomic regions and between coding and non-coding sequences. Frequency appears higher in non-coding regions because mutations in coding regions are more likely to be lethal and therefore fail to propagate in the gene pool. When comparing two chromosomes, nucleotide diversity can range from 3 to 50 SNPs per 10 kb of sequence (Chakravarti, 1999). The majority of SNPs are functionally insignificant, but a proportion can account for phenotypes including disease susceptibility, disease resistance and pharmacological response (Collins et al., 1999), (Kleyn and Vesell, 1998). SNPs can be used as genetic markers in identifying disease genes, providing opportunity for the early intervention and treatment of patients. An important focus of research is to identify SNPs that have direct functional effects on a pathology and demonstrating definite functional effects accountable to a single nucleotide variant would be vital if any prognostic value were to be derived (Figtree et al., 2009).

1.4.1 ERβ Polymorphisms and Disease Association Studies

1.4.1.1 ERB Polymorphisms and Chronic Wounds - Case-control Association Study

Although ER’s have been studied in the context of wounds in mice, there has been limited research into human ER’s and the role they have in the development of chronic wounds. Although many studies have found ERβ SNPs to be associated with various forms of cancer and multiple diseases, there has been limited investigation to this in the field of wound healing. Two previous studies from our lab by Ashworth et al were relevant to the topic of estrogen signalling and the development of chronic wounds (Ashworth et al., 2005; Ashworth et al., 2008). In these case-control association studies, it was shown that particular estrogen ERβ variants predispose individuals to the development of chronic wounds. In the first of these studies it was shown that
whilst no ERα alleles were significantly associated with venous ulceration, the CA*18 repeat allele of ERβ was significantly associated with venous ulceration \((n = 120, OR= 1.8, 95\% CI = 1.1–2.8, P = 0.02)\) (Ashworth et al., 2005). This indicated that specific variations in the ERβ gene (ESR2) could be associated with incidence of chronic wounds, whilst ERα gene (ESR1) seems unlikely to have any association.

In the second study, the area of association was narrowed down to a region spanning the ON promoter and exon in the 5'UTR of the ESR2 (Ashworth et al., 2008). Once again, this investigation used a case-control association design comparing subjects with active venous ulcers to a healthy control group. This study focussed on single nucleotide polymorphism (SNPs), which are defined as a base variation in a single DNA position amongst ≥1% of the population. This study found four SNPs in close proximity to upstream regulatory regions of the ERβ gene, including the ON exon and promoter (Figure 1.6a). The SNPs rs2978381, rs2987983, rs1887994 and rs3783736 were all seen to be significantly associated with a predisposition to the formation of venous ulcers. Furthermore, a major susceptibility haplotype made up of these four SNPs, carried by a significantly larger proportion of ulcer patients compared to control group, was significantly associated with elevated serum levels of tumour necrosis factor-alpha (TNF-α). Chronic wounds, including venous ulcers, are thought to be caused by a prolonged or inflated inflammatory response, which would be reflected in an elevated level of cytokines (including TNF-α) in the blood. TNF-α is widely expressed in granulocytes, macrophages, fibroblasts, and epithelial cells (Spriggs et al., 1992) and so it is likely that these cell types would be affected by alteration in ER signalling.

Venous ulcers are an example of a complex genetic disease influenced by a consortium of factors (both genetic and environmental) that can, in the correct combination, cause disease. One factor in isolation is insufficient, but can contribute to increased risk of a particular disease developing. Whilst ERβ gene polymorphisms are unlikely to be the main cause of a venous ulcer, they may have a functional affect that combines with other factors and tips the scales in the direction of disease formation.
Table 1.2 - Disease associated estrogen receptor beta single nucleotide polymorphisms

<table>
<thead>
<tr>
<th>dbSNP (rs)</th>
<th>Nucleotide Change</th>
<th>Chromosomal Position</th>
<th>Position relative to translational start site</th>
<th>Pathological association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1256120</td>
<td>G/T</td>
<td>64805001</td>
<td>-55297</td>
<td>Idiopathic scoliosis</td>
<td>(Zhang et al., 2009)</td>
</tr>
<tr>
<td>3020444</td>
<td>T/C</td>
<td>64791013</td>
<td>-41309</td>
<td>Bone mineral density</td>
<td>(Ichikawa et al., 2005)</td>
</tr>
<tr>
<td>2978381</td>
<td>T/C</td>
<td>64766652</td>
<td>-16948</td>
<td>Venous ulceration</td>
<td>(Ashworth et al., 2008)</td>
</tr>
<tr>
<td>2978381</td>
<td>C/T</td>
<td>64766652</td>
<td>-16948</td>
<td>Testicular germ cell tumour</td>
<td>(Kristiansen et al., 2012)</td>
</tr>
<tr>
<td>2987983</td>
<td>T/C</td>
<td>64763653</td>
<td>-13949</td>
<td>Prostate cancer</td>
<td>(Thellenberg-Karlsson et al., 2006)</td>
</tr>
<tr>
<td>2987983</td>
<td>A/G</td>
<td>64763653</td>
<td>-13949</td>
<td>Breast cancer</td>
<td>(Treek et al., 2009)</td>
</tr>
<tr>
<td>2987983</td>
<td>A/G</td>
<td>64763653</td>
<td>-13949</td>
<td>Venous ulceration</td>
<td>(Ashworth et al., 2008)</td>
</tr>
<tr>
<td>10483774</td>
<td>C/T</td>
<td>64763599</td>
<td>-13895</td>
<td>Hypospadias</td>
<td>(Beleza-Meireles et al., 2006)</td>
</tr>
<tr>
<td>3841304</td>
<td>_C</td>
<td>64761918</td>
<td>-12214</td>
<td>Prostate cancer</td>
<td>(Sun et al., 2005)</td>
</tr>
<tr>
<td>1271572</td>
<td>G/T</td>
<td>64761917</td>
<td>-12213</td>
<td>Ovarian cancer</td>
<td>(Lurie et al., 2009)</td>
</tr>
<tr>
<td>1271572</td>
<td>G/T</td>
<td>64761917</td>
<td>-12213</td>
<td>Cardiovascular disease</td>
<td>(Rexrode et al., 2007)</td>
</tr>
<tr>
<td>1271572</td>
<td>G/T</td>
<td>64761917</td>
<td>-12213</td>
<td>Epithelial ovarian carcinoma</td>
<td>(Lurie et al., 2009)</td>
</tr>
<tr>
<td>1271572</td>
<td>G/T</td>
<td>64761917</td>
<td>-12213</td>
<td>Prostate cancer</td>
<td>(Sun et al., 2005)</td>
</tr>
<tr>
<td>1271572</td>
<td>G/T</td>
<td>64761917</td>
<td>-12213</td>
<td>Myocardial infarction</td>
<td>(Domingues-Montanari et al., 2008)</td>
</tr>
<tr>
<td>8008187</td>
<td>C/G</td>
<td>64761805</td>
<td>-12101</td>
<td>Alters TF binding sites at promoter region</td>
<td>(Philips et al., 2012)</td>
</tr>
<tr>
<td>3829768</td>
<td>A/G</td>
<td>64761594</td>
<td>-11890</td>
<td>Alters TF binding sites at promoter region</td>
<td>(Philips et al., 2012)</td>
</tr>
<tr>
<td>35036378</td>
<td>T/G</td>
<td>64761121</td>
<td>-11417</td>
<td>Alters TF binding sites at promoter region</td>
<td>(Philips et al., 2012)</td>
</tr>
<tr>
<td>3832949</td>
<td>_C</td>
<td>64760813</td>
<td>-11109</td>
<td>Hypospadias</td>
<td>(Beleza-Meireles et al., 2006)</td>
</tr>
<tr>
<td>1887994</td>
<td>G/T</td>
<td>64760611</td>
<td>-10907</td>
<td>Venous ulceration</td>
<td>(Ashworth et al., 2008)</td>
</tr>
<tr>
<td>1887994</td>
<td>G/T</td>
<td>64760611</td>
<td>-10907</td>
<td>Hypospadias</td>
<td>(Beleza-Meireles et al., 2006)</td>
</tr>
<tr>
<td>7159462</td>
<td>G/A</td>
<td>64758876</td>
<td>-9172</td>
<td>Obesity</td>
<td>(Nilsson et al., 2007)</td>
</tr>
<tr>
<td>3783736</td>
<td>C/A</td>
<td>64751372</td>
<td>-1668</td>
<td>Venous ulceration</td>
<td>(Ashworth et al., 2008)</td>
</tr>
<tr>
<td>1256031</td>
<td>C/T</td>
<td>64746179</td>
<td>3525</td>
<td>Ventricular mass</td>
<td>(Peter et al., 2005)</td>
</tr>
<tr>
<td>1256031</td>
<td>C/T</td>
<td>64746179</td>
<td>3525</td>
<td>Bone mineral density</td>
<td>(Shearman et al., 2004)</td>
</tr>
<tr>
<td>1271573</td>
<td>A/G</td>
<td>64737471</td>
<td>12233</td>
<td>Alzheimer’s disease</td>
<td>(Pirskanen et al., 2005)</td>
</tr>
<tr>
<td>1256043</td>
<td>A/G</td>
<td>64734282</td>
<td>15422</td>
<td>Alzheimer’s disease</td>
<td>(Pirskanen et al., 2005)</td>
</tr>
<tr>
<td>1256049</td>
<td>C/T</td>
<td>64724051</td>
<td>25653</td>
<td>Late-life depression</td>
<td>(Ryan et al., 2011)</td>
</tr>
<tr>
<td>12435857</td>
<td>A/G</td>
<td>64723525</td>
<td>26179</td>
<td>Testicular germ cell tumour</td>
<td>(Kristiansen et al., 2012)</td>
</tr>
<tr>
<td>1256059</td>
<td>C/T</td>
<td>64710417</td>
<td>39287</td>
<td>Ventricular mass</td>
<td>(Peter et al., 2005)</td>
</tr>
<tr>
<td>1256059</td>
<td>C/T</td>
<td>64710417</td>
<td>39287</td>
<td>Alzheimer’s disease</td>
<td>(Pirskanen et al., 2005)</td>
</tr>
<tr>
<td>1256059</td>
<td>C/T</td>
<td>64710417</td>
<td>39287</td>
<td>Bone mineral density</td>
<td>(Shearman et al., 2004)</td>
</tr>
<tr>
<td>944050</td>
<td>A/G</td>
<td>64700045</td>
<td>49659</td>
<td>Endometrial cancer</td>
<td>(Ashton et al., 2009b)</td>
</tr>
<tr>
<td>944050</td>
<td>A/G</td>
<td>64700045</td>
<td>49659</td>
<td>Vascular dementia</td>
<td>(Xin et al., 2012)</td>
</tr>
<tr>
<td>4986938</td>
<td>A/G</td>
<td>64699816</td>
<td>49888</td>
<td>Breast cancer</td>
<td>(Yu et al., 2011)</td>
</tr>
<tr>
<td>4986938</td>
<td>A/G</td>
<td>64699816</td>
<td>49888</td>
<td>Vascular dementia</td>
<td>(Dresner-Pollak et al., 2009)</td>
</tr>
<tr>
<td>4986938</td>
<td>A/G</td>
<td>64699816</td>
<td>49888</td>
<td>Endometriosis</td>
<td>(Wang et al., 2004b)</td>
</tr>
<tr>
<td>4986938</td>
<td>A/G</td>
<td>64699816</td>
<td>49888</td>
<td>Premature coronary artery disease</td>
<td>(Mansur Ade et al., 2005)</td>
</tr>
<tr>
<td>928554</td>
<td>A/G</td>
<td>64694195</td>
<td>55509</td>
<td>Preeclampsia</td>
<td>(Maruyama et al., 2004)</td>
</tr>
<tr>
<td>1255998</td>
<td>C/G</td>
<td>64693871</td>
<td>55833</td>
<td>Endometrial cancer</td>
<td>(Ashton et al., 2009a)</td>
</tr>
</tbody>
</table>
1.4.1.2 Possible Effects of Polymorphisms/ SNP function in ERβ expression and Chronic wound formation

Mutations to the 5'UTR can affect gene expression at a number of levels in a number of different ways. At the transcriptional level it can influence transcription factor binding, miRNA binding, enhancer or silencer sites, epigenetic modification, splice site or promoter utilisation. At the translational level it can influence secondary mRNA structures, such as upstream open reading frames (uORF's), internal ribosome entry sites (IRES), iron responsive element (IRE) and the Kozak consensus sequence, which can effect mRNA stability, localisation and translational rate (Chatterjee, 2010). A large-scale study found that expression was altered to a functionally relevant extent in over a third of genes identified to have SNPs of the promoter region (Hoogendoorn et al., 2003).

The complex system of multiple untranslated exons and distinct promoters allows highly organised ER gene regulation, providing tailored responses in specific tissues and physiological processes (Alcazar et al., 2010). The 5'UTR exons of ESR2 are transcribed to form part of the mRNA and are thought to contain regulatory elements that dictate translation. Considering this, functional polymorphisms in the ERβ ON promoter region may influence downstream estrogen responsive genes by altering ERβ transcription levels, isoform variation or translation in inflammatory and wound related cells.

More than 1300 SNPs within the human ERβ gene have been listed by the National Centre for Biotechnology Information Database (http://www.ncbi.nlm.nih.gov/projects/SNP), most of which are found in non-coding regions. These polymorphisms can contribute to disease by influencing the expression or processing of the ERβ transcript, altering estrogen signalling (Nott et al., 2008). ERβ plays a dominant regulatory role in inflammatory and repair processes by modulating the immune system and has been implicated in several diseases of this nature, such as chronic inflammatory bowel disease and arthritis (Harris et al., 2003). In disease association studies the genetic variations of individuals with a disease are examined and compared to a control group without the disease, to establish whether any particular variant has significant association to disease state. An overview of ERβ associated disease studies can be seen in Table 1.2, whilst gene SNP locations can be seen in Figure 1.6b. The majority of these SNPs cluster across the ON promoter region, where the venous ulcer associated SNPs are also located. This indicates that this is an important regulatory feature of the gene and that dysfunction in this region is likely to impact upon downstream estrogen signalling. It has been shown that peripheral leukocytes, involved in the inflammatory response in chronic wounds, use the ON promoter exclusively to express ERβ (Hirata et al., 2001). Thus, changes caused by a particular SNP variant in this region could bring about functional changes in ERβ expression or action to alter function in these cell types. Equally the ON promoter may be important in a range of additional cell types leading to
functional changes in wound related cells such as fibroblasts, keratinocytes, macrophages and neutrophils.

A decrease in ERβ would change the dimer ratio and result in a change in target gene expression level. The ratio of ERβ to ERα would be important if a therapeutic SERM was used to treat chronic wounds. If the ratio of ERβ to ERα was below the selective specificity of the SERM, the estrogen treatment would signal predominantly through ERα, which has been shown to be detrimental to the wound healing process (Campbell et al., 2010). A recent study has identified a change in promoter usage for the transcription of ERβ between pre- and post-menopausal females (Smith et al., 2010b). Both the 0K and 0N promoter regions were shown to be used equally in pre-menopausal women, but this shifted to almost exclusively 0N usage in post-menopausal females. This suggests that the 0N promoter may be particularly utilised in elderly or estrogen deficient individuals. The effect of a functional polymorphism at this site would therefore be exaggerated in these individuals. This lack of understanding provides a major opportunity to functionally explore and elucidate the effects of ERβ alteration and the downstream effects in regards to estrogen signalling and wound healing.

1.4.1.3 Transcriptional Effects of SNPs

Transcription factors bind to specific consensus sequences at promoter regions and bring about the formation of the pre-initiation complex, required for transcription. The 0N promoter region contains binding sites for transcription factors such as elements that form a TATA box and initiator element (Inr) sequence. Polymorphisms in the promoter region which change the nucleotide sequence at their location may alter transcription factor binding. Tissue- or cell-specific transcription factors bind to non-core promoter elements, which in ERβ include sites for GATA, SP-1, AP1 and Oct1 (Li et al., 2000). A recent study identified three SNPs within the 0N promoter region that were predicted to alter transcription factor binding, one of which appeared to reduce promoter luciferase reporter activity (Philips et al., 2012).

Other gene regulatory elements include enhancer and silencer elements. Enhancers are specific DNA motifs that can sequester transcription factors to upregulate the formation of pre-initiation complex to the promoter, increasing transcription. They can be found up to 100kb upstream or downstream from the promoter, with the looping of chromatin playing an important role in this interaction (Riethoven, 2010). The Alu repeat sequence, located between -1416 and -1703, is an example of an enhancer sequence found in the ESR2 gene (Li et al., 2000). A disruption of an enhancer sequence by a SNP could result in a loss of function and a subsequent reduction in transcription. Silencer elements downregulate gene expression by binding transcription factor repressor proteins or acting as position-dependent negative regulatory elements (Riethoven,
A change in nucleotide sequence could disrupt a silencer element or create a novel silencer site, affecting overall transcription of the gene.

Epigenetic changes brought about by ERβ polymorphisms could have a downstream influence on estrogen signalling. The ON promoter region is G-C rich which provides susceptibility to methylation (Nott et al., 2008). Hypermethylation has been implicated in altered gene expression and increased risk of disease. Methylation decreases gene transcription and although the exact mechanism through which methylation inhibits transcription is unknown, it is thought that the methylation blocks promoter sites from transcription binding (Phillips, 2008). The level of methylation of the ON promoter region has been suggested to be inversely associated with the expression of ERβ in breast tumours (Zhao et al., 2003). The SNP rs2987983 which formed part of the disease-associated haplotype in venous ulceration is also associated with an increased risk of prostate cancer (Ashworth et al., 2008), (Thellenberg-Karlsson et al., 2006). A further study investigating ERβ expression during prostate cancer development demonstrated that progressive methylation of the ON promoter region led to transcriptional inactivation of ERβ (Zhu et al., 2004). This raises the possibility that this SNP, or others in the region, may increase ON methylation and contribute to disease development in both prostate cancer and venous ulceration. Another study found that epigenetic changes marked by DNA methylation of the ERβ promoter played a role in cardiovascular atherosclerosis and vascular aging (Kim et al., 2007). Atherosclerotic tissue showed higher methylation than normal tissue and inhibition of DNA-methyltransferase significantly increased ERβ expression. This suggests a link between cardiovascular disease and a deficiency in estrogen signalling. Polymorphisms in the ERβ gene that increase methylation could cause further estrogen signalling deficiencies in the vascular endothelial cells, increasing the risk of complications such as venous insufficiency, leading to venous ulceration.

1.4.1.4 Post-transcriptional Effects of SNPs

The functional influence of a SNP could be at the mRNA processing stage and translational stage. The previously described transcriptional regulation factors results in the production of pre-mRNA which then undergoes multiple processing stages before they become functional mRNA. Introns are removed and modifications made such as the addition of a 7-methyl-guanylate cap at the 5’ end and a poly(A) tail at the 3’ end (Mignone et al., 2002). A unique 5’UTR containing the ON exon is found in ERβ mRNA that has been transcribed from that promoter site. Any change in nucleotide sequence would therefore also be found in the primary mRNA. This could influence RNA and processing events, such as alternative splicing, mRNA stability, cellular localisation and translational regulation.
The 5'UTR of this mature mRNA can mediate regulation in several manners. Motifs in this region can interact with RNA-binding proteins (RBPs) which are governed by both the primary and secondary structure of the mRNA molecule (Mignone et al., 2002). These RBPs can form the fundamental translational machinery needed for successful translation, such as polyadenylate binding protein I (PABPI), or they can be regulators of expression with either promotive or inhibitory characteristics, such as human antigen R (HuR) (Araujo et al., 2012). Thus, an alteration in the binding sequence by a SNP could completely ablate gene translation or could increase or decrease subsequent protein expression.

Untranslated open reading frames (uORFs) are major regulatory elements that can be found in 5'UTRs and are present in the ERβ gene. These are sequences that have a start and stop site upstream of the coding region. Translation will be initiated at an upstream AUG to form an open reading frame. After the translation of this upstream ORF and detachment of the 60s ribosomal subunit, the 40s ribosomal subunit has the option to abandon the mRNA or it may retain the mRNA and reinitiate translation at the main ORF (Mignone et al., 2002). This will impact upon mRNA translational efficiency and stability. It has been suggested that uORF's have a role to play in keeping basal translational gene level low (Rogozin et al., 2001). The regulation or stability of the mRNA could be affected by polymorphisms and there is evidence that the upstream regions of ER genes can regulate translation (Pentecost et al., 2005).

Interactions between UTR regions and RNA-binding proteins have also been shown to provide cellular localisation signals which interact with the intracellular transport mechanisms of RNA (Martin and Ephrussi, 2009). Whilst most regulatory factors mediating mRNA localisation are found in the 3'UTR region, there has been evidence that elements of the 5'UTR can play a role. mRNA localisation involves the interaction between ‘zipcode’ elements and their interacting zipcode proteins of the UTR and their interaction with the cytoskeleton (Chabanon et al., 2004). Correct regulation here is vital for the appropriate translation of the gene.

There is evidence that dysregulation can be caused by mutations that effect pre-mRNA splicing (Cartegni et al., 2002). If a SNP falls at a boundary between an intron and exon or at particular binding sequences, such as exonic splicing enhancers (ESE) where spliceosome proteins can bind, alternative splicing can occur (Krawczak et al., 1992). Numerous ERβ splice isoforms can be formed, mediated by spliceosome assembly at specific splice sites (Schaal and Maniatis, 1999). ERβ polymorphisms at exon/intron boundaries may alter a splice site, favouring the formation of a particular ERβ splice isoform, or introduce a new splice site creating a non-functional protein. Alternative isoforms could play a role in disease. For instance, ERβcx isoforms, which has no functional activity, preferentially binds ERα inhibiting its ability to transcribe target genes (Ogawa et al., 1998).
1.5 Hypothesis and Aims

Chronic wounds of the elderly are a major social and financial problem for health services and represent a major area of unmet clinical need. Current treatments are largely ineffective due to poor understanding of the underlying causes and mechanisms.

It has been demonstrated that elderly or estrogen deficient individuals respond to estrogen treatment with accelerated wound healing and that estrogen protects against the formation of chronic wounds (Margolis et al., 2002), (Ashcroft et al., 1999). ERβ polymorphisms are associated with a number of diseases, with many clustering around the ON promoter region, highlighting the importance of this region. Moreover, ERβ receptor SNPs are associated with predisposition to chronic ulceration, with the implicated haplotype traversing the ON promoter region (Ashworth et al., 2008). These same SNPs have also been implicated in other disease predispositions, hinting at a functional significance (Table 1.2). Clearly there is an association, but there is no current understanding of the functional significance of ERβ SNPs in the wound healing environment.

We hypothesise that specific disease-associated SNP’s in ESR2 alter ERβ expression and cell function, ultimately contributing to the development of chronic healing pathology. This study will investigate the molecular and cellular consequences of ERβ polymorphisms across the ON promoter region, exploring ER function during wound repair and linking this to chronic wound pathology.

Specific aims include:

1: To characterise primary human skin cells harbouring venous ulcer-associated SNPs in wound related models. Estrogen signalling stimulates wound healing, but SNPs of the ERβ gene are associated with delayed healing. A direct link has not been determined between these findings. Therefore work in this thesis will characterise the effect of these SNPs on skin cells in wound relevant processes.

2: To identify the consequences and causes of venous ulcer-associated SNPs, including direct effects upon ERβ expression. It remains unclear whether all or one of these SNPs do indeed cause a change in ERβ expression. The effect of each SNP on gene regulation and expression will hence be investigated.

3: To identify changes in wound dynamics, including inflammatory signalling, in SNP harbouring cells. A large factor contributing to deficient healing in chronic wounds is an excessive inflammatory response. Gene expression analysis will be used to identify differences in
inflammatory profiles between wild type skin cells and cells harbouring venous ulcer-associated ERβ SNPs and how these affect interactions with inflammatory cells.

4: To identify the downstream signalling consequences of venous ulcer-associated SNPs and how this might influence wound healing. A transcriptome-wide analysis using RNA-seq will be employed to determine the exact downstream effects on gene expression by these SNPs and whether this alters pathways necessary for wound healing.
2. Chapter 2 - Materials and Methods

2.1 Cell culture

2.1.1 Cell culture conditions

Primary human keratinocytes were expanded in EpiLife® medium (Thermo Fisher Scientific Cat. MEPI500CA). To remove the intrinsic estrogen signalling of the media, cells were switched to phenol red-free EpiLife® medium (Thermo Fisher Scientific Cat. MEPICFPRF500), typically 48 hours before experimentation. Both medium were supplemented with HKGS (Human Keratinocyte Growth Supplement) (Thermo Scientific Cat. S0015), 60 µM calcium chloride (Sigma-Aldrich Cat. C7902) and 1% penicillin/streptomycin.

Primary human fibroblasts were expanded in complete DMEM (Dulbecco’s modified Eagle’s medium) (HyClone™ Cat no. SH30081.01) supplemented with 10% charcoal-stripped foetal bovine serum (FBS) and 1% penicillin/streptomycin and cultured in phenol red-free DMEM (Gibco® Cat. 31053-028) 48 hours before experimentation.

All cells were cultured at 37°C with 5% CO₂.

2.1.2 Scratch-wound activation

To wound activate cells, keratinocytes or fibroblasts were grown to 2 days post-confluence in 6 well-plates in estrogen-free media. Cells were washed in warm PBS and 500µl media added to each well. The cell monolayers were then scratched in 7 evenly spaced straight lines using a sterile p200 pipette tip, followed by 7 evenly spaced straight lines perpendicular to the first set. The cells were then washed twice with PBS to remove debris before being replaced with 3ml of appropriate media.

2.1.3 Migration assay

Keratinocytes or fibroblasts of low passage number were seeded in 96-well IncuCyte™ ImageLock™ Plates (Essen Bioscience, Cat no. 4379) at 15000 cells per well. The ImageLock™ plate incorporates a grid to ensure that images are taken at precisely the same position at each time point. These cells were grown to 4 days post-confluent in 250µl of estrogen-free media as previously described. 24 hours before wounding, cells were switched to drugged media consisting of ethanol control (vehicle), 100nM 17-β-estradiol (Sigma-Aldrich Cat no. E2758), 100nM 2,3-Bis(4-hydroxyphenyl)propionitrile (DPN) (Sigma-Aldrich, Cat no. H5915) or 100nM 1,3,5-Tris(4-
hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) (Sigma-Aldrich, Cat no. H6036). Before scratching, cells were washed twice with sterile PBS and the wells filled with 80µl of PBS for scratching. Scratch wounds were made using the 96-well WoundMaker™ (Essen Bioscience, Cat no. 4493). The WoundMaker™ allows the simultaneous and accurate scratching of 800 micron wounds in all 96 wells. Cells were washed twice more with PBS to remove debris, before 250µl of drugged media was placed into relevant wells. Culture plates were then placed into the IncuCyte™ FLR (Essen Bioscience) and the software programmed to take 2 phase contrast images of each scratch (at different positions) every 2 hours for 96 hours. Media was replenished after 72 hours. The IncuCyte software was used to calculate a range of variables including wound area width and wound area confluence (as a percentage of the initial wound area). Statistical analysis was calculated using one-way ANOVA with Bonferroni post hoc test.

2.1.4 Proliferation assay

Keratinocytes or fibroblasts of early passage number were seeded in 6-well Nunc™ tissue culture plates (Thermo Scientific, Cat no. 140685) at a low density (approx. 20000 per well for keratinocytes and 10000 per well for fibroblasts). There were 3 biological replicates for each group, each with at least n=4. Keratinocytes were grown in phenol red-free EpiLife® medium (Thermo Fisher Scientific Cat. MEPICFPREF500) supplemented with HKGS (Human Keratinocyte Growth Supplement) (Thermo Scientific Cat. S0015), 60 µM calcium chloride (Sigma-Aldrich Cat. C7902) and 1% penicillin/streptomycin. Fibroblast were grown in phenol red-free DMEM (Gibco® Cat. 31053-028) supplemented with 10% charcoal stripped foetal bovine serum (FBS) and 1% penicillin/streptomycin.

After 24 hours the media was replaced with drugged media. Cells were the treated with either 100nM 17β-estradiol (Sigma-Aldrich Cat no. E2758), 100nM 2,3-Bis(4-hydroxyphenyl)propionitrile (DPN) (Sigma-Aldrich, Cat no. H5915) or 100nM 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) (Sigma-Aldrich, Cat no. H6036). Drug stocks were dissolved in 100% ethanol and therefore control wells were also treated with media containing 1x10⁻⁵% ethanol. Culture plates were then placed into the IncuCyte™ FLR (Essen Bioscience) and the software set to take phase-contrast images (36 images per well) every 2 hours for 6 days, with media replenished after 72 hours. The IncuCyte software was used to calculate the percentage confluence of the cells over this period. The resultant growth curves were plotted and analysed using Prism Graph Pad software to determine doubling times. Statistical analysis was calculated using one-way ANOVA with Bonferroni post hoc test.
2.1.5 Mitomycin C treatment

Mitomycin C can be used to inhibit cell proliferation by covalently cross-linking DNA. Prior to assay, cells were treated with 8µg/ml Mitomycin C (Sigma Aldrich, Cat no. M4287) in normal media for 150 minutes at 37°C with 5% CO₂, washed twice with PBS and returned to normal media conditions.

2.1.6 Fibroblast and Keratinocyte co-culture with conditioned media

Fibroblasts were seeded at 300000 cells per well in 6 well plates in triplicate. The fibroblasts were grown to 2 days post-confluence in 25% phenol red-free DMEM (Gibco® Cat. 31053-028) supplemented with 10% charcoal-stripped foetal bovine serum (CS-FBS) and 1% penicillin/streptomycin and 75% phenol red-free EpiLife® medium (Thermo Fisher Scientific Cat. MEPICFPRF500) supplemented with HKGS (Human Keratinocyte Growth Supplement) (Thermo Scientific Cat. S0015), 60 µM calcium chloride (Sigma-Aldrich Cat. C7902) and 1% penicillin/streptomycin. 48 hours before wounding, cells were pre-drugged with 100nM 17-β-estradiol (Sigma-Aldrich Cat no. E2758) or with media containing media containing 1x10⁻⁵% ethanol as an estrogen negative control.

To wound activate, fibroblasts were washed in warm PBS and 500μl media added to each well. The cell monolayers were then scratched with 7 evenly spaced straight lines using a sterile p200 pipette tip, followed by 7 evenly spaced straight lines perpendicular to the first set. The cells were then washed twice with PBS before being replaced with 1.5ml of serum-free media with either 100nm estradiol or ethanol control. After 48 hours, media was removed and stored for use on keratinocyte scratch assays.

Keratinocytes were seeded in 96-well IncuCyte™ ImageLock™ Plates (Essen Bioscience, Cat no. 4379) at 15000 cells per well. Cells were grown to 4 days post-confluent in 250μl of estrogen-free media as previously described. 24 hours before wounding, cells were switched to drugged media consisting of either ethanol control, or 100nM 17-β-estradiol. Cells were scratched with the WoundMaker™ as previously described. 75% fresh phenol red-free EpiLife® medium (Thermo Fisher Scientific Cat. MEPICFPRF500) supplemented with HKGS (Human Keratinocyte Growth Supplement) (Thermo Scientific Cat. S0015), 60 µM calcium chloride (Sigma-Aldrich Cat. C7902) and 1% penicillin/ streptomycin was mixed with 25% conditioned media from the wound-activated fibroblasts (either estrogen positive or negative) and 250μl added to each well. Culture plates were then placed into the IncuCyte™ FLR (Essen Bioscience) and the software programmed to take 2 phase contrast images of each scratch (at different positions) every 2 hours for 96 hours.
Media was replenished after 72 hours. The IncuCyte software was used to calculate a range of variables including wound area width and wound area confluence (as a percentage of the initial wound area).

2.1.7 Monocyte and fibroblast co-culture

Fibroblasts were seeded at 300000 cells per well in 6 well plates in triplicate. They were grown to confluence in media containing 50% phenol red-free DMEM (Gibco® Cat. 31053-028) and 50% phenol red-free Iscove’s Modified Dulbecco’s Medium (IMDM) (Thermo Fisher Scientific, Cat no. 21056023), supplemented with 10% charcoal-stripped foetal bovine serum (CS-FBS) and 1% penicillin/streptomycin. When the fibroblasts reached confluence, cells were either treated with 100nM 17-β-estradiol or with 1x10⁻⁵% ethanol as a control. Once 2 days post-confluent, cell layers were scratch activated by creating 7 parallel scratch wounds with a sterile p200 pipette tip. Human blood derived CD14+ monocytes were obtained from our collaborators at the Singapore Immunology Network (SIgN). They were seeded into ThinCert™ Cell Culture Inserts (Greiner Bio-One, Cat no. 657610) at 1 million per well and transferred to the wells containing the scratch activated fibroblasts. The fibroblasts and CD14+ monocytes were co-cultured for 48 hours in IMDM supplemented with 10% charcoal-stripped foetal bovine serum (CS-FBS) and 1% penicillin/streptomycin. Media was treated with either 100nM 17-β-estradiol or with 1x10⁻⁵% ethanol as a control. As further controls, monocytes and fibroblasts were grown in isolation under the same conditions. After 48 hours, RNA was harvested as described below.

2.2 DNA and RNA preparation and genomic assays

2.2.1 DNA extraction

DNA was isolated using the Qiagen DNeasy Blood & Tissue Kit (Qiagen Cat. 69504) according to the manufacturer’s instructions. Briefly, media was removed and the cells washed twice with sterile PBS (Hyclone, Cat no. SH30256.01) before the addition of 0.05ml/cm² 0.05% trypsin, 0.5 mM EDTA (pH 8.0). Once cells were detached the trypsin was neutralised with the addition of 2 volumes of complete DMEM media. The cell suspension was transferred to a falcon tube and centrifuged at 700xg for 5 minutes. The media was removed and the pellet resuspended in 200μl of sterile PBS. The cells were then incubated at 56°C for 10minutes with 20μl proteinase K and 200μl Buffer AL before the addition of 420μl 100% ethanol. The solution was then added to a DNeasy spin column and placed in the centrifuge at 6000xg for 1min. The column was the washed with buffer AW1 at 6000xg, followed by buffer AW2 at 2000xg for 1 minute. The DNA was eluted with 200μl of buffer AE at 6000xg for 1 minute.
2.2.2 DNA quantification

The nanodrop (ND-1000) was blanked with elution buffer. DNA was quantified by placing 1μl of sample onto the pedestal of the nanodrop. The 260/280 ratio, 260/230 ratio and yield were recorded.

2.2.3 Tetra-primer ARMS-PCR genotyping

Cells were screened for venous ulcer-associated SNPs using the Tetra-primer ARMS-PCR genotyping method (Ye et al., 1992). This method provides a high throughput and inexpensive way to identify SNPs. Two pairs of primers were designed for each SNP according to the exemplar schematic bellow (Figure 2.1):

![Figure 2.1 – Tetra-primer ARMS-PCR method schematic](image)

Two outer primers (see Table 2.1) were designed to flank the SNP region and two inner primers were designed to each specifically bind one of the alternative alleles at the SNP site. Allele binding specificity was increased by inserting an additional mismatch into the 3rd 3’ terminal base of the inner primers. When all primers are included in a PCR reaction there are 3 possible products, distinguishable by their differing lengths, allowing identification of alleles. 50ng of keratinocyte DNA was amplified using the Invitrogen Accuprime Taq DNA polymerase system (Cat. 12339-016) under the following conditions:
**Reagents:**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1μl</td>
<td>Accuprime PCR Buffer I</td>
</tr>
<tr>
<td>2μl</td>
<td>Inner Primers (5μM) (1μl of each)</td>
</tr>
<tr>
<td>2μl</td>
<td>Outer Primers (1μM) (1μl of each)</td>
</tr>
<tr>
<td>1μl</td>
<td>Template DNA (50ng)</td>
</tr>
<tr>
<td>0.25μl</td>
<td>Accumprime Taq DNA polymerase</td>
</tr>
<tr>
<td>3.75μl</td>
<td>PCR grade H₂O (make to 10μl)</td>
</tr>
<tr>
<td>10μl</td>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

**Cycling Protocol:**

35 Cycles

- 72°C – 57°C for 15 cycles (touchdown PCR)
- 57°C for 20 cycles

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denature</td>
<td>1min</td>
<td>94°C</td>
</tr>
<tr>
<td>Denature</td>
<td>1min</td>
<td>94°C</td>
</tr>
<tr>
<td>Anneal</td>
<td>1min</td>
<td>72°C – 57°C (decrease 1°C per cycle)</td>
</tr>
<tr>
<td>Extend</td>
<td>1min</td>
<td>72°C</td>
</tr>
<tr>
<td>Final extension</td>
<td>2min</td>
<td>72°C</td>
</tr>
</tbody>
</table>

Touchdown-PCR was used in order to increase the specificity of the primer binding at the SNP site. Having a higher annealing temperature in the early rounds of PCR that gradually reduces means that any difference in Tₘ between correct and incorrect annealing will produce a twofold amplification advantage per cycle – leading to more accurate genotyping.

2.2.4 Gel electrophoresis

Agarose gels were made by heating 1x Tris-Borate-EDTA buffer (TBE pH8.3, 1st BASE, Singapore, Cat no. BUF-3013) to boiling point with 1.2% (w/v) agarose (Sigma-Aldrich, Cat no. A9539). When the temperature had dropped to 60°C, 1μl/50ml of SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Cat. S33102) was added to the solution and it was allowed to cool and solidify in a gel caste. 6x DNA loading dye (New England Biolabs Inc., USA) was then added to DNA samples and DNA amplicons were separated by gel electrophoresis at 75V in 1x TBE buffer. DNA bands were then visualised using the ChemiDoc™ MP imaging system (Bio Rad, Cat no. 1708280). Tetra-primer ARMS genotypes were assigned based on the sizes of the bands present (Table 2.1).

2.2.5 Sanger sequencing

To confirm the venous ulcer associated SNPs that were found via tetra-primer ARMS-PCR, Sanger sequencing was carried out. This was performed following the manufacturer’s instructions using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Cat no. 4337456) in 10μl reactions. The ExoSAP (Exonuclease I Shrimp Akaline Phosphotase) protocol was used to clean-up
samples before sequencing. Samples were incubated at 37°C for 30 mins, followed by 80°C for 15 mins with 0.95μl Exo I buffer, 0.05μl Exo I (Thermo Scientific Cat no. EN0581), 0.75μl SAP buffer and 0.25μl SAP (Affymetrix Cat no. 78390500). Samples were submitted to the DNA Sequencing Facility (IMCB, A*Star, Singapore) for analysis on the ABI PRISM 3730xl DNA Analyser. Electropherograms were analysed using 4Peaks freeware (Nucleobytes, The Netherlands).

2.2.6 RNA extraction

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen Cat no. 74106) according to the manufacturer’s instructions. Briefly, cells were washed twice with cold sterile PBS (Hyclone, Cat no. SH30256.01) before being lysed in situ with 35μl/cm² cold RLT buffer containing 10μl/ml β-mercaptoethanol (Sigma-Aldrich, Cat no. M6250-500ML). The lysate was then transferred to a 1.5ml Eppendorf tube and mixed with 1 volume of 70% ethanol before being transferred to a spin column and placed in a centrifuge for 30 seconds at 8000xg with flow-through discarded. 700μl of RW1 buffer was added to the column and placed in a centrifuge for 30 seconds at 8000xg and the flow-through discarded. 10μl of DNase I in 70μl of Buffer RDD (Qiagen RNase-free DNase set, Cat no. 79254) was added to the column and incubated at room temperature for 20 minutes. 500μl of RW1 buffer was added to the column and placed in a centrifuge for 30 seconds at 8000xg and the flow-through discarded. A further 700μl of RW1 buffer was added to the column and placed in a centrifuge for 30 seconds at 8000xg and the flow-through discarded. The column was then washed twice with RPE buffer for 2 minutes at 8000xg. RNA was eluted by repeatedly passing 50μl of RNase-free water through the membrane for 30 seconds at 8000xg. The quality and concentration of the RNA was determined using the Nanodrop ND-1000 as previously described.

2.2.7 cDNA synthesis (reverse transcription)

cDNA was synthesised using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Cat no. 04897030001) using 1μg of total RNA, according to the manufacturer’s instructions (Procedure B). Completed reactions were subjected to RNaseH treatment (Invitrogen, Cat no. 18021071) to remove mRNA by incubation with 1Unit of RNaseH for 20 minutes at 37°C.
2.2.8 Quantitative-PCR

Quantitative-PCR was carried out using the Lightcycler® 480 (Roche, Cat no. 05015278001) in 10μl reactions. Reactions were made up of 20ng cDNA, 0.03μM of each primer (see Table 2.3) and 1X LightCycler® 480 SYBR Green I Master Mix (Roche, Cat no. 04887352001). Reactions were subjected to the following cycles:

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denature</td>
<td>15min</td>
<td>95°C</td>
</tr>
<tr>
<td>Denature</td>
<td>10sec</td>
<td>95°C</td>
</tr>
<tr>
<td>Anneal</td>
<td>10sec</td>
<td>57°C (primer dependent)</td>
</tr>
<tr>
<td>Extend</td>
<td>15sec</td>
<td>72°C</td>
</tr>
<tr>
<td>Melt curve</td>
<td>5sec</td>
<td>95°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rr0.11°C/s</td>
</tr>
</tbody>
</table>

Expression levels were normalised to the housekeeping genes TATA-box binding protein (TBP) and/or ribosomal protein lateral stalk subunit P0 (RPLP0) and analysis carried out using the Lightcycler® 480 software. The melt curve was used to confirm that only a single amplicon was present.

2.2.9 Allele specific expression (ASE)

Primers were designed for each SNP with a common reverse primer and two allele specific primers at the SNP locus (Table 2.2). The allele specific forward primers were designed to end with the SNP loci as the end 3' terminal nucleotide. To further increase the primer specificity of the binding for one allele over the other, a further mismatch was introduced at the 3rd 3' nucleotide position (Liu et al., 2012). To validate primer function, qPCR reactions were used incorporating DNA of known ratios. DNA was extracted from cells that were homozygous for the Wild Type allele and the disease-associated allele. These DNA samples were then mixed to known ratios. ASE primers were then used to predict these ratios. If ratios were predicted to ±5% of the known ratios, primers were considered suitable for ASE assays. RNA was extracted and cDNA synthesised as previously described from cells heterozygous for the SNP in question. The ASE primer pairs were used in separate reactions in qPCR assays as described previously, in triplicates of cDNA that had been serial diluted (1, 1/4, 1/16, 1/32 and 1/64). Primer efficiencies (E) could be calculated by plotting RT-qPCR generated Ct values against log(concentration) and using the following:

\[
\text{Efficiency (E)} = 10^{\left(1/\text{slope}\right)}
\]
Once primer efficiencies (E) had been calculated and Ct values established, a normalised expression ratio of one allele (AS1) to the other (AS2) could be generated, which was also normalised against RPLP0 (ref) and normalised for primer efficiency discrepancies:

\[
\frac{(E_{\text{ref}})^{Ct_{\text{ref}}}}{(E_{AS1})^{Ct_{AS1}}} \div \frac{(E_{\text{ref}})^{Ct_{\text{ref}}}}{(E_{AS2})^{Ct_{AS2}}}
\]

2.3 Protein expression

2.3.1 Protein extraction

Cells were lysed in situ with ice cold RIPA buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% Na.deoxycholate, 1% Triton-X 10, 2mM EDTA and 1mM PMSF) with 1U/50ml Pierce™ protease and phosphatase inhibitor cocktail mini tablets (Thermo Scientific, Cat no. 88669) added immediately before use. Lysates were subsequently homogenised using the 550 Sonic Dismembrator (Fisher Scientific) with 3x10 second pulses interspersed by 5 second recovery intervals on ice. Protein concentrations of lysates were determined using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Cat no. 23225) according the manufacturer’s protocol.

2.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were mixed with 4X LDS buffer (Novex, Cat no. NP0007) and 10X Bolt reducing buffer (Novex, Cat no. B0009) before being boiled at 95°C for 7 minutes. Samples were loaded into 4-12% Bis-Tris Plus SDS polyacrylamide precast gels (Invitrogen, Cat no. NW04125) and separated by running through the gel at 140V for approximately 90 minutes in 3-(N-morpholino)propanesulfonic acid (MOPs) running buffer (Novex, Cat no. NP000102). Protein transfer was carried out using either the iBlot™ Dry Blotting system (Thermo Fisher Scientific) for 8 minutes at 20-25V or using the Mini Trans-Blot® Cell (Bio Rad, Cat no. 1703930) or wet transfer system at 100V for 90 minutes in cold Towbin transfer buffer (25mM Tris, 190mM glycine, 20% methanol) onto nitrocellulose membrane.
2.3.4 Western blotting

Non-specific antibody binding was blocked by incubating the nitrocellulose membrane in phosphate buffered saline with 0.1% Tween-20 (Sigma-Aldrich, Cat no. P9416) (PBS-T) containing 5% milk powder (Marvel, UK) for 1 hour with gentle agitation. Membranes were then incubated overnight at 4°C with primary antibody (Table 2.5) in blocking buffer. Membranes were then washed thrice with PBS-T for 7 minutes before being incubated with horseradish peroxidase–conjugated secondary antibody of goat anti-mouse IgG (H+L) (Promega, Cat no. W4021) diluted 1:2000 in blocking buffer at room temperature for 30 minutes. Membranes were then washed five times with PBS-T and once with PBS for 7 minutes before visualisation using Clarity™ ECL Western Blotting substrate (Bio Rad, Cat no. 1705061) on the ChemiDoc™ MP imaging system (Bio Rad, Cat no. 1708280) and analysed with the accompanying Image Lab™ software.

2.3.4 Immunocytochemistry

Cells were seeded on glass coverslips are fixed with 4% paraformaldehyde in PBS, pH7.4 (at room temperature) and permeabilised with 0.2% Triton for 10 minutes. Non-specific binding was blocked by incubation in PBS (pH7.4) with 0.1% Tween 20 (PBS-T) containing 5% goat serum. Coverslips were incubated with primary antibodies (Table 2.5) in blocking buffer at 4°C overnight and then washed thrice for 10 minutes with PBS-T. Cells were then incubated with the appropriate fluorophore-conjugated secondary antibodies (1:200 in blocking buffer) for 30 minutes at room temperatures and were again, washed thrice in PBS-T for 10 minutes. Lastly, cells were incubated with 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Cat no. D9542) at 1:100 in distilled water for 10 minutes, washed for 5 min with distilled water and mounted on glass slides with hydromount with 2.5% DABCO.

2.4 Electrophoretic Mobility Shift Assays (EMSA)

Electrophoretic Mobility Shift Assays (EMSA) were completed using the Pierce Light Shift Chemiluminescent EMSA Kit (Thermo Scientific, Cat no. 20148), as per the manufacturer’s instructions. Custom biotin-labelled DNA sequences representing disease-associated and healthy SNP variants were used as probes (Table 2.4) with nuclear extracts to determine whether transcription factors bound at these sequences. 6% native polyacrylamide gels were prepared and
pre-run in 0.5X TBE Buffer for at least an hour at 4°C and at 90V. Binding reactions for each probe were prepared in triplicates as follows:

<table>
<thead>
<tr>
<th></th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrapure H2O</td>
<td>16μl</td>
<td>15μl</td>
<td>13μl</td>
</tr>
<tr>
<td>10X Binding Buffer</td>
<td>2μl</td>
<td>2μl</td>
<td>2μl</td>
</tr>
<tr>
<td>Poly dl•dC/ Sonicated Salmon Sperm DNA</td>
<td>1μl</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Unlabelled Target DNA</td>
<td>---</td>
<td>---</td>
<td>2μl</td>
</tr>
<tr>
<td>Nuclear Extract</td>
<td>---</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Biotin-labelled Target DNA (1:100 dilution)</td>
<td>1μl</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Total</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
</tr>
</tbody>
</table>

Reactions were incubated at room temperature for 20 minutes before 5μl of Loading Buffer (Thermo Scientific, Cat no. R0611) was added to each binding reaction and 20μl of sample was added to each well of the polyacrylamide gel. The gel was run at 90V for approximately 1 hour at 4°C, until the dye front had progressed 2/3 of the length of the gel. Nylon membrane was acclimatised in 0.5X TBE Buffer for at least 10 minutes. The polyacrylamide gel was then sandwiched with the nylon membrane between high quality blotting paper and fresh transfer sponges (that had not been previously used in Western Blotting), in a clean electrophoretic transfer unit. Transfer took place at 4°C in 0.5X TBE at 60V for approximately 30 minutes. The nylon membrane was then exposed to a UV light source for 15 minutes to cross-link transferred DNA to the membrane. The membrane was then incubated, with gentle shaking, for 15 minutes with Blocking Buffer (Thermo Scientific) warmed to 37-50°C. Conjugate blocking buffer was prepared by the addition of Stabilized Streptavidin-horseradish Peroxidase Conjugate (Thermo Scientific) to Blocking Buffer (ratio of 1:300). The membrane was then incubated in Conjugate Blocking solution for a further 15 minutes with gentle shaking. The membrane was transferred to a clean container and washed with 1X Wash Buffer (Thermo Scientific) (warmed to 37-50°C) for 5 minutes and repeated four times. The membrane was then transferred to a clean container and incubated with Substrate Equilibrium Buffer (Thermo Scientific) for 5 minutes, with gentle shaking. Substrate Working Solution was prepared from 3ml of Luminol/enhancer Solution and 3ml of Stable Peroxidase solution (Thermo Scientific). The membrane was laid face-down in a puddle of this solution for 5 minutes, with no shaking. The moist membrane was then wrapped in plastic, placed in a film cassette and exposed to x-ray film (for 5+ minutes) to visualise the bands.
2.5 RNAseq preparation and analysis

A batch of 12 samples was sequenced at the Genome Institute of Singapore (GIS). This batch consisted of mRNA extracted from paired estrogen treated (E+) and untreated (E-) human keratinocyte cultures which were derived either from wild type donors ('WT') or ESR2 mutants ('D'). Keratinocytes were grown to confluence in phenol red-free EpiLife® medium (Thermo Fisher Scientific Cat. MEPICFPRF500) supplemented with HKGS (Human Keratinocyte Growth Supplement) (Thermo Scientific Cat. S0015), 60 µM calcium chloride (Sigma-Aldrich Cat. C7902) and 1% penicillin/streptomycin. 48 hours before collection, cell were treated with media containing either 100nM 17-β-estradiol (Sigma-Aldrich Cat no. E2758) (dissolved in 100% ethanol) or 1x10-5% ethanol (control solvent). 4 hours before collection, cells were wound activated with a sterile p200 pipette tip. Total RNA was extracted with the RNeasy Mini Kit (Qiagen Cat no. 74106) and RNA quality checked using the Bioanalyzer (Agilent 2100 Bioanalyzer) according to the manufacturer’s instructions. Samples were submitted to GIS and sequenced using an Illumina Hiseq 2500 in single-end 100bp mode and the reads were mapped to the human genome (GRCh38/hg20) with the STAR mapper (v2.5.1a) using the known transcriptome as a reference annotation (Ensembl GRCh38.83). All samples achieved mapping percentages over 95%. Gene expression was measured by counting the number of reads mapping to known genes with the Feature Counts package as implemented in the R package "Rsubread". This resulted in count tables for the sense strand reads, as a directional library preparation was used. Differential expression analyses were performed with edgeR with counts normalised using the trimmed mean of M-values normalization method (TMM). Strict pairwise comparison was used to generate lists of statistically significantly differentially regulated genes selected on a fold change ≥1.5, p≤0.05 and false discovery rate (FDR)≤0.05. Less stringent gene lists were selected for gene ontology (GO) analysis, with genes selected on a fold change ≥1.5 and ps≤0.05. Overrepresented gene ontology GO groups were identified using the online functional annotation tool PANTHER from the Gene Ontology Consortium (GOC). Significant GO terms were selected by Bonferroni corrected p-value (≤0.05). Pathway diagrams were adapted from KEGG pathways (Kanehisa et al., 2016).
### 2.6 Primers and antibodies

#### Table 2.1 - Tetra-ARMS-PCR primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Product size (bp)</th>
<th>Position</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1887994 outer primers</td>
<td>219</td>
<td>Forward</td>
<td>GAGAACCACGTCTGTTGAGACAATAACAGAGAAACCATAGGGAATAAAATGT</td>
</tr>
<tr>
<td>rs1887994 G allele</td>
<td>105</td>
<td>Reverse</td>
<td>ATTTAATCAAAGCATGGAATTTGAGCCATCC</td>
</tr>
<tr>
<td>rs1887994 T allele</td>
<td>167</td>
<td></td>
<td>CCAACTGCTCTGAAAGCTGTCGATGGGT</td>
</tr>
<tr>
<td>rs2978381 outer primers</td>
<td>409</td>
<td>Forward</td>
<td>TGTTTCTTCCTCTTTACCTCCCTCAAT</td>
</tr>
<tr>
<td>rs2978381 C allele</td>
<td>195</td>
<td>Reverse</td>
<td>ATGTTGTAATAATACCAACATGGAGGAGGA</td>
</tr>
<tr>
<td>rs2978381 T allele</td>
<td>269</td>
<td></td>
<td>CTCACATTGTTTCTCTCTTTTACCC</td>
</tr>
<tr>
<td>rs2978983 outer primers</td>
<td>295</td>
<td>Forward</td>
<td>CACCATATAGCAGCATGTTTCATCAAT</td>
</tr>
<tr>
<td>rs2978983 T allele</td>
<td>157</td>
<td>Reverse</td>
<td>CCCACAGAAGCTGTTTATTATAACAGGA</td>
</tr>
<tr>
<td>rs2978983 C allele</td>
<td>193</td>
<td></td>
<td>CTCAGTTTAATCGCAAGTGGAGGATGA</td>
</tr>
<tr>
<td>rs3783736 outer primers</td>
<td>195</td>
<td>Forward</td>
<td>AAAGTGTAATAGTATGGGCTTTCTATGAACCTCGACCAAATGATCGT</td>
</tr>
<tr>
<td>rs3783736 A allele</td>
<td>136</td>
<td>Reverse</td>
<td>TGGGAAAAGAAGTGATGAAATGCA</td>
</tr>
<tr>
<td>rs3783736 C allele</td>
<td>109</td>
<td></td>
<td>CAGTAGATAGAACAATTAATACATCTGAAGG</td>
</tr>
</tbody>
</table>

#### Table 2.2 - Allele Specific Expression (ASE) primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Product size (bp)</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASE rs1887994 Forward G allele</td>
<td>243</td>
<td>TACATCTTTCCAGGCCCTCA</td>
</tr>
<tr>
<td>ASE rs1887994 Forward T allele</td>
<td>243</td>
<td>TACATCTTTCCAGGCCCTCA</td>
</tr>
<tr>
<td>ASE rs1887994 Reverse</td>
<td>243</td>
<td>TACATCTTTCCAGGCCCTCA</td>
</tr>
<tr>
<td>ASE rs2978381 Forward T allele</td>
<td>220</td>
<td>TGATGTGGCAGCTGAGTCAGT</td>
</tr>
<tr>
<td>ASE rs2978381 Forward C allele</td>
<td>220</td>
<td>TGATGTGGCAGCTGAGTCAGT</td>
</tr>
<tr>
<td>ASE rs2978381 Reverse</td>
<td>220</td>
<td>TGATGTGGCAGCTGAGTCAGT</td>
</tr>
<tr>
<td>ASE rs2987983 Forward C allele</td>
<td>201</td>
<td>GCGACCTGAAACACCCACAG</td>
</tr>
<tr>
<td>ASE rs2987983 Forward T allele</td>
<td>201</td>
<td>GCGACCTGAAACACCCACAG</td>
</tr>
<tr>
<td>ASE rs2987983 Reverse</td>
<td>201</td>
<td>GCGACCTGAAACACCCACAG</td>
</tr>
<tr>
<td>ASE rs3783736 Forward C allele</td>
<td>198</td>
<td>TGGCAGC AAAAGGACAGTGGA</td>
</tr>
<tr>
<td>ASE rs3783736 Forward A allele</td>
<td>198</td>
<td>TGGCAGC AAAAGGACAGTGGA</td>
</tr>
<tr>
<td>ASE rs3783736 Reverse</td>
<td>198</td>
<td>TGGCAGC AAAAGGACAGTGGA</td>
</tr>
</tbody>
</table>
Table 2.3 - RT-qPCR primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Full gene name</th>
<th>Product size (bp)</th>
<th>Position</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL-2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>186</td>
<td>Forward Reverse</td>
<td>ATGTGTTGGAGAGGAGCTCAA TTCAGAGACAGGAGAGAAA</td>
</tr>
<tr>
<td>BCL-2(2)</td>
<td>B-cell CLL/lymphoma 2</td>
<td>171</td>
<td>Forward Reverse</td>
<td>CCCGGTGGATGAGCTGACCGAC GATGCAAGCTCCACCAAGCAG</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif chemokine ligand 2</td>
<td>183</td>
<td>Forward Reverse</td>
<td>AGTCTGGCCGCCCCCTTCT GCTCTTTTGGGACACTGCTT</td>
</tr>
<tr>
<td>CCL5</td>
<td>C-C motif chemokine ligand 5</td>
<td>162</td>
<td>Forward Reverse</td>
<td>CGGTTGATCCATCTTTGCTA ACTGCTGGGTTGAGCAC</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
<td>219</td>
<td>Forward Reverse</td>
<td>CAGGCTCCTTGTTGTTTGGT TGGAGAGCGGCAATGAGC</td>
</tr>
<tr>
<td>CXCL11</td>
<td>C-X-C motif chemokine 11</td>
<td>178</td>
<td>Forward Reverse</td>
<td>GTCAAGGCTTCCTCATGTT TGAGTTAGGACACTGTTT</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor alpha</td>
<td>132</td>
<td>Forward Reverse</td>
<td>GCTAGCAATGGGAGAATGAGA CAGCTCATGTCCTGAC</td>
</tr>
<tr>
<td>ERβ</td>
<td>Estrogen receptor beta</td>
<td>143</td>
<td>Forward Reverse</td>
<td>GGTACAGCATCATCAATATGTC TCAAGCTGAGGATATTCAAG</td>
</tr>
<tr>
<td>ERβ OK</td>
<td>Estrogen receptor beta OK promoter exon</td>
<td>259</td>
<td>Forward Reverse</td>
<td>GGTACAGCATCATCAATATGTC TCAAGCTGAGGATATTCAAG</td>
</tr>
<tr>
<td>ERβ ON</td>
<td>Estrogen receptor beta ON promoter exon</td>
<td>254</td>
<td>Forward Reverse</td>
<td>CAGGCGCGTGTGTTATCCT TGATGCGGATATTCAAG</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor 7</td>
<td>273</td>
<td>Forward Reverse</td>
<td>GGAGAAAGGAGACCCTAC TTTGAGGAGCTCGGTT</td>
</tr>
<tr>
<td>IL-12b</td>
<td>Interleukin 12b</td>
<td>142</td>
<td>Forward Reverse</td>
<td>GGTATTTGCGACCTCTCC CAGATTACATGTCCTGAC</td>
</tr>
<tr>
<td>IL-1a</td>
<td>Interleukin 1a</td>
<td>151</td>
<td>Forward Reverse</td>
<td>AGCTCTCTGAGAATGTGGA CAATTAGGGTACTCCAG</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
<td>210</td>
<td>Forward Reverse</td>
<td>GAACCTAAACATGGAGAGAGG TGGAGAGCTGACAATGAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
<td>162</td>
<td>Forward Reverse</td>
<td>CCAAGAGTGCTGAAAGAATGTC ACTACATGCAATGTCCTG</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
<td>83</td>
<td>Forward Reverse</td>
<td>GCCTCTCTGATTCTGCGAG GCTTTGACTGACACTAAGT</td>
</tr>
<tr>
<td>KGF</td>
<td>Fibroblast growth factor 7</td>
<td>194</td>
<td>Forward Reverse</td>
<td>TCAAAGGGGGGGAAGATGGA TCTTACACGACCTCTTTCG</td>
</tr>
<tr>
<td>KGF/FGF-7</td>
<td>Fibroblast growth factor 7</td>
<td>90</td>
<td>Forward Reverse</td>
<td>GGAGAAAAGGAGACTCAGAAGA TCTTACCACCCCTCTTTCG</td>
</tr>
<tr>
<td>KRT1</td>
<td>Keratin 1</td>
<td>126</td>
<td>Forward Reverse</td>
<td>AGAAGTGACCAACTCTAAAGACT AATTCTCTGATGGTCCTGGC</td>
</tr>
<tr>
<td>KRT5</td>
<td>Keratin 5</td>
<td>152</td>
<td>Forward Reverse</td>
<td>AGAGGTGGAGACAGTCACAT TGAGTAGATGCTTCTGCAG</td>
</tr>
<tr>
<td>KRT6a</td>
<td>Keratin 6a</td>
<td>89</td>
<td>Forward Reverse</td>
<td>CTGATGGGAGGAGGTTC GCGCACAAGACAGTGGC</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
<td>121</td>
<td>Forward Reverse</td>
<td>AGAACCGCTCTACAAGCAAG GAGTTGTCAAGGGCGATC</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metallopeptidase 9</td>
<td>153</td>
<td>Forward Reverse</td>
<td>TGCCAACCAACCAATACCATG GATGTCTGGCTCCGGGT</td>
</tr>
<tr>
<td>MRC1</td>
<td>Mannose receptor, C type 1</td>
<td>173</td>
<td>Forward Reverse</td>
<td>CCAGAGGTATGAAAGCAGCATG TCCAAATACCTGGTCTGAT</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells</td>
<td>242</td>
<td>Forward Reverse</td>
<td>CATTAACAGGAAGATGTTGCT CACCAGCAGCAGGAAAT</td>
</tr>
<tr>
<td>NRF1</td>
<td>Nuclear respiratory factor 1</td>
<td>208</td>
<td>Forward Reverse</td>
<td>AAGTGCAAGAGCTGACTAG TCCGGTCTTTTCTCGTTGCA</td>
</tr>
<tr>
<td>PDGFA</td>
<td>Platelet derived growth factor subunit A</td>
<td>177</td>
<td>Forward Reverse</td>
<td>GGAACCTCCAGCGACTCTT ACCGCTTGGTCTGGCAG</td>
</tr>
<tr>
<td>PGR</td>
<td>Progesterone receptor</td>
<td>204</td>
<td>Forward Reverse</td>
<td>CACCTTCACTTTGCTGCTGA TACAGGAGTCTGCCAGAC</td>
</tr>
<tr>
<td>RPLP0</td>
<td>Ribosomal protein lateral stalk subunit P0</td>
<td>98</td>
<td>Forward Reverse</td>
<td>CAGATGGTCTACAACTGATT GGAAGGGTGAATGCTGTCCTC</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte peptide inhibitor</td>
<td>144</td>
<td>Forward Reverse</td>
<td>CCCAGTCCTATGACAAAGAACTT ACCCCAGGCTCCTCTTCTT</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-box binding protein</td>
<td>89</td>
<td>Forward Reverse</td>
<td>CAGAACACAGAGGACAGTATT TTTCTTGCTGCGAGTCTGAGC</td>
</tr>
</tbody>
</table>
Table 2.4 - EMSA probes

<table>
<thead>
<tr>
<th>SNP</th>
<th>Sequence (5' &gt;3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rs2987983</td>
<td></td>
</tr>
<tr>
<td>Forward Healthy</td>
<td>GAATTGGAATAATCA C CCTCTCCACTCTGCA</td>
</tr>
<tr>
<td>Reverse Healthy</td>
<td>TGCAGAGTGAGAGGG G TGATTATTCCAATTC</td>
</tr>
<tr>
<td>Forward Disease-associated</td>
<td>GAATTGGAATAATCA C CCTCTCCACTCTGCA</td>
</tr>
<tr>
<td>Reverse Disease-associated</td>
<td>TGCAGAGTGAGAGGG A TGATTATTCCAATTC</td>
</tr>
<tr>
<td>Rs3783736</td>
<td></td>
</tr>
<tr>
<td>Forward Healthy</td>
<td>AAAGTGATGAATTAC C CCTCAGATATTTGTAT</td>
</tr>
<tr>
<td>Reverse Healthy</td>
<td>ATACAATATCTGAGG G GTAATTACACTCTT</td>
</tr>
<tr>
<td>Forward Disease-associated</td>
<td>AAAGTGATGAATTAC A CCTCAGATATTTGTAT</td>
</tr>
<tr>
<td>Reverse Disease-associated</td>
<td>ATACAATATCTGAGG T GTAATTACACTCTT</td>
</tr>
<tr>
<td>Rs1887994</td>
<td></td>
</tr>
<tr>
<td>Forward Healthy</td>
<td>GAAAGCTACTGGAAA G TTGGAAAATGACATG</td>
</tr>
<tr>
<td>Reverse Healthy</td>
<td>CATGCATTTTCCAA C TTTCAGTAGCTTT</td>
</tr>
<tr>
<td>Forward Disease-associated</td>
<td>GAAAGCTACTGGAAA A TTGGAAAATGACATG</td>
</tr>
<tr>
<td>Reverse Disease-associated</td>
<td>CATGCATTTTCCAA A TTTCAGTAGCTTT</td>
</tr>
</tbody>
</table>

Table 2.5 - Antibodies

<table>
<thead>
<tr>
<th>Target</th>
<th>Clone</th>
<th>Host species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERβ</td>
<td>14C8</td>
<td>Mouse</td>
<td>Abcam, UK (Cat no. ab288)</td>
</tr>
<tr>
<td>ERβ</td>
<td>MC10 (western)</td>
<td>Mouse</td>
<td>eBioscience (Cat no. 14-9336)</td>
</tr>
<tr>
<td>ERα</td>
<td>F-10</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology, USA (Cat no. sc-8002)</td>
</tr>
<tr>
<td>β-actin</td>
<td>AC-15</td>
<td>Mouse</td>
<td>Abcam, UK (Cat no. ab6276)</td>
</tr>
<tr>
<td>Secondary Ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-mouse HRP conjugated Ab</td>
<td>Goat</td>
<td>Promega, USA (Cat no. W4021)</td>
<td></td>
</tr>
<tr>
<td>Anti-mouse FITC conjugated Ab</td>
<td>Goat</td>
<td>Thermo Fischer Scientific, USA (Cat no. A-11001)</td>
<td></td>
</tr>
</tbody>
</table>
3. Chapter 3 - Functional characterisation of cells carrying venous ulcer associated SNPs

3.1 Introduction

The role of estrogen in the skin and wound healing is undeniably important with strong evidence showing that estrogen signalling can improve the rate of wound healing (Ashcroft et al., 1997a). Specific single nucleotide polymorphisms (SNPs) in estrogen receptor beta (ERβ) are associated with chronic, non-healing venous ulcers (Ashcroft et al., 1999) (Ashworth et al., 2008) and reduced ERβ expression is found in chronic wounds (Strudwick, 2006). What is unclear is how estrogen signalling brings about this improvement in wound healing and precisely what effect the presence of these SNPs might have in the wound environment. This chapter sets out to characterise the effects of specific SNPs on skin cell functions (receptor expression, cell proliferation and cell migration) and to explore how these changes might play a role in wound healing.

3.2 Cell acquisition and model system

3.2.1 Singapore Skin Cell Bank

In order to test the characteristics of the SNPs, skin cells harbouring the SNPs of interest needed to be identified and selected. The Institute of Medical Biology (IMB) maintains and supports a Skin Cell Bank of over 70 human cell samples collected for ethically approved research. Cells available include keratinocytes, fibroblasts and in some cases melanocytes from each patient. These cells are acquired from discarded tissue from Singaporean plastic surgery patients, with donor consent.

3.2.2 Genotyping for venous ulcer SNPs

In order to identify venous ulcer associated SNPs (rs2978381, rs2987983, rs1887994 and rs3783736), all cell samples in the IMB Skin Cell Bank were expanded and DNA harvested for genotyping. Initial genotype screens were carried out using tetra-primer amplification refractory mutation system-polymerase chain reaction (tetra primer ARMS-PCR) (Figure 3.1). This is an inexpensive and high throughput method in which primers for a specific allele of each SNP are used to form amplicons of differing sizes depending on the allele present in the sample. Once SNP harbouring cell lines were identified, Sanger sequencing was used to confirm the genotype.
Samples within the Skin Cell Bank largely mirror the Singaporean population in terms of ethnicity, with the majority of cell samples originating from Han Chinese patients and the remaining 20% comprising Malay and Indian patients and a single Caucasian sample. The original study that identified the venous ulcer-associated SNPs took place in Manchester, United Kingdom, and consisted almost entirely of Caucasian patients (Ashworth et al., 2008). Interestingly, the rs1887994 SNP variant is not found at all amongst Han Chinese and as such, could not be found in any of the IMB Skin Cell Bank samples. Therefore this SNP was excluded from this study, with the remaining 3 focussed upon.

<table>
<thead>
<tr>
<th>Cell Bank Identifier</th>
<th>Gender</th>
<th>Body site</th>
<th>Ethnicity</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-S-(K/F)-9</td>
<td>Female</td>
<td>Abdomen</td>
<td>Chinese</td>
<td>46</td>
</tr>
<tr>
<td>12-S-(K/F)-11</td>
<td>Female</td>
<td>Abdomen</td>
<td>Chinese</td>
<td>45</td>
</tr>
<tr>
<td>EBL(K/F)029</td>
<td>Female</td>
<td>Left breast</td>
<td>Malay</td>
<td>39</td>
</tr>
<tr>
<td>Disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-S-(K/F)-13</td>
<td>Female</td>
<td>Abdomen</td>
<td>Caucasian</td>
<td>44</td>
</tr>
<tr>
<td>EBL(K/F)013</td>
<td>Female</td>
<td>Abdomen</td>
<td>No details</td>
<td>No details</td>
</tr>
<tr>
<td>EBL(K/F)027</td>
<td>Female</td>
<td>Face</td>
<td>No details</td>
<td>No details</td>
</tr>
</tbody>
</table>

Table 3.1 - Primary cell patient and sample information. Information on primary keratinocyte and fibroblast source, provided by the Institute of Medical Biology, Skin Cell Bank - A*Star Singapore.
Figure 3.1 – Genotyping venous ulcer-associated SNPs through tetra-primer amplification refractory mutation system-polymerise chain reaction. Example electrophoresis agar gel showing the genotyping of 3 samples for the 4 venous ulcer SNPs and the resulting genotype derived. Upper bands show a control fragment whilst the lower two bands distinguish the alleles present. Genotype is determined by which bands are present at which size (see Figure 2.1).
3.2.3 Model for venous ulcer SNPs

From the IMB skin cell bank, 3 cell samples of corresponding keratinocytes and fibroblasts were selected for each group (Table 3.1). One group were homozygous for all 3 of the healthy SNP variant and was labelled as ‘Wild Type’ (WT). The second group were homozygous for the venous ulcer-associated SNP variants and was labelled as ‘Disease’ (D). Homozygous cells were selected because there was a strong venous-ulcer association with the haplotype and because these SNPs are often found together due to their strong linkage disequilibrium (supplementary Figure S.1). This resulted in model groups of fibroblasts and keratinocytes containing 3 biological replicates with distinct venous ulcer-associated haplotypes. These cells could then be used in vitro to study the effects of SNPs on receptor expression, cell proliferation and migration and to elucidate the molecular effects upon downstream estrogen signalling.

3.3 Specific dependence of ERβ levels on SNP genotype

The most obvious effect that the SNPs could be having is to alter the level of expression of ERβ in skin cells, either at the RNA level or the protein level. The SNPs are located in the 5’UTR of the gene, a region which often involved in expressional regulation (Araujo et al., 2012; Thellenberg-Karlsson et al., 2006). The presence of a different nucleotide at the SNP site could interrupt a transcription factor binding motif or introduce a new one. It may also fall within an enhancer or silencer region, diminishing the regions function. A further possibility is that the SNP may change the epigenetic regulation at the region. Alternatively, the SNPs could have an effect at the protein level by altering translation. mRNA often has regulatory sequences at its 5’ beginning that directs cellular compartmentalisation or will affect cytoplasmic stability. It was important to establish what is happening to ERβ at both levels to determine what affects the SNPs could be having, thus here we addressed SNP-induced changes in ERβ levels at both the RNA and protein level.

3.3.1 Histological and cytological investigation reveals difference in estrogen receptor expression

Staining for estrogen receptors in human skin (Figure 3.2a) reveals keratinocyte-expressed ERβ confined to the basal layer of the epidermis, in addition to the sebaceous gland and the hair follicle. There is very little staining in the dermis. ERα staining can also be seen in the sebaceous gland and hair follicle, but there is only very weak staining seen in the epidermis. Staining for ERβ in primary human skin cells that have been genotyped for venous ulcer-associated SNPs (Figure 3.2b) show that receptor is present in all cells, both when estradiol is present and when it is absent.
Figure 3.2 – Estrogen receptor expression in the skin. (A) Estrogen receptor beta and alpha staining in normal human skin wax histological section, counterstained with haematoxylin (blue). ERβ staining can be seen in the basal layer of the epidermis as well as the sebaceous glands and hair follicles, whilst ERα staining cannot be seen in the epidermis but can be seen in the sebaceous gland and hair follicle. (B) Immunocytochemistry staining of human primary keratinocytes and fibroblasts. Estrogen receptor beta could be detected (green) in both wild type (WT) and disease SNP-harbouring (D) keratinocytes and fibroblasts both with and without estradiol treatment. Nuclei are counterstained with DAPI (blue).
Figure 3.3 – RNA expression of the ERβ gene in Wild type and venous ulcer-SNP Disease keratinocytes with estradiol treatment. RT-qPCR for estrogen receptor beta from wild type (WT) and venous ulcer-SNP disease (D) keratinocytes treated with (+ve) or without (-ve) 17β-estradiol (100nM). Mean ±SEM, n=3 (biological replicates), *=p<0.05.

Figure 3.4 – ERβ protein expression in Wild type and venous ulcer-SNP Disease with estradiol treatment. (A) Representative Western blot for estrogen receptor beta with protein collected from wild type (WT) and venous ulcer-SNP disease (D) fibroblasts and keratinocytes treated with (+ve) or without (-ve) 17β-estradiol (100nM), accompanied by densitometry (B) quantification of each protein band normalised to actin. Mean ±SEM, n=3 (biological replicates).
3.3.2 ERβ expression at the transcriptional level

In terms of ERβ mRNA expression, no difference was seen in the venous ulcer-SNP harbouring keratinocytes when treated with estradiol (Figure 3.3). With the wild type keratinocytes, ERβ expression was significantly higher (approximately 3-fold) in the keratinocytes grown in the absence of estrogen than those treated with estradiol. This was also significantly higher than the expression seen in disease keratinocytes under both conditions. This could suggest that disease cells are not capable of increasing ERβ transcription in response to low estrogen levels and that disease keratinocytes have an innate problem in maintaining ERβ expression. In line with the finding that venous ulcer associated-SNP harbouring cells have reduced ERβ levels, human chronic wounds appear to demonstrate a lack of ERβ in both fibroblasts and keratinocytes of the venous ulcer (Strudwick, 2006).

3.3.3 ERβ expression at the translational level

ERβ is expressed in keratinocytes to a far greater degree than in fibroblasts (Figure 3.4a/b). The protein expression of ERβ appears to broadly follow the same pattern seen at the transcriptional level, in that receptor expression is increased in under low estrogen conditions in the wild type cells, but not to the same degree in the disease cells.

3.4. The Effects of ERβ SNPs on Proliferation

A key aspect of wound healing is cellular proliferation. During reepithelialisation, keratinocytes migrate from the wound margins as a sheet to cover the newly formed granulation tissue. The migrating cells do not proliferate and so cells adjacent to the wound edge must proliferation to replace the migrating cells. Keratinocytes start to proliferate 24 to 48 hours after wounding and it has been estimated that the rate of proliferation increases to roughly double that of the quiescent level (Safferling et al., 2013). Fibroblasts also proliferate in order to repopulate the dermis and to infiltrate and remodel the granulation tissue. Estrogen has been shown to stimulate DNA synthesis and to increase proliferation in both keratinocytes and fibroblasts (Campbell et al., 2010; Fishman et al., 1995; Stevenson and Thornton, 2007). If the ability of the cell to transduce estrogen signalling is curtailed, so too will be the rate of healing.

The level of proliferation of wild type and disease cell lines was measured in vitro through the continual monitoring of cell growth by the IncuCyte™ system (Essen Bioscience, USA). The area of
cell confluence was measured as a percentage using phase-contrast imaging at 2 hour intervals and growth curves plotted to enable the calculation of doubling time.

3.4.1 Keratinocyte proliferation is affected by SNP genotype

Keratinocyte confluence monitoring revealed that the disease cells, which harbour the venous ulcer-associated SNPs, have a significantly slower doubling time of 51.53 hours compared to 37.48 hours of the wild type in the absence of estrogen ligand (Figure 3.5a). This doubling time was reduced to 44.42 hours with the addition of estradiol, but was significantly slower than the 31.77 hours of the wild type cell lines. The disease and wild type cell lines responded similarly to estrogen treatment with a significant improvement of approximately 20% in doubling time in both cases (Figure 3.5a). Studies have shown that estradiol stimulates keratinocyte proliferation in wound healing (Zhou et al., 2016) and that this effect is possibly through ERα signalling (Verdier-Sevrain et al., 2004), but our own lab cannot corroborate these findings (Campbell et al., 2010).
Figure 3.5 – The effects of estrogen ligands on T Wild type and Disease keratinocytes and fibroblast doubling times. Doubling times of cultured primary (A) keratinocytes and (B) fibroblasts treated with estradiol (E2), DPN (ERβ specific agonist) and PPT (ERα specific agonist). Mean ±SEM, n=3 (biological replicates), *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.
3.4.2 Fibroblast proliferation is affected by SNP genotype in the presence of estradiol

Fibroblast proliferation assays revealed no significant difference between doubling times of disease and wild type cell lines in estrogen-free conditions (Figure 3.5b). However, there was a significant difference between wild type and disease fibroblasts when treated with estradiol. Estradiol treatment had no significant improvement on proliferation within each group, in agreement with previous findings (Stevenson et al., 2009) (Campbell et al., 2010).

3.4.3 Keratinocyte proliferation with ER selective agonist treatment is comparable to estradiol

In order to better understand the role of both receptors in estrogen signalling, and since ERα has previously been associated with increasing proliferation rate, selective receptor agonists were included in the proliferation assays. DPN is an ERβ agonist with a 70-fold selectivity over ERα, whilst PPT is an ERα agonist with a 410-fold selectivity over ERβ. Proliferation assays for keratinocytes showed that both DPN and PPT significantly reduced the doubling time of disease and wild type keratinocytes by a similar level (Figure 3.5a). Similar findings have been reported in human keratinocyte cell lines (Merlo et al., 2009), but no significant effect on proliferation by specific agonists is seen in murine keratinocytes (Stevenson et al., 2009) (Campbell et al., 2010).

3.4.4 Fibroblast proliferation with ER selective agonist treatment reveals genotype-dependent responses

The use of selective estrogen receptor agonists in fibroblasts revealed a significant increase in doubling time with both DPN and PPT for the wild type cell lines. By contrast, no significant difference was observed in the disease cell lines (Figure 3.5b). Previous studies have found no effect of specific-agonist treatment on in vitro proliferation of fibroblasts (Campbell et al., 2010).

3.5 The Effects of ERβ SNPs on Migration

The migration of both keratinocytes and fibroblasts is essential for successful wound healing. Fibroblasts must infiltrate the granulation tissue in order to remodel and construct a new dermis. Keratinocytes must reepithelialise the damaged epidermis to return the function and integrity of the skin barrier. In chronic wounds there is a dysfunction in the ability of cells to migrate and repopulate the wound site (Kirsner and Eaglstein, 1993). To determine the migrational capability of SNP harbouring cells, in vitro scratch assays were performed on both keratinocytes and
fibroblast cell lines. The Essen Incucyte system was used to monitor in vitro cell monolayer scratch assay progression at two hour intervals. Wound area confluence was measured in order to plot wound healing profiles of the differing cell types and conditions.

3.5.1 Keratinocyte migration is affected by SNP genotype

Wild type keratinocytes exhibit a far greater capacity to close the scratch assays compared to the disease cell lines (Figure 3.6a/b). By 16 hours post wounding, the wild type cell lines had closed 50% of the wound area, whereas the disease cell lines took 26 hours to close 50%. The mean complete wound closure time of the wild type keratinocytes was 50 hours, compared to 64 hours for the disease cell lines. Interestingly, treatment with 17-β-estradiol had no significant effect on migration in either cell group. This finding with primary human keratinocytes is at odds with the labs previous findings with mouse keratinocytes (Campbell et al., 2010) and with other published data using the NCTC 2544 keratinocyte cell line (Merlo et al., 2009).
Figure 3.6 – Wound closure of Wild type and Disease keratinocytes with estradiol treatment. (A) Wound closure profile of wild type and disease primary human keratinocytes in vitro in the presence (+ve) and absence (-ve) of 17β-estradiol (E2) (100nM), with percentage given as the area of the initial wound area covered by migrating cells measured at 2 hourly intervals. (B) Representative phase-contrast images of keratinocyte wound closure. Mean ±SEM, n=3 (biological replicates).
3.5.2 Keratinocyte migration with Mitomycin C treatment

Mitomycin C is an antibiotic that can be used as an inhibitor of proliferation in eukaryotic cells due to its ability to cross-link DNA halting its synthesis and preventing mitosis (Tomasz et al., 1987). Cells are briefly treated with a low dose of Mitomycin C (8μg/ml for 2 hours) immediately before scratching and then returned to normal media. This enables the migrational ability of the cells to be uncoupled from the proliferative capacity of the cells during scratch assays and to examine it in isolation.

As expected, Mitomycin C treatment significantly delayed keratinocyte scratch wound closure in both groups (Figure 3.7 and 3.10c/d), an effect that was particularly pronounced in the disease cell lines. Interestingly, the trajectory of closure was similar in wild type and disease cell scratch wounds for the first 20 hours post-wounding. However, after 20 hours disease cell migration reached a plateau while wild type cells continued to close the wound area (Figure 3.7). Once again, there was no significant difference seen between untreated and 17β-estradiol treated in either cell line (Figure 3.10c/d).

**Figure 3.7 - Scratch wound closure of Wild type and Disease keratinocytes with estradiol and mitomycin C treatment.** Wound closure profile of wild type and disease primary human keratinocytes *in vitro* in the presence (+ve) and absence (-ve) of 17β-estradiol (E2) (100nM) after pre-treatment of 8μg/ml mitomycin C for 2.5 hours, with percentage given as the area of the initial wound area covered by migrating cells measured at 2 hourly intervals. Mean ±SEM, n=3 (biological replicates).
3.5.3 Fibroblast migration is affected by SNP genotype

Similarly to keratinocytes, wild type fibroblasts migrate to close scratch wounds at a faster rate than their SNP-harbouring disease counterparts (Figure 3.8a/b). There is no significant difference in wound closure in response to 17-β-estradiol treatment in wild type cells (Figure 3.11a/b). Disease fibroblasts do respond to 17-β-estradiol treatment and by 48 hours, a significant increase in wound closure was observed only in disease cells (Figure 3.11b). Wild type fibroblasts (both control and 17-β-estradiol treated) close the 50% of the wound area in a mean time of around 32 hours. The disease group cells under estrogen treatment close 50% of the wound in a similar mean time of around 38 hours; however this time increases to a mean of 44 hours under low estrogen conditions.
Figure 3.8 - Wound closure of Wild type and Disease fibroblasts with estradiol treatment. (A) Wound closure profile of wild type and disease primary human fibroblasts in vitro in the presence (+ve) and absence (-ve) of 17β-estradiol (E2 (100nM)), with percentage given as the area of the initial wound area covered by migrating cells measured at 2 hourly intervals. (B) Representative phase-contrast images of keratinocyte wound closure. Mean ±SEM, n=3 (biological replicates).
3.5.4 Fibroblast migration with Mitomycin C treatment

As expected, fibroblasts displayed delayed scratch wound closure under Mitomycin C treatment, confirming an important role for proliferation in wound closure. Wild type cells with no estrogen treatment showed the most impaired wound healing capability (Figure 3.9 and 3.11c/d), whilst estrogen treated wild type cells showed a significantly better capability to close the wound area by 48 hours (Figure 3.9 and 3.11d). Disease cells closed the wound at a similar rate to the untreated wild type cells, shown by the lack of a significant difference at 24 and 48 hours (Figure 3.11c/d). There was no sensitivity to estrogen treatment demonstrated by the lack of significant difference between treated and untreated (Figure 3.9 and 3.11c/d).

![Fibroblast wound closure +MitomycinC](image)

**Figure 3.9 - Wound closure of Wild type and Disease fibroblasts with estradiol and Mitomycin C treatment.** Wound closure profile of wild type and disease primary human fibroblasts *in vitro* in the presence (+ve) and absence (-ve) of 17β-estradiol (E2) (100nM) after pre-treatment of 8μg/ml Mitomycin C for 2.5 hours, with percentage given as the area of the initial wound area covered by migrating cells measured at 2 hourly intervals. Mean ±SEM, n=3 (biological replicates).
3.5.5 Keratinocyte migration with ER selective agonist treatment reveals a beneficial role for ERα

The selective receptor agonists DPN and PPT were next used to dissect the contribution of each receptor to migration. At 12 hours post-wounding (Figure 3.10a) there was a significant improvement in both disease and wild type cells with PPT treatment. This indicates that signalling through the ERα receptor is more beneficial to wound healing in this scenario and the important receptor through which estrogen signalling is transduced. By 24 hours post-wounding (Figure 3.10b), this significant improvement in PPT treated wound closure was retained in disease cells, but not in wild type cells. This is somewhat surprising as our previous work has shown ERα signalling to have an inhibitory effect on wound healing, albeit with murine cells (Campbell et al., 2010), however a similar study with human cells did indicate a positive contribution to wound healing (Stevenson et al., 2009).

When Mitomycin C treatment is included in scratch wound closure assays this PPT healing promotion effect is lost (Figure 3.10c/d). This suggests that scratch wound closure is influenced by the proliferative effect of ERα signalling that rather than an increase in intrinsic migration. This data is supported by proliferation assays (Figure 3.5a) where PPT decreases the doubling time of both genotype groups of keratinocytes versus control. Moreover, several previous studies have identified ERα as a pro-proliferative agent (Helguero et al., 2005).

3.5.6 Fibroblast migration with ER selective agonist treatment reveals a beneficial role for ERβ

Selective estrogen receptor agonist treatments were also included in fibroblast scratch assays (Figure 3.11a-d). Here, at 48 hours post wounding a clear difference emerged in ligand response. In the disease group alone both estradiol and DPN treatment, but not PPT treatment significantly promoted closure versus control, indicating a positive effect of ERβ stimulation. When proliferation is inhibited by Mitomycin C (Figure 3.11c/d) wound closure is delayed and all ligand effects are lost, suggesting that ERβ signalling-mediated scratch wound closure in fibroblasts is via proliferative effects.
Figure 3.10 – Wound closure in Wild type and Disease primary keratinocytes with specific receptor agonist and Mitomycin C treatment. Wound closure of wild type (WT) and disease (D) primary human keratinocytes in vitro treated with (C and D) and without (A and B) Mitomycin C and with estradiol (E2) 17β-estradiol (E2) (100nM) DPN (100nM) (ERβ specific agonist) and PPT (10nM) at 12 hours (A), 24 hours (B and C) and 48 hours (D). Wound area confluence is given as a percentage of the original wound area. Mean ±SEM, n=3 (biological replicates), *=p<0.05, **=p<0.01, ***=p<0.001.
Figure 3.11 – Wound closure in Wild type and Disease primary fibroblasts with specific receptor agonist and Mitomycin C treatment. Wound closure of wild type (WT) and disease (D) primary human fibroblasts in vitro treated with (C and D) and without (A and B) Mitomycin C and with 17ß-estradiol (E2) (100nM) DPN (100nM) (ERβ specific agonist) and PPT (10nM) (ERα specific agonist) at 24 hours (A and C) and 48 hours (B and D). Wound area confluence is given as a percentage of the original wound area. Mean ±SEM, n=3 (biological replicates), *=p<0.05, **=p<0.01, ***=p<0.001.
3.5.7 Fibroblast and Keratinocyte co-culture reveals genotype-specific interactions

Wounds present a complex environment of different cells, signalling factors, and paracrine interactions. The previous scratch assays examined cell intrinsic responses, however, these cells do not act in isolation in a wound site and so it is important to explore the functional effects of interaction. To investigate this, fibroblasts were grown under estrogen positive and negative conditions and scratch wound activated. The conditioned media was removed after 48 hours and applied to keratinocyte scratch assays. Both wild type and disease keratinocytes were monitored for wound closure over a variety of conditioned media treatments (Figure 3.12).

Due to the unavoidable presence of sub-optimal fibroblast media (fibroblasts would not grow in purely keratinocyte media), keratinocytes closed the wounds at a reduced rate compared to scratch assays in optimised media (Figure 3.6). This also resulted in scratch assays that were far more variable within repeated assays. Similarly to the keratinocyte scratch assays in isolation, the WT cells closed the wound at a higher rate than the disease cells under all conditions (Figure 3.12a-e). Disease keratinocytes were relatively non-responsive to the conditioned media and show no statistically different migration rates in response to estrogen treatment or fibroblast-specific conditioned media. In contrast, the wild type cells were sensitive to conditioned media. Based on mean wound closure times, keratinocytes treated with estradiol positive WT conditioned media migrated at the highest rate. When treated with estrogen negative media from disease fibroblasts, the wild type keratinocytes closed the wound at a significantly slower rate compared to the control media (Figure 3.12c). Overall, treatment with WT fibroblast conditioned media appeared to be beneficial to keratinocyte wound closure, whilst disease fibroblast conditioned media was more detrimental, as demonstrated by the apparent, albeit not significant, differences in wound closure between the two (Figure 3.12d/c).
Figure 3.12 - Wound closure of Wild type and Disease keratinocytes treated with conditioned media from wound activated fibroblasts. (A) Disease keratinocytes (DK) and (B) wild type keratinocytes (WTK) were treated with wound activated media from fibroblasts (Fib) grown in the absence (-) or presence (+) of 17β-estradiol (E) (100nM). Wound closure profiles were generated (A-B) with wound area confluence is given as a percentage of the original wound area, measured at 2 hourly intervals – with 12 hour (C), 24 hour (D) and 48 hour (E) timepoints shown. Mean ±SEM, n=3 (biological replicates), *=p<0.05, **=p<0.01, ***=p<0.001.
3.6 Discussion

The 4 SNP haplotype examined in this study was originally linked to chronic wounds in a cohort of predominately Caucasian (EUR) patients from the Greater Manchester, UK area (Ashworth et al., 2008). By contrast, the primary skin cells available for this study were collectedly from the mainly Han Chinese (EAS) population of Singapore. Whilst rs2987983 and rs2978381 have similar incidence in both populations, the rs3783736 disease variant incidence is 2 fold lower in the EAS population whilst the rs1887994 disease variant does not occur at all (1000 Genomes Project (Genomes Project et al., 2015)). This limited the project to focusing upon the remaining 3 venous ulcer-associated SNPs. These SNPs are in linkage disequilibrium with one another (Supplementary Figure S.1). Cells harbouring these SNPs were selected from the IMB Skin Cell Bank after genotyping all keratinocyte samples. Three biological replicates of ‘Wild Type’ and venous ulcer-SNP harbouring ‘Disease’ (containing the nucleotide variants associated with venous ulcer patients) complementary fibroblasts and keratinocytes were selected (Table 3.1). Once identified, these cells could be used to test the effects of the SNPs on wound related processes. There are several drawbacks to using cells directly from patients in this manner. The SNPs are present in an otherwise varied genetic background amongst the samples. This makes it difficult to attribute any differences to the SNPs alone. Ideally these SNPs would be ‘knocked-in’ and tested in a neutral genetic background. Whilst this is possible using the CRISPR-cas system, it becomes very difficult and time-consuming and would be particularly problematic if 4 SNPs were to be inserted into the same cell (Singh et al., 2015). By using SNP-representative cells samples in triplicate, sourcing them from similar body sites to avoid further variation and by making observations based on specific responses to estrogen, these limitations can be negated. Primary cells have several advantages over immortalised cell lines. Primary cells of low passage more precisely reflect the phenotype of the primary tissue and are likely to maintain any epigenetic regulatory features and gene expression profiles. Whereas cell lines that are passaged many times are subject to genetic drift over time, selection based on media and are more prone to contamination with other cell lines and mycoplasma (Kaur and Dufour, 2012).

There are inconclusive and varied reports of estrogen receptor localisation in the skin. Whilst ERα has been detected in primates in all compartments of the skin, there seems to be scarce specific incidence other than in the sebaceous gland (Pelletier, 2000). ERβ has been detected in humans in the epidermis, dermis and capillaries (Thornton et al., 2003). Staining for estrogen receptors in chronic wounds reveals the presence of ERα, but an absence of ERβ (Strudwick, 2006). This study substantiates these findings (Figure3.2). ERα showed intense staining at the sebaceous gland, with somewhat strong staining at the hair follicle and very weak staining throughout the epidermis and dermal fibroblasts. ERβ also exhibited staining in the sebaceous gland and hair follicle, but with
more intense staining at the basal region of the epidermis but with no staining of the dermal fibroblasts. Immunohistochemistry study of the SNP model cells revealed the presence of ERβ in the both the wild type and disease keratinocytes and fibroblasts. Examining ERβ expression at the gene transcript level identified that whilst the disease keratinocytes both with and without estrogen treatment and wild type keratinocytes with estrogen treatment all showed similar levels of transcript, the wild type cells in the absence of estrogen treatment presented with a 3-fold greater level of transcript (Figure 3.3). At the protein level a similar pattern is present (Figure 3.4). Wild type cells grown in the absence of estrogen show a markedly higher level than disease and wild type estrogen positive cells. This seems to indicate that the effect on expression by the SNPs is at the gene transcription level, rather than at the protein translation level. This aligns with a recent report which showed that the rs35036378 SNP of the ERβ 0N region caused a 50% reduction in the promoter activity (Philips et al., 2012).

Estrogen is well known to increase keratinocyte and, to a lesser extent, fibroblast proliferation (Brincat et al., 1987) (Martin, 1997) (Tomaszewski et al., 2003) (Luo et al., 2014) (Emmerson et al., 2009) (Stevenson et al., 2008). In this study, the concentration of 100nM 17β-estradiol was used to treat cells as it falls within the normal physiological levels and after dose titration in scratch assays revealed it to be the optimum concentration (Supplementary Figure S2). There are numerous estrogen-responsive genes so it is likely that this occurs through a number of pathways. This study has looked at keratinocyte and fibroblast proliferation in vitro and therefore in isolation, but estrogen can increase keratinocyte proliferation both intrinsically and extrinsically. Estrogen increases the expression of KGF, which is especially increased upon wounding, and has been demonstrated to increase keratinocyte proliferation (Andreadis et al., 2001; Verdier-Sevrain et al., 2004; Zhang et al., 1998). However, it has been suggested that KGF is not extensively produced by epidermal cells, but rather by mesenchymal cells signalling in a paracrine fashion (Rubin et al., 1995). This indirect regulation of keratinocyte proliferation by estrogen is therefore not present in these assays. It has recently been reported that estrogen regulation of the extracellular signal-regulated kinase/protein kinase B (ERK/Akt) signalling pathway can accelerate wound healing by promoting keratinocyte proliferation (Zhou et al., 2016), whilst another study suggests that the effect of estrogen on keratinocyte proliferation is most likely mediated in part by activation of a non-genomic, membrane-associated signalling pathway involving kinases 1 and 2 and by genomic signalling through activation of nuclear receptors (Verdier-Sevrain et al., 2004). This provides evidence for the intrinsic role of estrogen in the regulation of keratinocyte proliferation.

In both the wild type and disease-SNP keratinocytes, a clear increase in proliferation can be seen when treated with 17β-estradiol demonstrating that estrogen signalling is occurring (Figure 3.5), in line with published studies (Zhou et al., 2016). Wild type cells proliferate at a greater rate than
disease cells both in the presence and absence of estradiol, indicating that they have a greater innate proliferative capability, possibly due to greater level of ERβ seen in the wild type cells. In the wild type cells, treatment with both ERα and ERβ is comparable to estradiol treatment. In the disease cells, this affect is greater suggesting that stimulation of single receptors is superior to signalling through heterodimers. It has traditionally been thought that ERα has the main promotive effect upon cell proliferation and that ERβ inhibits ERα action, but can compensate for its action when ERα is absent (Pettersson and Gustafsson, 2001). This is indeed what is seen with disease keratinocytes, but not to the same extent in wild type cells. When estrogenic ligands are removed, proliferation is severely reduced in the disease keratinocytes but not wild type. This is somewhat divergent from the literature, which suggests hyperproliferative keratinocytes are present at the wound margin of chronic wounds (Pastar et al., 2014). However, there are various other extrinsic factors at the wound site that are not represented in these simple assays and the proliferation rate of keratinocytes is important at the onset of a wound (Usui et al., 2005).

The literature notes that estradiol treatment has a limited effect on dermal fibroblast proliferation in vitro (Stevenson et al., 2009), which is corroborated by this study (Figure 3.5) where no significant effect was seen in either group. As with the keratinocytes, the wild type fibroblasts had a significantly higher proliferation rate than the disease cells, but only when treated with estradiol. Both DPN and PPT treatment showed a significant reduction in proliferative capability of wild type cells. This however, is not corroborated by the literature, which shows no effect of DPN and PPT treatment in human fibroblasts (Stevenson et al., 2009). Although, this study focuses on specific subsets of fibroblast based on genotype (and ERβ level), which may have different phenotypes compared to those tested by Stevenson et al. Our findings suggest that disease fibroblasts are significantly less proliferative than wild type cells when treated with estradiol and that this may be due to differences in estrogen receptor expression.

Cell migration, another important feature of wound healing, is promoted by estrogen in both fibroblasts and keratinocytes (Campbell et al., 2010) (Emmerson et al., 2009) (Stevenson et al., 2009). Ovariectomised mice which are lacking estrogen show a severe delay in reepithelialisation that can be rescued by estrogen treatment (Ashcroft et al., 1997a) and human chronic wounds show a reduced expression of ERβ (Strudwick, 2006). The elderly are more prone to the development of chronic wounds than the young, but topical and systemic estrogen treatment can provide protection from the development of these wounds (Ashcroft et al., 1999) (Margolis et al., 2002). Venous ulcer-associated SNPs of ERβ appear to reduce the level of available receptor which could impact upon estrogen signalling and the ability of skin cells to migrate.

Whilst estrogen has previously been shown to increase keratinocyte migration in vivo and in vitro (Ashcroft et al., 1997a) (Campbell et al., 2010), here there was no significant effect seen by
estrogen treatment in either group or at any time-point (Figure 3.10a/b). There was, however, a significant increase in keratinocyte migration when ERα-specific agonist PPT was used in both wild type and disease cells. In addition, once the proliferative ability of the keratinocytes was removed using Mitomycin C treatment, this significant increase by PPT was eliminated (Figure 3.10c/d). This suggests that the increased ability of the cells to close the wound was due to their increased proliferative capability, rather than an increase in migration capability. This is supported by the proliferation assay results which showed significant decreases in doubling times upon estradiol treatment (Figure 3.5a). This is in stark contrast to previous work by the lab (Campbell et al., 2010). The activity of estradiol was confirmed by dose dependent treatment, with 100nM being selected as the optimum concentration (Supplementary Figure S.2). This study focused upon human cells in vitro while most previous work from this lab studied murine cells both in vivo and in vitro. The difference seen could be as a species differences or due to the limitations of in vitro experimentation.

Keratinocytes of wild type genotype were consistently significantly faster at closing scratch wounds than disease keratinocytes, suggesting an innate ability to migrate faster that is independent of their proliferative capacity (Figure 3.10). Under Mitomycin C treatment, the migration of disease type keratinocytes virtually ceases after 24 hours and no further migration continues, whereas the wild type cells continue to migrate. This is interesting as it broadly reflects what is seen in chronic wounds and suggests that the presence of ERβ SNPs could play a role in the cessation of reepithelialisation in these wounds. ERβ SNPs appear to inhibit the expression of the receptor (Figure 3.3 and 3.4), as is seen in chronic wounds (Strudwick, 2006), and ERβ is the conduit through which the beneficial effects of estrogen upon wound healing are transduced (Campbell et al., 2010). This deficiency in ERβ would lead to a reduction in downstream signalling and gene activation that is necessary for keratinocyte migration.

The migration of wild type fibroblasts was not significantly affected by estradiol treatment or specific ER agonists, whereas disease fibroblasts migration was increased by both estradiol and ERβ-specific agonist, but not significantly by ERα-specific agonist (Figure 3.11b). This indicates a role for ERβ signalling in SNP harbouring fibroblasts and that a lack of signalling results in delayed scratch closure. A similar experiment conducted by Stevenson et al on human fibroblasts showed a significant increase in migration under estradiol treatment and ERα-specific agonist treatment, but not with ERβ-specific agonist treatment (Stevenson et al., 2008). As seen with the keratinocytes, wild type fibroblasts close scratch wounds at a significantly faster rate than disease fibroblasts in the absence of estradiol. Removing fibroblasts proliferative capability through Mitomycin C treatment eliminates statistically significant differences between treatments in both groups indicating a disproportionately large contribution of proliferation to the scratch wound closure. The results here are unclear and it does not appear that the action of the cells
corresponds to the SNP status of the cells or a response to estrogen signalling. It is important to note here that estrogen can also signal through membrane bound receptors through kinase pathways and that this effect may be distorting the relative influence that the nuclear estrogen receptors are having on cell migration.

The timely healing of a wound requires the coordinated action of several cell types with complex communications and regulation of signalling pathways between them. As such, characterisation of the action of skin cells in isolation, whilst allowing for specific observations, can give limited results in the context of modelling wound behaviour. Keratinocytes and fibroblasts signal between each other in a paracrine and autocrine fashion in the wound environment. Keratinocytes release TGFα and PDGF as well as TGFβ isoforms and fibroblasts release FGF2, KGF, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF) and TGFβ1 amongst other signalling molecules (Singer and Clark, 1999) (Werner et al., 2007). The impact of venous ulcer-associated SNPs could therefore be amplified by disruption of a signalling axis between cell types. Treating keratinocytes with conditioned media from fibroblasts wounded in the presence and absence of estrogen allows for the interaction between fibroblast signalling and keratinocyte migration to be explored. Once again, the keratinocytes closed wounds at a faster rate than their disease counterparts (Figure 3.12). It was apparent that the media from the wild type fibroblasts treated with estradiol elicited the greatest rate of scratch wound closure in both wild type and disease keratinocytes and that media from disease fibroblasts in the absence of estradiol significantly delayed scratch wound closure in wild type keratinocytes. Wild type cells closed the wound at a significantly higher rate in response to wild type-conditioned media with estradiol treatment than with wild type-conditioned media without estradiol (Figure 3.12c). In contrast, Keratinocytes were not responsive to estradiol treatment in isolation (Figure 3.10), suggesting that paracrine signalling from fibroblasts in response to estrogen signalling is important for keratinocyte migration. Whilst conditioned media from wild type fibroblasts was beneficial to keratinocyte migration, condition media from disease fibroblasts significantly delayed wild type keratinocyte wound closure regardless of estradiol treatment at the 12 hour timepoint (Figure 3.12c). However, this significant difference was not seen at later timepoints. This suggests that the ERβ SNPs convey a growth factor and cytokine expression profile in fibroblasts that could be detrimental to keratinocyte migration. This is likely to compound the already deficient innate keratinocyte migrational capability and amplify the delay in reepithelialisation.

In summary, data here suggest that venous ulcer-associated SNPs do have an effect upon wound-relevant skin cell functions. Wild type fibroblasts and keratinocytes proliferate at a significantly
higher rate than disease cells. This would have an important impact upon a wound as higher proliferative cells could repopulate and resolve the wound in a more timely fashion. Estrogen treatment increases the proliferation of keratinocytes but not fibroblasts, suggesting differing roles for estrogen signalling in these two cell types. Wild type fibroblasts and keratinocytes close scratch assays at a faster rate than SNP-harbouring disease cells, which reflects the delayed reepithelialisation seen in venous ulcer patients. Keratinocytes were not affected by estrogen treatment, but wound closure rate was increased with the treatment of an ERα agonist. This points to a more important role for ERα in wound healing in humans than previously thought. This increase in wound closure disappears when the proliferative capability of cells is removed, indicating that the increase in wound closure seen here is may be facilitated by the action of ERα upon proliferation rather than migration. Disease fibroblasts do respond positively to estrogen treatment in regards to scratch wound closure representing a SNP specific difference in estrogen action. Incorporating wounded fibroblast signalling into keratinocyte scratch assays revealed interplay between the two skin cells. WT fibroblasts, in the presence of estrogen, gave out pro-healing signals to the keratinocytes, whereas disease fibroblasts in the absence of estrogen caused delayed keratinocyte scratch wound closure at early healing timepoints. This indicates that venous ulcer-SNP genotype in fibroblasts could have an indirect effect upon keratinocyte reepithelialisation in addition to the innate SNP-causing deficiencies within the keratinocytes, that could amplify the effects of the SNPs upon wound healing.

Whilst it is clear that ERβ SNPs are associated with venous ulcers, the mechanisms and specific functional effects of their action remained unclear. Here, it can be seen that ERβ expression is lower in SNP harbouring cells and that these SNPs directly reduce both keratinocyte and fibroblast migration and proliferation. Fibroblasts also indirectly reduce keratinocyte migration, possibly through an alteration in growth factor expression. The reduction in receptor likely reduces signalling by ERβ, limiting the regulation of protein activation and gene expression that is beneficial for wound healing. Due to its’s wide ranging beneficial effects on wound healing, estrogen is being considered as a treatment for chronic wounds. Whilst these ERβ SNPs provide an excellent biomarker for the predisposition to the formation of venous ulcers, it is unclear what role they would have in response to estrogen treatment. However, it appears from data presented here that targeting ERβ with specific agonists could address the deficiency in ERβ signalling. Validation that these disease-associated SNPs directly influence cell function strongly supports further investigation.
4. Chapter 4 - The effects of venous ulcer SNPs on ERβ transcriptional regulation

4.1 Introduction

Estrogen signalling activates an extensive range of downstream target genes, demonstrated by the 1069 human genes identified in the ‘Estrogen Responsive Genes Database’ and the ‘KnowledgeBase for Estrogen Responsive Genes’ (Tang et al., 2004; Tang et al., 2007) and the 3665 unique estrogen receptor binding sites identified by genomic profiling (Carroll et al., 2006). Any dysfunction in estrogen receptor regulation and expression would therefore have a substantial effect on downstream signalling and cellular function.

Knockout mice have been successfully generated for both ERα and ERβ (Krege et al., 1998; Lubahn et al., 1993). ERα null mice are infertile in both genders with a reduced bone mass, upregulated follicle stimulating hormone (FSH) and luteinising hormone (LH) and decreased sexual behaviour (Curtis Hewitt et al., 2000). Interestingly, a human subject has been identified with an ERα mutation which renders the receptor completely dysfunctional. He is tall with bilateral axillary acanthosis nigricans, but otherwise normal in appearance. Further analysis revealed a below average bone density and high serum levels of estradiol, FSH and luteinising hormone LH (Smith et al., 1994). ERβ null mice are characterised as fertile but the females have fewer than average pups and a high bone density (Curtis Hewitt et al., 2000).

Relating to the skin, ERα null mice have an increased level of collagen accompanied by a decreased level of MMP-15 whilst ERβ null mice have decreased skin collagen content accompanied by an increase in MMP8 and MMP15 (Markiewicz et al., 2013). Castrated ERα and ERβ null mice treated with estradiol display delayed reepithelialisation, while ERβ null alone have fewer M2 macrophages (Gilliver et al., 2010). This suggests that ERα transduces inhibitory effects of estrogen upon wound healing. Further wound studies in ovariectomised ER knock-out mice revealed that in the absence of ERβ, estrogen significantly delayed healing, while in ERα null mice, estrogen promoted repair. These data are supported by ER agonist studies, where the ERβ agonist DPN substantially improved healing in wild type ovariectomised mice (Campbell et al., 2010). Therefore, any dysregulation or reduction in ERβ and its signalling capacity would most likely alter the balance from a pro-healing phenotype to a delayed healing phenotype.

SNPs that are found in noncoding regulatory sequences can influence gene expression through post-transcriptional, transcriptional and post-translational means. Transcription depends on interactions between protein transcription factors and DNA at specific regulatory sequences, whilst the accessibility to these sequences is dependent on chromatin structure governed by posttranslational histone modifications, such as acetylation and methylation at specific sites. These methods of dysregulation are demonstrated with ERβ by the association of specific ERβ
SNPs with prostate cancer and the regulation at the 0N promoter region through methylation hotspots in prostate cancer cells (Thellenberg-Karlsson et al., 2006; Zhang et al., 2007). Moreover, the rs35036378 SNP of the 0N promoter region leads to a 50% reduction in receptor gene expression (Philips et al., 2012). Due to the three dimensional folding of DNA, distant regulatory signals can be brought into contact with promoter regions. This means that changes brought about by a SNP at any point in the 5’UTR could have an effect via multiple regulatory processes. This function can be predicted based on features such as histone modifications, open chromatin architecture and transcription factor binding sequences (Edwards et al., 2013). This chapter aims to determine the effect of venous ulcer-associated SNPs of the 5’UTR upon gene transcription and translation and in what manner cause these effects.

4.2 Allele Specific Expression assay (ASE)

Allele specific expression assays (ASE) are a tool used to test the functionality of SNPs in non-coding regions identified by genetic association studies (Bjornsson et al., 2008; Knapp et al., 2014). Here, the gene expression ratio between alleles containing the variant nucleotides of a particular SNP is measured. Using cells that are heterozygous for the SNP of interest enables the measurement of both alleles at the same time in an otherwise identical genetic and cellular environment. This direct comparison within the same sample nullifies possible trans-regulatory elements (as both alleles are subject to them equally) and enables a clear assessment of cis-regulatory actions (the SNP of interest) upon gene expression.

This assay made use of allele specific primer design in combination with RT-qPCR to measure allelic ratio. For each SNP, a common primer was designed of around 20bp in length and approximately 200-250bp from the SNP site. Two primers were designed at the SNP site itself with a slightly longer length to aid DNA binding. In each case the SNP was located as the very last 3’ base. A deliberate mismatch to the reference genome was also incorporated into each primer at the 3’rd 3’ position to aid in allele specific binding (Liu et al., 2012). A primer will bind to a DNA sequence despite there being a single mismatch, albeit in a suboptimal manner. However, if there are two mismatches at the 3’ end, the primer will not bind and no target sequence will be amplified. Therefore, each primer pair will specifically measure one allele over the other in a heterozygous cDNA pool. Comparing these two measurements reveals the allele specific expression ratio of each SNP.
4.2.1 Cell selection and isolation

Cells of the IMB Skin Cell Bank were genotyped for the venous ulcer-associated SNPs and heterozygous cells for each SNP selected. RNA was harvested from these cells under normal cell culture and media conditions and reverse transcribed to form cDNA. Cells from the IMB Skin Cell Bank are available for ethically approved studies within the Institute, including genetic analysis.

4.2.2 Assay validation

In order to validate assay accuracy and primer design, genomic DNA (gDNA) from cells known to be homozygous for each SNP variant were mixed in known ratios and the assay used to predict these ratios (Tables 4.1-4.3). RT-qPCR reactions were set up in triplicates for each gDNA mix and ASE primer pair with the Ct (threshold cycle) calculated for each. Since the ratio for 1:1 should contain the same amount for each allele, the Ct values should be equal. Any difference here would likely be due to pipetting error or difference in primer efficiency. Therefore the ΔCt of the 1:1 ratio was calculated and applied to the ΔCt of the remaining ratios Ct values to form the ΔΔCt. This ΔΔCt value could then be used to estimate the allele frequency in the sample gDNA pool:

\[
\text{Frequency of allele (\%)} = \frac{1}{(2^{\Delta\Delta Ct} + 1)} \times 100
\]

This calculated experimental value could then be compared to the theoretical values of the known ratios to give an indicator of the accuracy of the assay. The rs3783736 ASE assay had a mean percentage error of 2.01% across all ratios measured, with the greatest single error of 3.15% for the 1:2 ratio (Table 4.1). The rs2987983 ASE assay was far less accurate at predicting the correct allelic ratio with a mean percentage error of 6.58% and with the largest single error calculation of 10.18% for the 3:1 ratio (Table 4.2). The rs2978381 ASE assay returned a mean percentage error of 1.08% across all measured ratios with a maximum error of 2.23% in the 9:1 ratio (Table 4.3). Although the percentage error was rather large for the rs2987983 SNP, overall this validation confirmed the suitability of this assay allele specific expression in cell samples.
Table 4.1 – **rs3783736 Allele Specific Expression assay validation.** DNA from homozygous samples of either the A allele or C allele were mixed in known ratios before the ASE assay was used to predict these ratios through qPCR, Ct= threshold cycle, Δ = change/difference in value.

<table>
<thead>
<tr>
<th>DNA Ratio (A/C)</th>
<th>Allele</th>
<th>Ct mean</th>
<th>Stdev.</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>Theoretical %</th>
<th>Observed %</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>A</td>
<td>25.21</td>
<td>0.24</td>
<td>-0.05</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>25.26</td>
<td>0.05</td>
<td>0.05</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>A</td>
<td>26.06</td>
<td>0.26</td>
<td>1.16</td>
<td>1.21</td>
<td>33.33</td>
<td>30.18</td>
<td>3.15</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>24.90</td>
<td>0.09</td>
<td>-1.16</td>
<td>-1.21</td>
<td>66.67</td>
<td>69.82</td>
<td>-3.15</td>
</tr>
<tr>
<td>1:3</td>
<td>A</td>
<td>26.42</td>
<td>0.22</td>
<td>1.71</td>
<td>1.76</td>
<td>25.00</td>
<td>22.79</td>
<td>2.21</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>24.71</td>
<td>0.08</td>
<td>-1.71</td>
<td>-1.76</td>
<td>75.00</td>
<td>77.21</td>
<td>-2.21</td>
</tr>
<tr>
<td>1:9</td>
<td>A</td>
<td>27.71</td>
<td>0.10</td>
<td>3.36</td>
<td>3.41</td>
<td>10.00</td>
<td>8.62</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>24.35</td>
<td>0.24</td>
<td>-3.36</td>
<td>-3.41</td>
<td>90.00</td>
<td>91.38</td>
<td>-1.38</td>
</tr>
<tr>
<td>3:1</td>
<td>A</td>
<td>24.51</td>
<td>0.23</td>
<td>-1.71</td>
<td>-1.66</td>
<td>75.00</td>
<td>75.96</td>
<td>-0.96</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>26.22</td>
<td>0.04</td>
<td>1.71</td>
<td>1.66</td>
<td>25.00</td>
<td>24.04</td>
<td>0.96</td>
</tr>
<tr>
<td>9:1</td>
<td>A</td>
<td>24.71</td>
<td>0.38</td>
<td>-2.88</td>
<td>-2.83</td>
<td>90.00</td>
<td>87.67</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>27.59</td>
<td>0.07</td>
<td>2.88</td>
<td>2.83</td>
<td>10.00</td>
<td>12.33</td>
<td>-2.33</td>
</tr>
</tbody>
</table>
Table 4.2 – rs2987983 Allele Specific Expression assay validation. DNA from homozygous samples of either the T allele or C allele were mixed in known ratios before the ASE assay was used to predict these ratios through qPCR, Ct= threshold cycle, Δ = change/difference in value.

<table>
<thead>
<tr>
<th>DNA Ratio (T/C)</th>
<th>Allele</th>
<th>Ct mean</th>
<th>Stdev.</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>Theoretical %</th>
<th>Observed %</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>C</td>
<td>24.36</td>
<td>0.28</td>
<td>-0.66</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>25.02</td>
<td>0.49</td>
<td>0.66</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>C</td>
<td>24.78</td>
<td>0.02</td>
<td>0.85</td>
<td>1.51</td>
<td>33.33</td>
<td>25.94</td>
<td>-7.39</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>23.93</td>
<td>0.22</td>
<td>-0.85</td>
<td>-1.51</td>
<td>66.67</td>
<td>74.06</td>
<td>7.39</td>
</tr>
<tr>
<td>1:3</td>
<td>C</td>
<td>25.28</td>
<td>0.05</td>
<td>1.47</td>
<td>2.14</td>
<td>25.00</td>
<td>18.53</td>
<td>-6.47</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>23.80</td>
<td>0.05</td>
<td>-1.47</td>
<td>-2.14</td>
<td>75.00</td>
<td>81.47</td>
<td>6.47</td>
</tr>
<tr>
<td>1:9</td>
<td>C</td>
<td>25.97</td>
<td>0.19</td>
<td>2.52</td>
<td>3.18</td>
<td>10.00</td>
<td>9.92</td>
<td>-0.08</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>23.45</td>
<td>0.16</td>
<td>-2.52</td>
<td>-3.18</td>
<td>90.00</td>
<td>90.08</td>
<td>0.08</td>
</tr>
<tr>
<td>3:1</td>
<td>C</td>
<td>23.71</td>
<td>0.09</td>
<td>-1.55</td>
<td>-0.88</td>
<td>75.00</td>
<td>64.82</td>
<td>-10.18</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>25.26</td>
<td>0.01</td>
<td>1.55</td>
<td>0.88</td>
<td>25.00</td>
<td>35.18</td>
<td>10.18</td>
</tr>
<tr>
<td>9:1</td>
<td>C</td>
<td>23.67</td>
<td>0.04</td>
<td>-2.78</td>
<td>-2.11</td>
<td>90.00</td>
<td>81.23</td>
<td>-8.77</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>26.45</td>
<td>0.14</td>
<td>2.78</td>
<td>2.11</td>
<td>10.00</td>
<td>18.77</td>
<td>8.77</td>
</tr>
</tbody>
</table>

rs2987983
### Table 4.3 – rs2978381 Allele Specific Expression assay validation

DNA from homozygous samples of either the T allele or C allele were mixed in known ratios before the ASE assay was used to predict these ratios through qPCR. Ct = threshold cycle, Δ = change/difference in value.

<table>
<thead>
<tr>
<th>DNA Ratio (T/C)</th>
<th>Allele</th>
<th>Ct mean</th>
<th>Stdev.</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>Theoretical %</th>
<th>Observed %</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>C</td>
<td>27.07</td>
<td>0.10</td>
<td>-0.75</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>27.82</td>
<td>0.15</td>
<td>0.75</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>C</td>
<td>27.73</td>
<td>0.03</td>
<td>0.24</td>
<td>1.00</td>
<td><strong>33.33</strong></td>
<td><strong>33.38</strong></td>
<td><strong>0.05</strong></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>27.49</td>
<td>0.21</td>
<td>-0.24</td>
<td>-1.00</td>
<td><strong>66.67</strong></td>
<td><strong>66.62</strong></td>
<td><strong>-0.05</strong></td>
</tr>
<tr>
<td>1:3</td>
<td>C</td>
<td>28.24</td>
<td>0.30</td>
<td>0.74</td>
<td>1.50</td>
<td><strong>25.00</strong></td>
<td><strong>26.16</strong></td>
<td><strong>1.16</strong></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>27.49</td>
<td>0.08</td>
<td>-0.74</td>
<td>-1.50</td>
<td><strong>75.00</strong></td>
<td><strong>73.84</strong></td>
<td><strong>-1.16</strong></td>
</tr>
<tr>
<td>1:9</td>
<td>C</td>
<td>29.77</td>
<td>0.31</td>
<td>2.26</td>
<td>3.01</td>
<td><strong>10.00</strong></td>
<td><strong>11.02</strong></td>
<td><strong>1.02</strong></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>27.51</td>
<td>0.07</td>
<td>-2.26</td>
<td>-3.01</td>
<td><strong>90.00</strong></td>
<td><strong>88.98</strong></td>
<td><strong>-1.02</strong></td>
</tr>
<tr>
<td>3:1</td>
<td>C</td>
<td>26.89</td>
<td>0.11</td>
<td>-2.41</td>
<td>-1.66</td>
<td><strong>75.00</strong></td>
<td><strong>75.92</strong></td>
<td><strong>0.92</strong></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>29.30</td>
<td>0.52</td>
<td>2.41</td>
<td>1.66</td>
<td><strong>25.00</strong></td>
<td><strong>24.08</strong></td>
<td><strong>-0.92</strong></td>
</tr>
<tr>
<td>9:1</td>
<td>C</td>
<td>26.71</td>
<td>0.14</td>
<td>-3.60</td>
<td>-2.84</td>
<td><strong>90.00</strong></td>
<td><strong>87.77</strong></td>
<td><strong>-2.23</strong></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>30.30</td>
<td>0.67</td>
<td>3.60</td>
<td>2.84</td>
<td><strong>10.00</strong></td>
<td><strong>12.23</strong></td>
<td><strong>2.23</strong></td>
</tr>
</tbody>
</table>
4.2.3 Primer efficiency and assay normalisation

ASE assays were performed on cDNA samples derived from cells heterozygous for each SNP in triplicate. Normalisation of the values to the reference gene RPLP0 initially demonstrated that the wild type allele was expressed to a 3.35-fold higher degree for rs3783736, 1.78-fold higher for rs2987983 and 0.86-fold lower for rs2978381 (Figure 4.1a). However, as previously mentioned the primers used in these assays are sub-optimal and differ by a single base and as such, one would expect there to be a discrepancy in their efficiencies. The PCR efficiency, which is given as a number between 1 and 2 (2 indicates 100% efficiency), can be defined as the increase in amplicon per cycle and can be influenced by a number of factors such as template concentration and polymerase source (Booth et al., 2010). In this case, the only variable factor in the assay is the primer design. Ct values cannot be directly compared without this difference in efficiency being accounted for. Efficiency can be calculated by using qPCR to generate Ct values of serially diluted sample cDNA and plotting these values against the log of the concentration (Figure 4.1b). Generated slopes can then be used to determine the efficiency (Efficiency (E) = 10(-1/slope)). Efficiencies of the rs3783736 alleles were A=1.823 and C=1.825, rs2987983 alleles T=1.915 and C=1.951 and rs2978381 alleles T=1.919 and C=1.834.

These efficiency values can then be factored into the allele specific expression ratio calculations to provide an adjusted, accurate comparison. This was carried out using the following equation:

\[
\text{Allele specific expression ratio} = \frac{(E_{\text{ref}})^{\text{Ct}_{\text{ref}}}}{(E_{AS1})^{\text{Ct}_{AS1}}} \pm \frac{(E_{\text{ref}})^{\text{Ct}_{\text{ref}}}}{(E_{AS2})^{\text{Ct}_{AS2}}}
\]

\(AS1=\text{specific allele 1}, AS2=\text{specific allele 2}, E=\text{efficiency}, \text{ref}=\text{reference gene}, \text{Ct}=\text{threshold cycle}\)
Figure 4.1 – rs3783736 Allele Specific Expression assay. Cells heterozygous for the SNP were used in qPCR reaction to measure (A) the initial allele ratio and (B) the primer efficiencies of the primer pairs in order to identify their efficiencies. The calculated efficiencies were then used to normalise the initial ratios for the differences in efficiencies to derive the true allele ratio (C). Dashed grey lines represent the 95% confidence interval of the slope.
Figure 4.2 – rs2987983 Allele Specific Expression assay. Cells heterozygous for the SNP were used in qPCR reaction to measure (A) the initial allele ratio and (B) the primer efficiencies of the primer pairs in order to identify their efficiencies. The calculated efficiencies were then used to normalise the initial ratios for the differences in efficiencies to derive the true allele ratio (C). Dashed grey lines represent the 95% confidence interval of the slope.
Figure 4.3 – rs2978381 Allele Specific Expression assay. Cells heterozygous for the SNP were used in qPCR reaction to measure (A) the initial allele ratio and (B) the primer efficiencies of the primer pairs in order to identify their efficiencies. The calculated efficiencies were then used to normalise the initial ratios for the differences in efficiencies to derive the true allele ratio (C). Dashed grey lines represent the 95% confidence interval of the slope.
4.2.4 Allele specific expression assays reveal that venous ulcer-associated SNPs effect ERβ expression

Once efficiencies had been accounted for, the true allele specific expression ratios could be identified. The rs3783736 allele expression ratio was initially 3.35:1 in favour of the WT SNP variant (Figure 4.1a), but after adjustment was calculated as 2.77:1 in favour of the WT SNP variant (Figure 4.1c). The rs2987983 allele expression ratio was 1.77:1 initially (Figure 4.2a), but this was reduced to 1.03:1 after efficiency correction (Figure 4.2c). This can be considered as an equal level of expression between alleles. With the final rs2978381 SNP, the initial allele expression ratio was 1.16:1 in favour of the venous ulcer-associated (disease) allele (Figure 4.3a). Upon efficiency correction, this ratio altered to 4.75:1 in favour of the disease allele. This result suggests that the disease variant has a functional effect by increasing ERβ expression. Linkage disequilibrium (LD) could play a role in the results here. Linkage disequilibrium can be defined as the non-random association of alleles at different loci - SNPs are inherited together to a greater degree than would be expected by chance. The functional effect of an increase of decrease in expression could therefore be caused by a SNP in LD with the tested SNP rather than by the tested SNP itself.

4.3 Transcription factor binding

4.3.1 Bioinformatic predictions

Transcription factor binding software can be used to predict transcription factors that might bind to a given sequence using databases of experimentally validated binding motifs and positional weight matrices. Incorporating the alternative nucleotides at a SNP position in this analysis can identify potential SNP-specific differences in transcription factor binding. RegSNP is a tool that is designed to produce transcription factor binding site likelihood scores at positions of single nucleotide polymorphisms, employing the MATCH™ algorithm (Quandt et al., 1995) and using the public Transfac (Matys et al., 2006) and Jaspar (Mathelier et al., 2016) transcription factor binding databases. This can give an indication of possible functional difference in gene regulation. This tool was used alongside information from the UCSC human Genome Browser (Kent et al., 2002) to build up a picture of how gene regulation could be effected by the venous ulcer-associated SNPs.

Applying this strategy to the rs3783736 SNP reveals the presence of regulatory features (UCSC Genome Browser; Figure 4.4a). The rs3783736 SNP lays immediately upstream (1569bp) of the
first coding exon for ERβ. This region is elevated for the H3K27Ac histone mark, determined by chromatin immunoprecipitation sequencing (ChIP-seq) experiments, particularly in normal human epidermal keratinocytes (NHEK) (Ernst et al., 2011). The H3K27Ac is the acetylation of lysine 27 of the H3 histone protein which is thought to enhance transcription by blocking the repressive H3K27Me3 and changing the chromatin architecture to make the DNA more accessible for transcription. The SNP also falls within an experimentally identified DNaseI hypersensitivity site (Figure 4.4a). These areas of chromatin are vulnerable DNase action, losing their compact structure to expose the DNA to transcriptional mechanisms (Gross and Garrard, 1988). The RegSNP transcription factor binding prediction tool (Quandt et al., 1995) identified 2 potential transcription factors that may differentially bind depending on the nucleotide present (Figure 4.4b). The software provides an experimentally derived positional weight matrix score for each allele. The difference between alleles gives a ratio which indicates how potentially influential the SNP is with respect to the transcription factor binding. In this case the software predicts the presence of a SNP-dependent homeobox binding motif and particularly for the homeobox-containing transcription factor Nkx2-5.

Examining the genetic landscape at the rs2987983 SNP site reveals a number of relevant features which implicates the region as an important regulatory hub (Figure 4.5a). The rs2987983 is located upstream of the 0N untranslated exon and promoter region and downstream of the 0K untranslated exon and promoter. There is a high degree of H3k27Ac mark identified across the region, particularly in human umbilical vein endothelial cells (HUVEC) and to a lesser extent NHEKs and K562 cells (immortalised myelogenous leukaemia line) (Ernst et al., 2011). It also falls within a DNaseI hypersensitivity site and at the binding location of several transcription factors, including the activator protein 1 (AP1) binding complex and GATA2 (Wang et al., 2012). AP1 regulates cell growth and is particularly important in skin homeostasis and regeneration. In wound healing, AP1 is downstream of cytokine signalling and regulates the balance between keratinocyte proliferation and differentiation (Angel et al., 2001). RegSNP transcription factor binding prediction software identifies several transcription factor binding motifs at the SNP site (Figure 4.5b). The significant motif, downstream core element (DCE-S-III) is involved in regulating recruitment of RNA-polymerase for gene transcription (Smale and Kadonaga, 2003) (Lewis et al., 2000). A plethora of other transcription factor binding motifs are found at the SNP site with differential positional weight matrices dependent upon the SNP.

The rs2978381 SNP is further upstream of the 0N exon and promoter region, between the X3 and X4 cassette exons. This site lacks histone markers and is in the vicinity of two DNaseI hypersensitivity sites, but not directly within them (Figure 4.6a). It does not fall at a site that has been identified to bind any transcription factors by ChIP-seq (Angel et al., 2001). However,
RegSNP transcription factor binding prediction identifies possible differentially binding for GATA1, GATA2 and heat shock factor (HSF) transcription factors.
Figure 4.4 – Transcription factor binding differences revealed by bioinformatic and electrophoretic mobility shift assay analysis of rs3783736. (A) Data compiled by the ENCODE Genome Browser (Kent et al., 2002) reveals rs3783736 to lie in a DNase I hypersensitivity region with a slight enrichment of H3K27Ac in NHEK cells. (B) SNP transcription factor binding difference prediction using REGSNP software where start and end denote the sequence position of the transcription factor motif, strand indicates to which strand of the DNA the motif is found, allele high score indicates the higher positional weight matrix score of the two alleles, while score difference indicates the position weight matrix score difference between the two alleles and the ratio is formed from these two scores. (C) Electrophoretic mobility shift assay using biotin labelled probe representing wild type (WT) sequence and disease-associated (D) sequence, HeLa nuclear extract and unlabelled ‘cold’ competitor probe, reveals a loss of transcription factor binding with the disease variant.
Transcription factor binding differences revealed by bioinformatic and electrophoretic mobility shift assay analysis of rs2987983.

(A) Data compiled by the ENCODE Genome Browser (Kent et al., 2002) reveals rs2987983 to lie in a DNase I hypersensitivity region with an enrichment of H3K27Ac, and at the binding site of numerous transcription factors and RNase polymerase II. (B) SNP transcription factor binding difference prediction using REGSNP software where start and end denote the sequence position of the transcription factor motif, strand indicates to which strand of the DNA the motif is found, allele high score indicates the higher positional weight matrix score of the two alleles, while score difference indicates the positional weight matrix score difference between the two alleles and the ratio is formed from these two scores. (C) Electrophoretic mobility shift assay using biotin labelled probe representing wild type (WT) sequence and disease-associated (D) sequence, HeLa nuclear extract and unlabelled ‘cold’ competitor probe, reveals a difference in transcription factor binding between SNP variants.
Figure 4.6 – Transcription factor binding differences revealed by bioinformatic and electrophoretic mobility shift assay analysis of rs2978381. (A) Data compiled by the ENCODE Genome Browser (Kent et al., 2002) reveals rs2978381 to lie in a region of little gene regulation activity. (B) SNP Transcription factor binding difference prediction using REGSNP software where start and end denote the sequence position of the transcription factor motif, strand indicates to which strand of the DNA the motif is found, allele high score indicates the higher positional weight matrix score of the two alleles, while score difference indicates the position weight matrix score difference between the two alleles and the ratio is formed from these two scores. (C) Electrophoretic mobility shift assay using biotin labelled probe representing wild type (WT) sequence and disease-associated (D) sequence, HeLa nuclear extract and unlabelled ‘cold’ competitor probe, reveals similar transcription factor binding between SNP variants, but with a differing degree of specificity in the binding.
Electrophoretic mobility shift assays (EMSAs) reveal differential transcription factor binding

To determine if transcription factors (TF) do differentially bind to these specific SNP sites, electrophoretic mobility shift assays (EMSA) were employed (Gurevich et al., 2010). Biotin-labelled probes were designed to both the healthy and disease-associated variant of each SNP (31bp sequences with centrally located SNP nucleotide). These were then 3’ end labelled with biotin and annealed to their complimentary strand. EMSA assays were carried out using HeLa nuclear extract which provides a large array of transcription factors common in keratinocytes and fibroblasts. Transcription factor bound probe is revealed by retarded migration during gel electrophoresis.

The rs3783736 SNP showed a difference in transcription factor binding between wild type and disease alleles. When labelled wild type probe is incubated with nuclear extract, a band can be seen after gel separation (Figure 4.4c). This band is diminished when excess of the same unlabelled or ‘cold’ probe is incorporated, indicating that this is a specific binding event. When this is repeated with the disease probe, which has a single nucleotide difference, there is no band present in the gel. This indicates that the transcription factor that binds to the motif with the wild type variant of the SNP, can no longer bind once the nucleotide sequence has changed.

The rs2987983 SNP also shows a difference in transcription factor binding between the wild type and disease representative probes (Figure 4.5c). The wild type probe shows 2 bands indicating that 2 separate transcription factors can bind to the sequence. However the intensity of the band is not reduced to a great degree upon the addition of ‘cold’ probe, suggesting that this binding may be non-specific. These 2 bands are seen once again when disease probe in incubated with the nuclear extract. In this case though, a 3rd band is also present. This band is significantly reduced upon addition of ‘cold’ probe suggesting a direct competition for binding and therefore specificity. This assay indicates that a motif, for an otherwise unbound transcription factor, is introduced by the rs2987983 SNP.

The EMSA for the rs2978381 SNP also showed 2 bands after gel electrophoresis, suggesting the possible binding of 2 transcription factors (Figure 4.6c). These 2 bands were seen with both the wild type and disease variant, although more intense in the wild type. When excess ‘cold’ probe was incorporated with the reaction, in both cases the bands intensities were reduced indicating specific binding. In this instance the differing band intensities may indicate functional significant alteration in transcription factor binding affinity between wild type and SNP motifs.
4.4 Discussion

Once a SNP or haplotype has been identified as being associated with a particular phenotype or disease type, it can be difficult to find the functional link. This is because the identified SNP could be in linkage disequilibrium with an as yet unidentified functional SNP, and thus merely reporting on a functional effect elsewhere. Even if the responsible SNP is identified, it can still be difficult to identify the specific functional effect because there are diverse potential levels of action. Non-coding SNPs of the untranslated region can influence both transcription and translation in a number of different ways. Due to the localisation across the ON promoter region, it can be reasonably considered that the venous ulcer-associated SNPs in this study may have an effect upon the transcriptional regulation of ERβ.

Allele specific expression assays have successfully been used in a number of studies to determine the functional effect of a SNP upon gene expression (Bjornsson et al., 2008; Knapp et al., 2014; Sun et al., 2010). By measuring the relative abundance of the allele specific transcript, one can determine the functional effect of the change in nucleotide upon gene expression. Using a cell heterozygous for the SNP in question removes the influence of trans-acting regulation, and considers cis-acting regulation in isolation. Although the SNPs are present in the 5'UTR region of the gene, they will be incorporated into the pre-mRNA providing that they are downstream of the promoter. This situation is complicated by the multiple promoters present in the ERβ gene. The SNPs rs29878381 and rs2987983 are downstream of the OK promoter but upstream of the ON promoter, whilst rs3783736 is downstream of both promoters. Tissue specific utilisation of the alternate promoters has been suggested. In the prostate, for example, ERβ1 appears to be transcribed predominantly from promoter ON (Zhang et al., 2007). Both promoters are expressed to a somewhat similar degree in keratinocytes (Figure 5.1). This limits the effectiveness of the allele specific expression assay because there are two possible sources of transcript which are disparately regulated. Depending on the position of the SNP, the assay could report on transcripts from one or both promoters and won’t necessarily account for the regulatory function of the SNP being tested. However, the SNPs in question are in a high level of linkage disequilibrium (LD) with one another and so can be used as reporters for the effects of upstream SNPs (see Supplementary Figure S.1).

The allele specific expression assay of the rs3783736 SNP, which is located the furthest downstream of the promoters, indicated that the WT variant was transcribed 2.8-fold more than the disease variant (Figure 4.1c). This indicates that the rs3783736 SNP or a SNP in strong LD with it has a direct effect upon gene transcription, with the disease variant introducing a change that significantly reduces expression. The rs2987983 SNP allele specific expression assay revealed that both transcripts were present in equal abundance, as would be expected if there was no
difference in gene regulation (Figure 4.2c). However, because this SNP is upstream of the 0N promoter region, the transcripts detected cannot be transcribed from that promoter, but rather by the 0K promoter. This means that this SNP could be having an effect on the regulation of the 0N promoter and thus impact upon the overall transcript production of ERβ, which is not reported in this assay. The allele specific expression assay for rs2978381 SNP revealed a 4.7-fold lower abundance of WT transcript compared to the disease variant (Figure 4.3c). Once again, this SNP is upstream of the 0N promoter and hence its effect upon this promoter cannot be discerned from this assay. However, it does appear that this SNP, or a SNP is strong LD has an effect to increase the expression of ERβ from the 0K promoter.

Exploring the genetic landscape at a SNP site can provide information to appraise whether it is likely to affect gene regulation and expression (Schaub et al., 2012). Combining data for DNase I hypersensitivity cluster (Consortium, 2012) (Thurman et al., 2012), ChIP-seq validated transcription factor binding data (Gerstein et al., 2012) (Wang et al., 2013) (Wang et al., 2012) and H3K27Ac marker enrichment (Consortium, 2012) from the ENCODE consortium at each SNP loci enables the ASE assay data to be put into genetic context.

The rs3783736 SNP (increased 2.8 fold in WT) lies in a DNase I hypersensitivity region (Figure 4.4a). These regions are susceptible to cleavage by the DNase I enzyme which reflects a reduced nucleosome occupancy, loss of condensed chromatin structure and exposed DNA as a consequence of transcription dependent nucleosome turnover (Mito et al., 2007) (Workman, 2006). These are thought to be areas of gene regulation and sites of transcription factor binding (Gross and Garrard, 1988). The rs522444 SNP, in a DNase I hypersensitivity of the kinesin-associated protein 3 (KIFAP3) gene, has been experimentally validated to reduce gene expression (Landers et al., 2009) highlighting that SNPs can have a functional effect upon gene expression at these sites. There is a slightly elevated enrichment of H3K27Ac, particularly in NHEK cells at the vicinity of rs3783736, which also suggests a region of regulatory importance (Figure 4.4a).

Transcription factor binding prediction software recognises motifs for homeobox proteins, particularly Nk2 homeobox 5 (Nkx2-5), with different positional weight matrix scores between the SNP alleles (Figure 4.4b). Nkx2-5 is best known for its essential role in cardiomyogenesis (Ueyama et al., 2003), but it is also expressed in the skin and may play a role in the shift between cell proliferation and differentiation (Hwang et al., 2009). Electrophoretic mobility shift assays confirm specific transcription factor binding to the DNA sequence immediately surrounding the rs3783736 SNP in the wild type variant alone (Figure 4.4c). Allele specific expression assays revealed that the WT transcript was expressed 2.7-fold over the disease variant. Considering that the rs3783736 SNP falls in a minor regulatory region of the gene and in silico and in vitro evidence suggests allele specific transcription factor binding, it could be concluded that rs3783736 has a direct functional effect on ERβ transcription. However, the rs3783736 SNP is downstream of the main 0K and 0N
promoter regions making it unlikely to affect their function, but upstream of the recently suggested E1 promoter (Smith et al., 2010b). However, the E1 promoter has only been demonstrated in cancer cell lines and its utilisation was negligible compared to the ON promoter utilisation, making it unlikely that the SNP plays a role in ERβ expression through this promoter. Many DNase I hypersensitivity regions contain enhancer elements and one possibility is that the SNP is located in an enhancer site. Indeed it is possible for a SNP at an enhancer site to effect gene expression, demonstrated by the rs12821256 SNP which alters lymphoid enhancer-binding factor 1 (LEF1) transcription factor binding at an enhancer region, reducing the expression of KIT ligand KITLG and producing a phenotypic difference in hair pigmentation (Guenther et al., 2014). Another possibility is that the SNP is in close LD with another functional SNP, or acts in combination with other SNPs. It is clear however that the disease variant rs3783736 SNP is associated, whether directly or indirectly, with a reduction in ERβ gene expression.

rs2987983 also appears to lay in a regulatory region of the ERβ gene (Figure 4.5a). It is located in a DNase I hypersensitivity region and in a region which is highly enriched in histone H3K27Ac signifying an active enhancer region (Creyghton et al., 2010). Furthermore the SNP falls at the site of several ChIP-seq validated transcription factor binding sites (Angel et al., 2001), including members of the AP-1 forming families, pre-B-cell leukaemia homeobox 3 (PBX3), GATA2 and DNA-directed RNA polymerase II subunit RPB1 (POLR2A) which is the largest subunit of RNA polymerase II, responsible for RNA synthesis. Transcription factor binding prediction (Figure 4.5b) identified several possible differentially binding transcription factors at the SNP site. The downstream core element subelement III (DCE_S_III) is a transcriptional core promoter sequence that contains a binding motifs for RNA polymerase (Smale and Kadonaga, 2003). This has the highest allele positional matrix score and also the largest score difference between alleles. This is in consensus with the ENCODE compiled ChIP-seq data showing the binding of the RNA polymerase subunit RPB1 at the SNP site and suggests that the ability of RPB1 to bind at the site would be adversely affected by the rs2987983 SNP. The correct recruitment of RNA polymerase is essential for gene expression and any change in its binding caused by the change in sequence of rs2987983 would be predicted to decrease ERβ gene expression. Elements of AP1 such as c-Fos, c-Jun and BATF are also shown by ChIP-seq data to bind at the SNP site. The AP1 complex shares similar actions to estrogen receptor beta in the skin, being downstream of many cytokine signalling pathways and is involved in skin homeostasis and keratinocyte proliferation (Takahashi et al., 2002) (Shi and Isseroff, 2005) and differentiation (Adhikary et al., 2004) (Angel et al., 2001) and one of its constituents JUNB is important for timely wound repair (Florin et al., 2006). Disruption of ERβ expression could negate the effects of AP1 upon skin homeostasis and wound repair. Electrophoretic mobility shift assays revealed the potential binding of 2 transcription factors to the WT SNP variant and 3 binding to the disease-associated variant (Figure 4.5c). This suggests that the disease-SNP changes the DNA sequence to form a motif recognised by a factor
that would not otherwise bind. This could either increase transcription or reduce transcription by competing with the conventional transcription factors and interfering with the fine-tuned regulatory apparatus. The allele specific expression assay revealed no change in transcript expression (Figure 4.2c), but as previously described, this assay is not a true reflection of the effect of rs2987983 upon the 0N promoter region and that the downstream rs3783736 ASE assay, of which rs2987983 is in strong LD with, is a better reporter for any dysregulation at the 0N promoter by rs2987983.

Further to venous ulcers, rs2987983 has been identified as being associated with breast cancer (Treeck et al., 2009) and prostate cancer (Thellenberg-Karlsson et al., 2006), with authors speculating that the SNP could play a role in gene expression and subsequent cancer risk. The importance of this region for regulation is also highlighted by the finding that the methylation of a CpG island upstream of the 0N promoter can lead to the transcriptional inactivation of ERβ during prostate cancer development (Zhu et al., 2004). Other studies similarly found that methylation at the 0N promoter is associated with reduced expression and ERβ isoform expression differences in breast and ovarian cancers (Suzuki et al., 2008) (Zhao et al., 2003). If epigenetic silencing of the gene can occur at this region, other SNP-instigated changes in regulation would also be expected to influence gene expression. Another study took a bioinformatics approach to identify SNPs that could have an effect upon ERβ regulation (Philips et al., 2012). Although the authors did not identify rs2987983, they did identify a nearby SNP in the TATA box that was shown to reduce promoter activity and expression of ERβ by half in luciferase assays. This data, in partnership with supporting evidence from other studies, demonstrates the importance of the regulatory region in which the rs2987983 SNP lies and suggests that an alteration in transcription factor binding is likely to alter gene expression as seen in the downstream rs3783736 ASE assay.

The rs2978381 SNP (reduced 4.7 fold in WT) is located further upstream of the 0N promoter between cassette exons X3 and X4. It is in a relatively unremarkable region of the gene that does not fall in a DNase I hypersensitivity region or at the binding site of a validated transcription factor (Figure 4.6a). Furthermore, there is no H3K27Ac enrichment or any other regulatory feature at the locus. Transcription factor binding prediction did identify binding motifs for GATA1, GATA2 and the heat shock factor (HSF) family of transcription factors (Figure 4.6b). Two transcription factors were seen to bind in the EMSA assay (Figure 4.6c) with both the WT and D variant which could reflect those identified by the prediction software. However, the transcription factors that bind to the WT probe are not out-competed by the ‘cold’ probe, suggesting that the binding is not specific for this variant of the DNA sequence. Curiously, the ASE assay reveals that the WT variant is transcribed to an almost 5-fold lesser degree than the disease variant. Due to the SNPs location upstream of the 0N promoter, this assay is likely to report upon the activity of the 0K promoter. This appears irreconcilable with the ASE assay result for the nearby rs2987983 SNP which
indicated almost 1.8-fold more WT transcript. It could be possible that the SNPs are on opposite alleles to one another, distorting the result, or that the mRNA is processed differently between their locations (i.e. mRNA splicing). Transcription factor binding differences between the alleles could be further investigated using a recently developed method ‘reverse ChIP’ (Déjardin et al., 2008) (Mittler et al., 2009). This in vivo method uses a DNA probe to pull down proteins associated with a targeted DNA locus which can then be identified via mass spectrometry.

In summary, allele specific expression assays provide clear evidence that venous ulcer-associated SNPs affect gene transcription. Bioinformatic investigation, alongside electrophoretic mobility shift assays, indicate that SNPs also appear to directly alter transcription factor binding. rs2987983, is particularly exciting, located in an important regulatory region of the gene, and likely influencing ERβ transcription as reported by the rs3783736 ASE assay. ERβ is a global promoter of wound healing and any dysfunction in its regulation or expression that reduced the amount of receptor protein produced in the skin would diminish estrogen signalling and likely have a detrimental effect on the skin homeostasis and its ability to coordinate a healing response.
5. Chapter 5 - Venous ulcer-associated SNPS affect ERβ signalling during wound progression and alter the inflammatory profile

5.1 Introduction

Strong evidence for the importance of ERβ comes from the ERβ⁻/⁻ mouse which exhibits a delayed healing phenotype in the presence of estradiol ligand (Campbell et al., 2010). This study also found that signalling through ERα is detrimental to healing and that different phenotypes are observed as a result of ERβ signalling depending on the presence or absence of ERα. This suggests an important role for ER heterodimer dynamics in ERβ signalling. ERβ⁻/⁻ null mouse wounds are also characterised by a significant increase in neutrophils signifying that a loss of signalling through ERβ leads to an amplified inflammatory response. If estrogen receptor beta expression were strongly SNP regulated then one would expect downstream effects on estrogen regulated target genes, many of which are important in wound healing. It could be hypothesised that growth and migratory processes would be curtailed, and dysregulated signalling molecules such as cytokines and growth factors would result in an inappropriate inflammatory response.

Cytokine and growth factor signalling are important for the orchestration and timely resolution of wound healing (reviewed (Barrientos et al., 2008)). Upon wounding, keratinocytes express TGFα and TGFβ along with PDGF, FGF-2 and various cytokines of the IL family. Fibroblasts also upregulate these growth factors and cytokines along with EGF, FGF-7 and VEGF, whilst infiltrating macrophages express a large range of both inflammatory and anti-inflammatory agents depending on the phase of wound healing and their polarisation. Macrophages that are polarised towards M1 are pro-inflammatory, whereas macrophages polarised to the M2 phenotype are thought to be anti-inflammatory and pro-healing (reviewed (Ferrante and Leibovich, 2012)). The signalling and interplay of keratinocytes, fibroblasts and cells of the immune system relies on the precise temporal production of these factors at the correct levels throughout the wound healing process. The importance of these factors and the suggestion that SNPs could alter this signalling dynamic was hinted at in scratch assays of keratinocytes treated with conditioned media from fibroblasts with the healthy and disease variants of the venous ulcer associated SNPs (Figure 3.12). Fibroblast conditioned media appeared to regulate keratinocyte healing outcome in a SNP status-dependent manner. This chapter aims to characterise the effects of venous ulcer-associated SNPs on the cytokine and growth factor production of skin cells in a wound environment, and how this might in turn affect inflammatory cells in a chronic wound environment.
5.2 Estrogen receptor beta expression during wounding

To examine the relationship between venous ulcer SNPs and wound healing-related gene expression in keratinocytes, *in vitro* scratch assays were performed with RNA and protein collected at different time-points post-wounding. The expression of ERβ was analysed at the mRNA and protein level in both WT and disease cells to determine whether there were SNP-dependent differences in receptor expression over the time-course (Figure 5.1a and 5.2).
Figure 5.1 – Estrogen receptor beta and promoter mRNA expression wound time-course in keratinocytes in the presence and absence of estradiol. mRNA expression measured by qPCR relative to TBP reference gene across scratch assay time-points in disease and wild type keratinocytes in the absence (-ve) and presence (+ve) of estradiol (E2) treatment for total (A) ERβ, (B) 0K promoter exon containing transcript and (C) 0N promoter exon containing transcripts (as means ±SEM of n=3 (biological replicates)).
5.2.1 Estrogen receptor beta mRNA expression in wounded keratinocytes

Initially, ERβ mRNA is expressed to a similar degree in both WT and disease keratinocytes when treated with estradiol. However when estradiol is removed, a wound-responsive elevation in ERβ expression by approximately 2-fold is seen in the WT cells that is not mirrored in the disease cells (Figure 5.1a). ERβ expression 2 hours post-wounding is elevated by 1.43-fold in the WT estradiol-negative keratinocytes, 2.14-fold in the disease estradiol-negative keratinocytes and 1.68-fold in the disease estradiol treated keratinocytes, but receptor expression is not increased at all in the WT estrogen treated keratinocytes. Expression is quickly curtailed by 6 hours post-wounding and returns to the baseline level in all samples by 12 hours post-wounding. Interestingly, ERβ expression in WT keratinocytes does increase throughout wound healing and is 1.77-fold higher than baseline levels by 48 hours post-wounding.

To determine if specific promoter utilisation in the expression of ERβ varied between the SNP model cell lines, RT-qPCR was employed targeting sequences specific to the 0K and 0N untranslated exons that are found in mRNA transcripts and exclusively associated with each promoter (Figure 5.1b/c). These promoters have previously been found to be differentially dominant depending on tissue and disease state, although reports are conflicting (Zhao et al., 2003) (Lee et al., 2013) (Smith et al., 2010a) (Zhang et al., 2007). Here, transcriptional activity was detected in keratinocytes from both the 0N and 0K promoters.

Their expressional wound profiles broadly mirror overall ERβ transcript expression, but with interesting subtle differences. Before wounding, the 0N promoter is utilised to a greater extent in wild type cells compared to disease cells both with estradiol treatment and without. The 0K promoter is also utilised to a greater extent in wild type cells compared to disease cells, but only in the absence of estradiol. This indicates that 0K promoter utilisation is dependent on estradiol treatment, that 0N promoter utilisation is independent of estradiol treatment and that the utilisation of both promoters is reduced in cells harbouring venous ulcer SNPs.

Disease cell promoter utilisation is uniform throughout wound closure, other than a small increase in 0N promoter utilisation immediately after wounding in estradiol treated cells. In untreated wild type cells, 0K and 0K promoter utilisation is upregulated immediately post-wounding before sharply reducing, whilst estradiol treated cells gradually increase 0N utilisation throughout wound closure. Estradiol treated wild type show an initial reduction in 0K utilisation, which gradually increases throughout wound closure. This demonstrates that wild type cells have a more dynamic regulation of ERβ expression during wounding.
5.2.2 Estrogen receptor beta protein expression in wounded keratinocytes

The expression profile of ERβ at the protein level during keratinocyte in vitro wound healing was also examined (Figure 5.2). Interestingly, WT keratinocytes appear to have a higher degree of post-transcriptional modification than disease associated keratinocytes (Figure 5.2). Specifically, bands of higher molecular weight were detected at greater intensity in WT samples, particularly in the absence of estradiol ligand. The lower band represents the ERβ1 isoform at 59kDa and whilst the monoclonal antibody used does recognise all of the ERβ isoforms, ERβ1 is the largest isoform making it unlikely that the higher band is another ERβ isoform (Smith et al., 2010b). Negative controls (data not shown) indicate that this is not an artefact of the secondary antibody. Changes in intensity across time points of the same cells indicate that this is unlikely to be non-specific binding of the primary antibody. This makes it likely that the heavier band represents a post-translational modification of ERβ. Note that this modification is almost absent in the disease keratinocytes suggesting that the regulation of this modification is effected by the presence of venous ulcer-associated SNPs. ERβ expression was found to be consistently lower in disease SNP harbouring keratinocytes than wild type cells.
Figure 5.2 – Estrogen receptor beta protein expression wound time-course in keratinocytes in the presence and absence of estradiol. Representative Western blot and derived relative intensity bar graph for estrogen receptor beta (ERβ) expression across scratch assay time-points in disease and wild type keratinocytes, in the absence (E-) and presence (E+) of estradiol treatment, with corresponding beta-actin blot, as means ±SEM of n=3 (biological replicates) independent experiments.
5.3 Venous ulcer SNPs influence keratinocyte cytokine and growth factor expression

To further characterise the effects of venous ulcer associated ERβ SNPs on wound dynamics, the expression profile of several wound associated keratinocyte signalling molecules were examined across scratch assay time points in the absence and presence of estradiol treatment (Figure 5.3). Expression varied greatly across cell lines in each group resulting in a lack of significant findings, but some dissimilarity could be detected. Keratin 6a (KRT6a) expression was seen to increase post-wounding, indicating that the keratinocytes were in a wound responsive state (Figure 5.3).

The main wound source of the cytokine IL-6 is neutrophils and monocytes (Barrientos et al., 2008), yet it is also produced by keratinocytes (Kupper et al., 1989). IL-6 is necessary for normal wound repair (Gallucci et al., 2000), can indirectly induce keratinocyte migration through STAT3 activation (Gallucci et al., 2004), but is found at high levels in chronic non-healing wounds (Trengove et al., 2000). It is thought that IL-6 production is downregulated by estrogen, demonstrated by the increased IL-6 levels in estrogen deprived postmenopausal women (Rachon et al., 2002). WT keratinocytes responded to wounding with an increase of approximately 2-fold in IL-6 expression at 2 hours post-wounding, which quickly returned baseline within 6 hours (Figure 5.3). Surprisingly, estradiol treatment did not alter expression. Disease cells expressed approximately 100-fold less IL-6, which may underpin the delayed scratch assays wound healing in these cells (Figures 3.6, 3.7 and 3.12).

Transforming growth factor alpha (TGFα) is expressed as an autocrine and paracrine growth factor by keratinocytes (Klein et al., 1992), stimulating reepithelialisation and enhancing wound healing (Schultz et al., 1987). Transforming growth factor beta (TGFβ) is expressed by numerous cells in the wound environment (Amjad et al., 2007) where it modulates immune cell infiltration (Pakyari et al., 2013) and induces keratinocyte integrin expression to promote reepithelialisation (Gailit et al., 1994). Increased TGFβ levels in chronic wounds correlate with healing (Gohel et al., 2008) and evidence indicates that TGFβ expression is induced by estrogen signalling in wounds (Ashcroft et al., 1997a). Intriguingly, the mean expression of both TGFα and TGFβ was approximately 50% less in disease keratinocytes than wild-type across all time points measured (Figure 5.3).

Tumour necrosis factor alpha (TNFα) is an injury induced inflammatory agent (Gragnani et al., 2013) that modulates actin cytoskeleton regulators and integrins to enhance keratin migration (Banno et al., 2004). Of direct relevance serum levels of TNFα were elevated in patients carrying the venous ulcer-associated ERβ haplotype (Ashworth et al., 2008), whilst estrogen inhibits TNFα in a range of cells and tissues (Xing et al., 2007) (Takao et al., 2005). Here, TNFα levels were unchanged between WT and disease keratinocytes (Figure 5.3).
Figure 5.3 – Wound-related mediator mRNA expression wound time-course in keratinocytes in the presence and absence of estradiol. mRNA expression measured by qPCR relative to TBP reference gene, across scratch assay time-points of disease (D) and wild type (WT) keratinocytes in the absence (E-) and presence (E+) of estradiol (E) treatment, for interleukin-6 (IL6), transforming growth factor alpha (TGFα), transforming growth factor beta (TGFβ), tumour necrosis factor alpha (TNFα) and keratin 6a (KRT6a) (as means ±SEM of n=1-3 (biological replicates)).
5.4 Venous ulcer SNP effects on cytokine and growth factor expression in fibroblasts

The effects of venous ulcer associated ERβ SNPs on important wound cytokine and growth factor expression in fibroblasts was investigated by measuring gene expression in vitro at 2 hours post-wounding in the absence and presence of estradiol treatment (Figure 5.4). In brief, while all cytokines and growth factors measured were induced by estradiol treatment in WT fibroblasts, they were unaltered by estradiol treatment in disease fibroblasts. This provides strong evidence that ERβ SNPs influence fibroblast responsiveness to estrogen signalling.

Fibroblast growth factor 2 (FGF2) is a growth factor that is present in numerous tissues, including the wounded skin where it is expressed by keratinocytes, fibroblasts mast cells, smooth muscle cells and endothelial cells (Gonzalez et al., 1996) (Barrientos et al., 2008). FGF2 in upregulated upon wounding where it upregulates keratinocyte and fibroblast migration and ECM formation, whilst the absence of FGF2 results in delayed healing (Ortega et al., 1998) (Sogabe et al., 2006) (Kashpur et al., 2013) (Schreier et al., 1993) (Buckley-Sturrock et al., 1989). The expression of estrogen receptors is correlated with FGF-2 synthesis (Smith et al., 2002). FGF2 expression was not statistically significantly increased by estradiol treatment in disease fibroblasts, but was significantly increased by estradiol in wild type cells. Expression was statistically significantly higher in wild type fibroblasts compared to disease fibroblasts under estradiol treatment, but not in the absence of estradiol.

Vascular endothelial growth factor (VEGF) is expressed by fibroblasts, amongst other cells, in the wound (Barrientos et al., 2008) (Frank et al., 1995). It is an inducer of vascular permeability (Brkovic and Sirois, 2007), an endothelial cell mitogen (Leung et al., 1989) and can stimulate reepithelialisation and collagen deposition (Bao et al., 2009). It is also a target of estrogen signalling which induces VEGF expression in a range of estrogen regulated tissues (Mueller et al., 2000). WT fibroblast reflects this in the significant increase of VEGF expression subsequent to estradiol treatment (Figure 5.4). This significant increase is not repeated in disease fibroblasts, suggesting a dysfunction in estrogen signalling.

TGFα and TGFβ are both pro-healing factors in acute wounds (Schultz et al., 1987) (Gailit et al., 1994), with evidence showing that TGFβ is upregulated by estrogen (Ashcroft et al., 1997a). TGFα mRNA levels are significantly higher in WT fibroblasts compared to disease fibroblast, whilst expression is significantly increased by estradiol treatment in WT but not disease fibroblasts. This pattern is repeated with TGFβ, although WT untreated cells express TGFβ to a similar degree to disease cells. This again suggests at a dysfunction of estrogen signalling in disease fibroblasts.
Finally, matrix metallopeptidase 9 (MMP9) is an extracellular matrix protease that is necessary to allow cell migration and tissue remodelling in wound healing (Salo et al., 1994), but prolonged and excessive expression can be found in chronic wounds and is a predictor of poor healing (Liu et al., 2009). There is evidence that estrogen can both downregulate (Campbell et al., 2010) and upregulate (Oh et al., 2012) MMP-9 during wound healing. Here, the effect of estradiol followed the pattern of the other wound related molecules and growth factors (Figure 5.3). WT fibroblasts significantly upregulated MMP-9 expression in comparison to disease cells and upon estradiol stimulation.
Figure 5.4 – Wound-related mediator mRNA expression in fibroblasts in the presence and absence of estradiol 2 hours post-wounding. mRNA expression measured by qPCR relative to TBP reference gene, at 2 hours post-wounding of disease (D) and wild type (WT) fibroblasts (F) in the absence (E-) and presence (E+) of estradiol (E) treatment, for fibroblast growth factor-2 (FGF2), vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGFα), transforming growth factor beta (TGFβ), and matrix metallopeptidase 9 (MMP9) (as means ±SEM of n=3 biological replicates, *p<0.05, **p<0.01, ***p<0.001).
5.5 Venous ulcer SNP effects on the inflammatory profile of monocyte co-cultures

Studying the effects of ERβ SNPs on the production of signalling molecules in isolation can reveal important information about individual cell types, but a more physiologically relevant understanding can be acquired by examining the interaction of wound healing cells. To this end, fibroblasts harbouring either the disease associated SNP variants or WT SNP variants were wounded in co-culture with human peripheral blood derived mononuclear cells (PBMCs) and gene expression measured at 48 hours post-wounding. The experiment was performed under low estrogen conditions to mimic the reduced levels in venous ulcers.

5.5.1 Fibroblast inflammatory markers in co-culture with monocytes

Fibroblasts are responsible for repopulating and regenerating the damaged dermis by proliferating and infiltrating the nascent granulation tissue and remodelling the extracellular matrix. In the granulation tissue, they encounter and interact with cells of the immune system such as neutrophils and macrophages. Macrophages, which are derived from monocytes extravasating from the bloodstream, are responsible for the release of many inflammatory mediators in the wound. The interaction between fibroblasts and macrophages can determine the extent of the release of these inflammatory mediators and whether they are pro- or anti-inflammatory in nature. The co-culture of monocytes with wounded fibroblasts revealed a difference in the expression of wound relevant genes between the WT and SNP-harbouring disease fibroblasts (Figure 5.5). Disease fibroblasts showed a greater level of transcription of IL-1, IL-6, TNFα, MIF and MMP-9.

IL-1 a pro-inflammatory cytokine that is expressed by fibroblasts (Kumar et al., 1993) and can improve the wound healing quality in acute wounds (Thomay et al., 2009). However, inhibition of the IL-1β isoform in chronic wounds can improve wound healing by inducing a healing associated macrophage phenotype (Mirza et al., 2013). Fibroblasts with ERβ SNPs present with a greater IL-1 expression than the WT fibroblasts, which could induce a pro-inflammatory phenotype in the monocytes (Figure 5.5). As previously discussed, IL-6 is an inflammatory cytokine that is pro-healing in acute wounds, but is found at excessive levels in chronic wounds. IL-6 is found to be expressed to a greater extent in the disease fibroblasts, which while beneficial for acute wounding, could be problematic in venous ulcers. MIF is an inflammatory mediator that can be expressed by fibroblasts during wound healing (Abe et al., 2000) which can delay wound healing (Emmerson et al., 2009).

MIF has pro-inflammatory roles in lymphocyte activation and nitric oxide synthesis, counter-regulates the anti-inflammatory effects of glucocorticoids, leading to increased expression of IL-6,
IL-1 and IL-8 (Calandra and Bucala, 1995) and is found at high concentrations in chronic wounds (Grieb et al., 2012). Estrogen levels are inversely associated with MIF expression, modulating wound healing by downregulating MIF (Ashcroft et al., 2003). Microarray study determined that MIF affected a large proportion of the effects of reduced estrogen on wound repair (Hardman et al., 2005). In coculture with monocytes, disease fibroblasts revealed the greater expression level of MIF. This would prime the wound to a pro-inflammatory, non-healing phenotype and indicates the lack of susceptibility to estrogen signalling (Figure 5.5).

MMP-9 expression is higher in disease fibroblast than WT fibroblasts when cultured with monocytes (Figure 5.5) in direct contrast to the expression pattern when fibroblasts are grown in isolation (Figure 5.4). MMP-9 is important in the early phases of wound healing, but high levels are associated with a poor healing prognosis.

Secretory leukocyte protease inhibitor (SLPI) is an enzyme that protects epithelial tissues from serine proteases and regulates the inflammatory response by suppressing NFκB activation and subsequent cytokine and chemokine expression (Song et al., 1999) (Ashcroft et al., 2000) (Lentsch et al., 1999). SLPI is expressed in the wound (Wingens et al., 1998) with the SLPI null mouse acting as a model for severe delayed wound healing (Ashcroft et al., 2000). Estrogen has the ability to upregulate the expression of SLPI in various tissues (Chen et al., 2004) (Fahey et al., 2008) (McKiernan et al., 2011) and estrogen deficient post-menopausal women present with reduced SLPI levels (Shimoya et al., 2006). Hence, SLPI can be used as a marker of wound healing potential and estrogen receptor action. Here we see the WT fibroblasts expressing SLPI whilst it is almost undetectable in disease fibroblasts (Figure 5.5).
Figure 5.5 – Wound-related mediator mRNA expression in fibroblasts co-cultured with human monocytes at 48 hours post-wounding under low-estrogen conditions. mRNA expression measured by qPCR relative to TBP reference gene, at 48 hours post-wounding of disease (D) and wild type (WT) fibroblasts (F), co-cultured with monocytes (Mo) in the absence (E-) of estradiol (E) treatment, for interleukin-1 (IL-1), interleukin-6 (IL-6), macrophage migratory inhibitory factor (MIF), matrix metallopeptidase 9 (MMP9) and secretory leukocyte protease inhibitor (SLPI) (as means ±SEM of n=3 (biological replicates)).

5.5.2 Monocyte inflammatory factors in co-culture with fibroblasts

Examining the profiles of monocyte wound relevant gene expression, in co-culture with scratch-activated WT and disease fibroblasts in low estrogen against monocytes cultured in isolation conditions, revealed differences in inflammatory response (Figure 5.6). This suggests that ERβ regulation in fibroblasts can exert paracrine influences on inflammatory cells.

Both ERα and ERβ are expressed by CD14+ human monocytes (Ashcroft et al., 2003) (Pioli et al., 2007) and it is clear that estrogen has a direct role in the modulation of monocyte and macrophage immune function (reviewed (Bouman et al., 2005)). It is thought that the anti-inflammatory protective effects of estrogen are transduced via ERα rather than ERβ (Polanczyk et al., 2003) (Liu et al., 2003). In a wound context, the alternative polarisation of macrophages towards a healing phenotype has been demonstrated to be promoted by ERα accompanied by an altered cytokine profile (Campbell et al., 2014), whilst both receptors can have a potent overall anti-inflammatory effect in the wound (Campbell et al., 2010). The importance of ERα in macrophage polarisation implies that the ratio of estrogen receptors present in macrophages could have an important outcome in regards to inflammation and wound healing. Here, we see
that monocyte expression of ERβ decreases when paired with WT fibroblasts, but increases when paired with disease fibroblasts, whilst this trend is reversed for ERα (Figure 5.6). In the context of the literature, this would imply that monocytes in co-culture with WT fibroblasts would have more potential to be polarised towards an anti-inflammatory, pro-healing phenotype due to the higher ERα to ERβ ratio.

The estrogen modulated inflammatory mediator MIF was expressed by monocytes to a similar degree when incubated with wound activated WT and disease fibroblasts (Figure 5.6). IL-2 is an inflammatory cytokine that increases wound ingress of fibroblasts and inflammatory cells (DeCunzo et al., 1990), that improves wound healing (Barbul et al., 1986) and has been proposed as a treatment in chronic wounds due to its underrepresentation in these wounds (Chan, 2015). Monocytes interacting with WT fibroblasts upregulate IL-2 by 60-fold, while it is barely detectable in monocytes interacting with disease fibroblasts. IL-6 is also pro-healing in acute wounds (Gallucci et al., 2000), but increased concentrations are associated with non-healing wounds (Trengove et al., 2000). Whilst IL-6 expression is increased with both fibroblasts sets compared to monocytes in isolation, its expression is almost 250-fold greater with disease fibroblasts than with WT fibroblasts. TNFα expression is increased in wound healing where it has inflammatory effects and encourages reepithelialisation, but is associated with chronic wounds (Mast and Schultz, 1996). TGFβ is involved in all aspects of wound healing, is upregulated upon acute injury, but is found at reduced levels in chronic wounds (Barrientos et al., 2008). Expression levels were similar between the two groups, with the mean expression level being slightly higher with the WT fibroblasts. MMP-9 expression is approximately 2-fold higher in monocytes exposed to wounded WT fibroblasts in comparison to disease fibroblasts.
Figure 5.6 – Wound-related gene expression of human monocytes co-cultured with fibroblasts at 48 hours post-wounding under low-estrogen conditions. mRNA expression measured by qPCR relative to TBP reference gene, of human monocytes (Mo) co-cultured with disease (D) and wild type (WT) fibroblasts (F) at 48 hours post-wounding in low-estrogen conditions, for estrogen receptor beta (ERβ), interleukin-2 (IL-2), interleukin-6 (IL-6), macrophage migratory inhibitory factor (MIF), estrogen receptor alpha (ERα), transforming growth factor beta (TGFβ), tumour necrosis factor alpha (TNFα) and matrix metallopeptidase 9 (MMP9) (as means ±SEM of n=2 (biological replicates)).
5.5.3 M1/M2-like polarisation is dependent on fibroblast SNP genotype

Circulating peripheral blood monocytes can be recruited to the wound where they differentiate to macrophages in response to their surroundings (reviewed (Brancato and Albina, 2011)). Previous studies have successfully investigated the effects of specific cell types in co-culture with human monocyte on monocyte differentiation and polarisation (Hollmen et al., 2015). Considering the differing inflammatory profiles of monocytes co-cultured with wound activated WT and disease fibroblasts and the ability of estrogen receptors to modulate macrophage polarisation (Campbell et al., 2014), markers for M1 (inflammatory) and alternatively activated M2 (anti-inflammatory/pro-healing) macrophage polarisation were examined (Figure 5.7). Markers were chosen based on the most differentially expressed gene markers in transcriptional profiling assays of macrophages that have been polarised to either a M1 phenotype, by treatment with IFNγ and lipopolysaccharides, or a M2 phenotype, by treatment with IL-4 (Martinez et al., 2006). These markers were polarised artificially in vitro with specific signalling markers and therefore may not reliably reflect the true state of polarisation seen in vivo.

C-C chemokine receptor type 7 (CCR7), which is a membrane receptor responsible for lymph node traffic, is recognised as a marker of M1 polarisation with transcriptional profiling suggests that it is transcribed 107-fold more in M1 polarised macrophages compared to M2 polarised macrophages (Martinez et al., 2006). CCR7 is significantly increased in both monocytes co-cultured with WT wound activated fibroblasts and disease fibroblasts compared to monocytes in isolation. However, there is no significant difference in monocyte CCR7 expression between WT and disease fibroblast co-cultures.

C-X-C motif chemokine ligand 11 (CXCL11) is a marker of M1 polarisation that transcriptional profiling finds is expressed to a 212 fold greater degree in M1 polarised macrophages compared to M2 polarised macrophages (Martinez et al., 2006). CXCL11 is not significantly upregulated in monocytes co-cultured with WT wound activated fibroblasts compared to monocytes cultured in isolation (Figure 5.7). CXCL11 expression is however upregulated in monocytes cultured with wound-activated disease fibroblasts compared to both monocytes in isolation and to monocytes cultured with WT wound-activated fibroblasts.

Mannose receptor C type 1 (MRC1) is a phagocytic membrane bound lectin receptor that can recognise glycan bound microorganisms and mediate ingestion. MRC1 is a recognised M2 polarisation marker (Stein et al., 1992) which transcriptional profiling reveals is expressed 43-fold more in M2 polarised macrophages compared to M1 polarised (Martinez et al., 2006). Whilst MRC1 is upregulated significantly compared to isolated monocytes in both WT and disease co-
cultured monocytes, MRC1 is expressed significantly higher in monocytes co-cultured with WT fibroblasts compared to disease fibroblasts, indicating a greater degree of M2 polarisation.

The significantly increased monocyte expression level of the CXCL11 M1 polarisation marker with disease fibroblasts and the significantly increased level of the MRC1 M2 polarisation marker with WT fibroblasts suggest that fibroblasts with ERβ SNPs prime human monocytes to an M1, inflammatory phenotype and that WT fibroblasts prime human monocytes to a M1 pro-healing phenotype.
Figure 5.7 – Macrophage M1/M2 polarisation marker gene expression in human monocytes co-cultured with fibroblasts at 48 hours post-wounding under low-estrogen conditions. mRNA expression measured by qPCR relative to TBP reference gene, of human monocytes (Mo) co-cultured with disease (D) and wild type (WT) fibroblasts (F) at 48 hours post-wounding in low-estrogen conditions, for the M1 marker C-C chemokine receptor type 7 (CCR7), the M1 marker C-X-C motif chemokine 11 (CXCL11) and the M2 marker mannose receptor C type 1 (MRC1) (as means ±SEM of n=3 (biological replicates), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).
5.6 Discussion

The wound is a complex environment of inflammation, cell growth, cell migration, extracellular matrix synthesis and remodelling. The successful initiation and resolution of these phases involves coordination of multiple cell types via signalling molecules, growth factors, cytokines and chemokines. Estrogen is a pleiotropic promoter of wound healing with effects on all major wound cell types. Here, we demonstrate that ERβ SNPs associated with venous ulceration have different intrinsic effects on keratinocytes and fibroblasts and fascinating paracrine influence on wound healing. Functional SNPs of ERβ would therefore likely have an effect on the expressional profiles of skin cells and their interactions with cells of the immune system.

Scratch wound expressional profiling in keratinocytes revealed differences in ERβ mRNA expression between WT and venous ulcer-associated SNP harbouring disease keratinocytes (Figure 5.1). Expression in disease keratinocytes did not alter due to estradiol treatment suggesting insensitivity to estrogen signalling, but was reduced in estradiol treated WT cells. Promoter utilisation also varies between cells groups. The ERβ promoters are used in a tissue and disease specific manner (Hirata et al., 2001) (Springwald et al., 2010), with silencing of the ON promoter linked to the progression of cancer (Gao et al., 2016) (Zhang et al., 2007). It appears that whilst both WT and venous ulcer SNP harbouring disease cells utilise both the ON and OK promoters, WT cells can employ the ON promoter to a greater and more dynamic degree both with and without estrogen ligand, than the disease cells. This would support the hypothesis that venous ulcer-associated ERβ SNPs of the ON promoter region have a functional effect on transcription.

Concurrent analysis of ERβ expression at the protein level in keratinocyte scratch assays supported this in revealing that ERβ is expressed to a greater degree in WT cells, both with and without estradiol treatment (Figure 5.2). Western blotting also suggested that post-translational modifications of ERβ are reduced in WT keratinocytes, although this remains to be confirmed. This observation could well be functionally significant, as ERβ can be regulated by phosphorylation (Tremblay et al., 1999), ubiquitination (Picard et al., 2008) and palmitoylation (Galluzzo et al., 2007). These modifications are thought to influence the formation of membrane-bound estrogen receptors which play a role in the rapid non-genomic effects of estrogen signalling (Levin, 2009). Phosphorylation of Ser124 and Ser106 can enhance ligand independent transcriptional activity of ERβ (Tremblay et al., 1999), which may underpin ligand independent differences in target regulation between WT disease cells.

Although fibroblasts express less ERβ than keratinocytes, they are still estrogen responsive, displaying increased proliferation and migration (Campbell et al., 2010). In this study, in contrast to keratinocytes, fibroblasts displayed clear and significant differences in the expression of wound
related factors upon estradiol stimulation (Figure 5.4). Fibroblast gene expression was measured at the 2 hour post-wound time-point to maximise the change in expression induced by wound healing and due to the elevated gene expression seen in keratinocytes at this timepoint. However, what was observed in keratinocytes may not hold true to fibroblasts and examining such a small window post-wounding limits the information that can be garnered from this assay. Some genes may have different induction times or be regulated at different points during wound healing. Estradiol increased expression of FGF-2, VEGF, TGFα, TGFβ and MMP9 in WT fibroblasts, while disease fibroblasts were entirely not responsive. All of these factors act to promote acute wound healing and are found at decreased levels in chronic wounds (Barrientos et al., 2008). Thus, our data have identified specific defects in the induction of multiple relevant targets in disease fibroblasts (Figure 3.12). Interestingly, in the context of paracrine signalling FGF-2, VEGF, TGFα and TGFβ are all known to increase keratinocyte migration (Peplow and Chatterjee, 2013). Clearly, ulcer-associated ERβ SNPs induce an altered expression profile of wound mediators (including FGF-2, VEGF, TGFα and TGFβ) that is less conducive to wound healing.

Chronic wounds are characterised by an inappropriate, excessive and prolonged inflammatory response. Unfortunately, for this project we were unable to obtain immune cells harbouring the ERβ SNPs. Instead we assessed the indirect effects through scratch activated SNP specific fibroblasts cells on wild type monocytes in a co-culture setting (Figure 5.6). This model closely mimics the in vivo situation where newly extravasated monocytes are found in close proximity with fibroblasts in the wound granulation tissue. Indeed, fibroblast-monocyte co-cultures have been demonstrated as suitable models to examine complex cell-cell signalling in a manner more representative of in vivo interactions than with mono-cultures or with conditioned media (Holt et al., 2010).

The inflammatory cytokines IL-1, IL-6 and MIF were expressed to a far greater degree in disease fibroblasts compared to WT fibroblasts when co-cultured with monocytes (Figure 5.7). Both IL-1 and IL-6 are found at elevated levels in chronic wounds (Trengove et al., 2000). MIF is a pro-inflammatory mediator that is downregulated by estrogen signalling which is found in chronic wounds (Grieb et al., 2012) and can increase inflammatory cytokine expression, including IL-6 (Calandra and Bucala, 1995). A relatively high expression level indicates a lack of down-regulation through estrogen receptors and the potential to prime macrophages to a pro-inflammatory phenotype. Interestingly, the estrogen receptor ratio within monocyte became switched upon co-culture with WT and disease scratch activated fibroblasts. WT fibroblasts induced a ratio in favour of ERα whilst disease fibroblasts induced a ratio in favour of ERβ. This data implies that interaction with wounded fibroblasts can directly regulate the expression of both estrogen receptors in monocytes. The polarisation of wound macrophages towards a pro-healing
phenotype with an altered cytokine profile, which is beneficial for timely wound healing, is promoted by the action of ERα (Campbell et al., 2014).

Fibroblasts with the WT SNP variants induced increased expression of IL-2, TGFβ, TNFα and MMP9 compared to monocytes in isolation, whilst they were downregulated by fibroblasts with disease variant SNPs. IL-2 and TGFβ are pro-healing wound factors that are protective against chronic wounds and are associated with positive healing outcomes (Singer and Clark, 1999) (Chan, 2015) and MMP9 is important in acute wound healing but can be detrimental in chronic wounds (Liu et al., 2009). TNFα can have positive effects on acute wound healing (Ashcroft et al., 2012), but is associated with hyper-inflammatory pathologies including with patients harbouring venous ulcer-associated ERβ SNPs (Ashworth et al., 2008). Elevated systemic TNFα levels would most likely chiefly be caused by activated macrophages (Zhao et al., 1998) (Baer et al., 1998) and thus it would be expected that monocytes in co-culture with disease fibroblasts would also express elevated TNFα. However, this is not the case which might suggest a larger role for ERβ SNPs within the monocytes themselves. The mostly pro-healing cytokine IL-6 is upregulated in monocytes in co-culture with disease cells. IL-6 is downregulated by estrogen signalling and upregulated by MIF. The elevated levels of IL-6 seen here are likely due to the elevated level of MIF by the disease fibroblasts (Figure 5.6) and the lack of downregulation from estrogen signalling.

Macrophage polarisation is important in wound healing. In the early phases of wound healing, macrophages that enter the wound site are polarised towards a M1 inflammatory phenotype. These cells have an enhanced anti-microbial capacity and express high levels of pro-inflammatory cytokines including TNFα, IL-1, IL-6 and IL-23. As wound healing progresses and the wound is cleared of infection, this level of inflammation is unnecessary and counterproductive. At this stage the macrophage wound load becomes polarised to a pro-healing M2 phenotype which dampen pro-inflammatory mediators and secrete extracellular matrix components. It is thought that an imbalance in this paradigm can lead to excessive inflammation and chronic wounds. This has been demonstrated in the wounds of a diabetic mouse model (Mirza and Koh, 2011) and in human venous ulcer patients (Sindrilaru et al., 2011). The M1 macrophage marker CCR7 was significantly increased in monocytes after co-culture with both wounded disease fibroblasts and WT fibroblasts compared to monocytes in isolation, but with no significant difference between co-culture groups. The M1 macrophage marker CXCL11 was expressed to a significantly higher degree in monocytes co-cultured with disease fibroblasts compared to wild type fibroblasts, while the M2 macrophage marker MRC1 was expressed to a significantly higher degree in monocytes co-cultured with WT fibroblasts compared to with disease fibroblasts. This indicates that fibroblasts harbouring venous ulcer-associated ERβ SNPs have an innate ability to polarise
monocytes towards a M1 phenotype, while fibroblasts harbouring the WT SNP variants polarise monocytes towards a pro-healing M2 phenotype in low estrogen conditions.

In summary, venous ulcer-associated ERβ SNPs alter the expression of ERβ in keratinocytes during wound healing and under low estrogen conditions, resulting in distinct wound signalling profiles. A distinct SNP-specific wound healing profile is again seen in wounded fibroblasts both in isolation and in co-culture with human monocytes. This altered signalling profile allows for the indirect influence upon monocyte inflammatory cytokine expression and the polarisation towards a pro-inflammatory M1 phenotype rather than a pro-healing M2 phenotype, which is seen in the absence of the SNPs.

The absence of estrogen appears to exacerbate the altered signalling profiles in venous ulcer SNP-containing skin cells. This altered signalling profile has a reduced expression of pro-wound healing growth factors and mediators that would likely reduce reepithelialisation and extracellular matrix formation in a wound scenario which, combined with the pro-inflammatory effect conveyed to monocytes, would create conditions analogous to a chronic wound. This could have broader implications in a patients harbouring estrogen receptor beta SNPs that can functionally effect expression. Dysfunctional regulation of inflammation is associated with various pathologies and indeed, estrogen receptor polymorphisms are associated with several diseases, such as arthritis (Sato et al., 2012), cancer (Sun et al., 2005) and neurodegeneration (Pirskanen et al., 2005). This connection is further reinforced by the identification of the venous ulcer SNP rs2987983 also being associated with prostate and breast cancers (Thellenberg-Karlsson et al., 2006) (Treeck et al., 2009). These SNPs potentially act as bio-markers for several pathologies and it should be considered that any treatment designed for venous ulcers could also have a prophylactic effect against other inflammatory disorders.
6.1 Introduction

Estrogen is a widely recognised regulator of wound healing, influencing almost all aspects of the repair process. A dearth of estrogen signalling, whether through lack of ligand or lack of available receptor, can lead to suboptimal wound healing. Specifically, both ovariectomised (Ashcroft et al., 1997a) and estrogen receptor null mice (Campbell et al., 2010) display delayed acute wound healing. Further to this, in these models the positive effects of estrogen on wound healing are transduced exclusively through ERβ and not ERα (Campbell et al., 2010). These findings are replicated in humans where the estrogen component of hormone replacement therapy can protect against the development of chronic wounds (Margolis et al., 2002). Chronic wounds are further linked to the action of estrogen by the discovery of a haplotype of SNPs in the ON promoter region of the ERβ gene that is significantly associated with predisposition to the formation of venous ulcers (Ashworth et al., 2008). We have reported that these ERβ SNPs can lead to reduced receptor expression in keratinocytes and fibroblasts, delayed in vitro wound healing and altered cytokine and growth factor expression (Chapters 3 and 5).

Estrogen regulates gene expression through the estrogen nuclear hormone receptors ERα and ERβ. These are transcription factors that dimerise and become activated upon the binding of estrogen ligand. Once activated by estrogen ligand they can translocate to the nucleus where they directly induce gene expression by binding to estrogen response element (ERE) sequences in the promoters of target genes, or indirectly enhance gene expression through interaction with other transcription factors, such as the AP1 complex (Kushner et al., 2000). They can also become activated by phosphorylation through the action of mitogen-activated protein kinase 1 (MAPK) (Kato et al., 1995) (Bhatt et al., 2012). Estrogen can additionally rapidly signal through membrane-bound estrogen receptors. These receptors have the same genetic origin as the nuclear receptors, but have been trafficked to the membrane where they can associate with G-protein coupled receptors (GPCRs). Activation induces cAMP generation and recruits second messengers such as the protein kinases phosphatidylinositol-3-kinase (PI3K), serine-threonine kinase (Akt) and MAPK. Estrogen receptors can also induce phosphorylation of Src and SH2 which activate Ras, Raf, extracellular signal regulated kinases (Erk), c-Jun n-terminal kinase (JNK) and p38, to invoke transcriptional regulation (Levin, 2009).

Several microarray studies, and more recently RNAseq studies, have reported the effects of estrogen signalling on the transcriptome in various cells and tissues including prostatic stromal cells (Bektic et al., 2004), breast cancer cells (Yamaga et al., 2013) (Wang et al., 2004a),
endometrial cells (Ren et al., 2015) and cerebral cortex (Humphreys et al., 2014). There is evidence that estrogen receptors can regulate the expression of at least 800 different genes (Tang et al., 2004) (Frasor et al., 2003), many of which are involved in cell homeostasis, proliferation, survival and growth factor expression. The gene expression pattern depends greatly on the ratio of ERα to ERβ within a cell (Gong et al., 2014). In the context of the skin and wounds, a microarray study found that the differences in gene expression between elderly and young male wounds are almost exclusively estrogen regulated (Hardman and Ashcroft, 2008).

Although RNAseq analysis has been applied to identify changes in keratinocyte gene expression during wounding as a consequence of factors such as IL-4 (Serezani et al., 2016), it has not yet been applied to study the effects of estradiol on human keratinocyte gene expression. Here we have used RNAseq to transcriptionally profile in vitro wounded keratinocytes harbouring venous ulcer-associated ERβ SNPs versus wild type cells. Three biological replicates of Wild type (WT) and venous ulcer-associated ERβ SNP containing disease (D) keratinocytes were grown in the absence of estrogen or with 100nM 17β-estradiol treatment for 48 hours prior to harvest. These cells were scratch activated before RNA collection at 4 hours post-wounding. The Illumina Hiseq2500 was used for single-end sequencing.

6.2 Establishing sample variance

Multidimensional scaling (MDS) was used to visually evaluate the degree of variance between the sample groups (Figure 6.1). Data indicate that estrogen treatment led to minimal difference in expression profile, with the treated and untreated sample of each cell source clustering together. There did however appear to be a greater degree of variance based on genotype, with divergent clusters for WT and disease samples. Clearly, disease sample 2 displayed a phenotype that was more similar to wild type that disease samples 1 and 3. Independent, hierarchical clustering using the 200 most differentially regulated genes again demonstrates clear divergence based on genotype, with little variance between treated and untreated gene expression profiles (Figure 6.1). Again, disease sample 2 clustered with wild type samples more closely than disease. However, disease sample 1 and wild type sample 3 display similar profiles, distinct from the remaining disease and wild type groups, indicating a somewhat heterogeneous effect of SNPs on gene expression.
Figure 6.1 – Clustering of venous ulcer-associated SNP disease cells and healthy cells. Multidimensional Scaling Analysis (MDS) plot (left) of disease cells (D) (samples 1-3) treated with estrogen(+) (red), without estrogen(-) (orange) and wild type (WT) cells (samples 1-3) treated with estrogen(+) (dark green) and without estrogen(-) (light green). Samples cluster based on their patient source (small circles) and based on their genotype (large circles). Heat map of hierarchical clustering (right) of WT and D cells treated with (E+) or without (E-) estrogen, based on the 200 most differentially expressed genes. Samples appear to cluster based on their genotype and not estrogen treatment, other than disease sample 2 which shows greater similarity to WT samples.
6.3 Differentially regulated genes by genotype and by estradiol treatment

To determine which genes were differently regulated between groups (estrogen positive vs estrogen negative or WT vs D), strict filtering criteria was implemented on fold-change (Fc) (≥±1.5), and statistical significance (P<0.05 and FDR<0.05). The ten most significantly differentially expressed genes for each group comparison can be found in Table 6.1.

In accordance with the lack of distinction seen between estrogen treated and untreated samples in the MDS plot and the hierarchical analysis (Figure 6.1a/b), there were no significantly differentially estrogen-regulated genes identified with global comparison across both genotypes. Paired analysis, splitting the dataset into two genotype specific sets, resulted in the identification of appreciably more genes responding to estrogen treatment. Curiously, the most statistically significant estrogen-upregulated genes in WT genotype were pseudogenes. Pseudogenes are gene copies that do not have the ability to code for protein. Though insufficiently understood, recent conjecture is that pseudogenes have roles in gene regulation through the generation of IncRNAs, siRNAs or miRNA-binding sites (Kalyana-Sundaram et al., 2012) (Milligan and Lipovich, 2014). The most statistically significant estrogen-downregulated genes in WT cells were mostly transfer-RNAs and pseudogenes. Only two significantly estrogen-upregulated genes were identified in disease cells which were collagen VI (COL6A3) and eukaryotic translation elongation factor 1 pseudogene (EEF1A1P29). Considerably more genes were down-regulated by estrogen in disease cells, including golgin genes (GOLGA8A and GOLGA8B), which are involved in tethering vesicles to the Golgi, and mitochondrial genes (MT-ND6 and MT-TE).

Comparing by genotype, irrespective of estrogen treatment (global comparison), revealed a significant upregulation in WT samples of genes involved in the cell cycle (PLK5), metabolism (IDH2) and numerous immune regulators (NLRP10, S100A9 and CD274) (Table 6.1). There were also many immune-related genes significantly upregulated in disease cells, especially members of MHC Class I antigen presenting proteins (HLA-F, -A, -G and the pseudogene HLA-H). The cell adhesion molecule CADM1 was also found to be upregulated. Comparing by genotype with and without estrogen treatment gave broadly similar results (Table 6.1).
Table 6.1 – Top ten most statistically significant differentially regulated genes by estradiol treatment and genotype. Selected on p-value (<0.05) with fold-change >1.5 fold-change and sorted by FDR (<0.05).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold-change</th>
<th>p-value</th>
<th>FDR</th>
<th>Gene function/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated by estrogen (global)</strong></td>
<td>None significant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Downregulated by estrogen (global)</strong></td>
<td>None significant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated by estrogen in WT (WTE+ vs WTE-)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eukaryotic translation elongation factor 1 alpha 1 pseudogene 12</td>
<td>EEF1A1P12</td>
<td>2.097</td>
<td>2.190E-34</td>
<td>6.645E-30</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Y box binding protein 1 pseudogene 2</td>
<td>YBX1P2</td>
<td>1.542</td>
<td>9.645E-14</td>
<td>5.781E-10</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta pseudogene 5</td>
<td>YWHAZP5</td>
<td>1.583</td>
<td>1.048E-13</td>
<td>5.781E-10</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Eukaryotic translation elongation factor 1 alpha 1 pseudogene 4</td>
<td>EEF1A1P4</td>
<td>1.587</td>
<td>4.226E-13</td>
<td>1.973E-09</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Ferritin, heavy polypeptide 1 pseudogene 2</td>
<td>FTH1P2</td>
<td>1.623</td>
<td>8.137E-13</td>
<td>3.526E-09</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>SERPINE1 mRNA binding protein 1 pseudogene 6</td>
<td>SERBP1P6</td>
<td>1.558</td>
<td>1.335E-09</td>
<td>5.781E-10</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Fatty acid binding protein 5 pseudogene 11</td>
<td>FABP5P11</td>
<td>1.583</td>
<td>3.288E-09</td>
<td>7.673E-06</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Ribosomal protein L36a pseudogene 13</td>
<td>RPL36AP13</td>
<td>1.517</td>
<td>8.895E-09</td>
<td>1.861E-05</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Cytoplasmic polyadenylation element binding protein 1</td>
<td>CPEB1</td>
<td>3.567</td>
<td>1.533E-07</td>
<td>2.448E-04</td>
<td>RNA binding translation factor</td>
</tr>
<tr>
<td>MT-RNR2-like 8</td>
<td>MTRNR2L8</td>
<td>1.651</td>
<td>3.620E-05</td>
<td>2.468E-02</td>
<td>Antiapoptotic factor with neuroprotective action</td>
</tr>
<tr>
<td><strong>Downregulated by estrogen in WT (WTE+ vs WTE-)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrially encoded tRNA glycine</td>
<td>MT-TG</td>
<td>4.460</td>
<td>8.307E-76</td>
<td>5.040E-71</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Mitochondrially encoded tRNA histidine</td>
<td>MT-TH</td>
<td>3.175</td>
<td>5.924E-32</td>
<td>1.198E-27</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Mitochondrially encoded tRNA tryptophan</td>
<td>MT-TW</td>
<td>2.766</td>
<td>4.696E-27</td>
<td>7.123E-23</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Ribosomal protein L37 pseudogene 2</td>
<td>RPL37P2</td>
<td>4.506</td>
<td>1.432E-23</td>
<td>1.448E-19</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Mitochondrially encoded tRNA aspartic acid</td>
<td>MT-TD</td>
<td>2.776</td>
<td>1.043E-20</td>
<td>9.042E-17</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Mitochondrially encoded tRNA isoleucine</td>
<td>MT-TI</td>
<td>1.959</td>
<td>2.820E-16</td>
<td>1.901E-12</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Ribosomal protein S26 pseudogene 47</td>
<td>RPS26P47</td>
<td>1.533</td>
<td>1.321E-08</td>
<td>2.671E-05</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Ribosomal protein S26 pseudogene 31</td>
<td>RPS26P31</td>
<td>1.513</td>
<td>3.580E-08</td>
<td>6.582E-05</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Ribosomal protein SA pseudogene 3</td>
<td>RPSAP3</td>
<td>1.782</td>
<td>7.805E-08</td>
<td>1.353E-04</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Interleukin 1 receptor-like 1</td>
<td>IL1RL1</td>
<td>1.529</td>
<td>2.486E-07</td>
<td>3.428E-04</td>
<td>Member of the interleukin 1 receptor family that can be induced by proinflammatory stimuli</td>
</tr>
<tr>
<td><strong>Upregulated by estrogen in D (DE+ vs DE-)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen, type VI, alpha 3</td>
<td>COL6A3</td>
<td>1.965</td>
<td>6.166E-05</td>
<td>1.871E-02</td>
<td>Encodes the alpha-3 chain of type VI collagen</td>
</tr>
<tr>
<td>Eukaryotic translation elongation factor 1 alpha 1 pseudogene 29</td>
<td>EEF1A1P29</td>
<td>1.801</td>
<td>6.458E-05</td>
<td>1.917E-02</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Gene name</td>
<td>Gene symbol</td>
<td>Fold-change</td>
<td>p-value</td>
<td>FDR</td>
<td>Gene function/Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>-------------</td>
<td>------------</td>
<td>------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Poly(A) binding protein, cytoplasmic 1-like</td>
<td>PABPC1L</td>
<td>1.509</td>
<td>1.965E-20</td>
<td>2.980E-16</td>
<td>mRNA polyadenylation</td>
</tr>
<tr>
<td>GABPB1 antisense RNA 1</td>
<td>GABPB1-AS1</td>
<td>1.582</td>
<td>4.189E-19</td>
<td>4.841E-15</td>
<td>Surrogate indicator of stress</td>
</tr>
<tr>
<td>BTB (POZ) domain containing 19</td>
<td>BTBD19</td>
<td>1.620</td>
<td>1.538E-12</td>
<td>7.777E-09</td>
<td>Protein-protein binding</td>
</tr>
<tr>
<td>Leukocyte receptor cluster (LRC) member 8</td>
<td>LENG8</td>
<td>1.552</td>
<td>3.678E-12</td>
<td>1.594E-08</td>
<td>Protein binding</td>
</tr>
<tr>
<td>Golgin A8 family, member B</td>
<td>GOLGA8B</td>
<td>1.559</td>
<td>4.517E-12</td>
<td>1.827E-08</td>
<td>Vesicle-mediated transport</td>
</tr>
<tr>
<td>Nuclear paraspeckle assembly transcript 1</td>
<td>NEAT1</td>
<td>1.651</td>
<td>6.151E-12</td>
<td>2.333E-08</td>
<td>Long non-coding RNA forming the core structural component of the paraspeckle sub-organelles</td>
</tr>
<tr>
<td>Golgin A8 family, member A</td>
<td>GOLGA8A</td>
<td>1.551</td>
<td>1.164E-11</td>
<td>4.156E-08</td>
<td>Maintaining Golgi structure</td>
</tr>
<tr>
<td>Mitochondrially encoded NADH dehydrogenase 6</td>
<td>MT-ND6</td>
<td>1.789</td>
<td>3.069E-11</td>
<td>9.801E-08</td>
<td>Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase</td>
</tr>
<tr>
<td>Mitochondrially encoded tRNA glutamic acid</td>
<td>MT-TE</td>
<td>2.394</td>
<td>1.631E-10</td>
<td>3.958E-07</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Metastasis associated lung adenocarcinoma transcript 1</td>
<td>MALAT1</td>
<td>1.625</td>
<td>1.192E-08</td>
<td>1.809E-05</td>
<td>Molecular scaffold for ribonucleoprotein complexes</td>
</tr>
<tr>
<td>DNA-damage-inducible transcript 3</td>
<td>DDIT3</td>
<td>10.755</td>
<td>2.931E-13</td>
<td>1.617E-09</td>
<td>Dominant-negative inhibitor member of the CCAAT/enhancer-binding protein (C/EBP) family</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 2 (NADP+)</td>
<td>IDH2</td>
<td>6.475</td>
<td>2.460E-12</td>
<td>1.148E-08</td>
<td>Intermediary metabolism and energy production</td>
</tr>
<tr>
<td>Psoriasis susceptibility 1 candidate 2</td>
<td>PSORS1C2</td>
<td>114.965</td>
<td>3.365E-12</td>
<td>1.416E-08</td>
<td>Correlates with psoriasis susceptibility</td>
</tr>
<tr>
<td>CD274 molecule</td>
<td>CD274</td>
<td>13.044</td>
<td>4.370E-12</td>
<td>1.657E-08</td>
<td>Involved in the costimulatory signal, essential for T-cell proliferation and cytokine production</td>
</tr>
</tbody>
</table>

**Downregulated by estrogen in D (DE+ vs DE-)**

**Upregulated in WT vs D (global)**
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold-change</th>
<th>p value</th>
<th>FDR</th>
<th>Gene function/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major histocompatibility complex, class I, F</td>
<td>HLA-F</td>
<td>14.422</td>
<td>9.019E-22</td>
<td>2.736E-17</td>
<td>Involved in the presentation of foreign antigens to the immune system</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, H (pseudogene)</td>
<td>HLA-H</td>
<td>6.632</td>
<td>9.874E-20</td>
<td>1.498E-15</td>
<td>Pseudogene, possibly derived from HLA-A</td>
</tr>
<tr>
<td>Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 gamma</td>
<td>PIK3C2G</td>
<td>17.412</td>
<td>5.219E-15</td>
<td>3.958E-11</td>
<td>Roles in signaling pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking</td>
</tr>
<tr>
<td>Spectrin repeat containing, nuclear envelope 1</td>
<td>SYNE1</td>
<td>16.165</td>
<td>1.187E-11</td>
<td>4.002E-08</td>
<td>Spectrin repeat that localizes to the nuclear membrane</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, A</td>
<td>HLA-A</td>
<td>5.717</td>
<td>2.127E-11</td>
<td>6.452E-08</td>
<td>Involved in the presentation of foreign antigens to the immune system</td>
</tr>
<tr>
<td>Actin related protein 2/3 complex, subunit 1B, 41kDa</td>
<td>ARPC1B</td>
<td>5.347</td>
<td>2.419E-11</td>
<td>6.671E-08</td>
<td>Regulation of actin polymerization</td>
</tr>
<tr>
<td>Phospholipid transfer protein</td>
<td>PLTP</td>
<td>21.436</td>
<td>1.099E-10</td>
<td>2.222E-07</td>
<td>Lipid transfer proteins that may be involved in cholesterol metabolism</td>
</tr>
<tr>
<td>Cell adhesion molecule 1</td>
<td>CADM1</td>
<td>16.263</td>
<td>2.018E-10</td>
<td>3.711E-07</td>
<td>Mediates homophilic cell-cell adhesion in a Ca(2+) -independent manner</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, G</td>
<td>HLA-G</td>
<td>13.156</td>
<td>2.319E-10</td>
<td>4.020E-07</td>
<td>Involved in the presentation of foreign antigens to the immune system</td>
</tr>
</tbody>
</table>

<p>| MIR2117 host gene                                                        | MIR2117HG   | 132.824     | 1.486E-23    | 9.019E-19    | microRNA                                                                                  |
| NLR family, pyrin domain containing 10                                   | NLRP10      | 3.451       | 1.026E-08    | 1.245E-04    | Likely regulatory mediator of the innate immune system                                     |
| Ankyrin repeat domain 35                                                | ANKRD3S     | 4.769       | 2.564E-08    | 2.593E-04    | Possible regulator of apoptosis                                                           |
| DNA-damage-inducible transcript 3                                       | DDI1T3      | 3.319       | 1.909E-07    | 1.447E-03    | Dominant-negative inhibitor member of the CCAAT/enhancer-binding protein (C/EBP) family   |
| Pregnancy specific beta-1-glycoprotein 5                                | PSG5        | 8.761       | 2.504E-07    | 1.688E-03    | Carcinoembryonic antigen involved in embryonic cell adhesion                              |
| Polo-like kinase 5                                                       | PLK5        | 17.296      | 3.567E-07    | 2.164E-03    | Serine/threonine-protein kinase that plays a role in cell cycle progression                |
| S100 calcium binding protein A9                                           | S100A9      | 2.972       | 5.513E-07    | 3.041E-03    | Regulator of inflammatory response, cell cycle, differentiation and adhesion             |
| Isocitrate dehydrogenase 2 (NADP+)                                       | IDH2        | 2.538       | 7.513E-07    | 3.626E-03    | Intermediary metabolism and energy production                                            |
| Peptidoglycan recognition protein 4                                      | PGLYR4P4    | 2.871       | 1.341E-06    | 5.085E-03    | Pattern receptor that binds to murein peptidoglycans (PGN) of gram-positive bacteria      |
| Keratin 77, type II                                                     | KRT77       | 10.875      | 2.223E-06    | 7.099E-03    | Type II keratin involved in the structural integrity of epithelial cells                  |</p>
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold-change</th>
<th>p value</th>
<th>FDR</th>
<th>Gene function/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major histocompatibility complex, class I, F</td>
<td>HLA-F</td>
<td>3.722</td>
<td>2.005E-11</td>
<td>6.084E-07</td>
<td>Involved in the presentation of foreign antigens to the immune system</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, H (pseudogene)</td>
<td>HLA-H</td>
<td>2.406</td>
<td>2.154E-09</td>
<td>3.267E-05</td>
<td>Pseudogene, possibly derived from HLA-A</td>
</tr>
<tr>
<td>Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 gamma</td>
<td>PIK3CG</td>
<td>3.824</td>
<td>1.440E-07</td>
<td>1.248E-03</td>
<td>Roles in signaling pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 5</td>
<td>CXCL5</td>
<td>18.874</td>
<td>1.826E-06</td>
<td>6.452E-03</td>
<td>Chemokine that recruits and activates neutrophils</td>
</tr>
<tr>
<td>Spectrin repeat containing, nuclear envelope 1</td>
<td>SYNE1</td>
<td>3.957</td>
<td>1.914E-06</td>
<td>6.452E-03</td>
<td>Spectrin repeat that localizes to the nuclear membrane</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, W (pseudogene)</td>
<td>HLA-W</td>
<td>422.499</td>
<td>3.069E-06</td>
<td>8.464E-03</td>
<td>Pseudogene</td>
</tr>
<tr>
<td>Actin related protein 2/3 complex, subunit 1B, 41kDa</td>
<td>ARPC1B</td>
<td>2.258</td>
<td>4.308E-06</td>
<td>1.017E-02</td>
<td>Regulation of actin polymerization</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, A</td>
<td>HLA-A</td>
<td>2.327</td>
<td>4.358E-06</td>
<td>1.017E-02</td>
<td>Involved in the presentation of foreign antigens to the immune system</td>
</tr>
<tr>
<td>Cell adhesion molecule 1</td>
<td>CADM1</td>
<td>3.975</td>
<td>7.856E-06</td>
<td>1.444E-02</td>
<td>Mediates homophilic cell-cell adhesion in a Ca(2+)-independent manner</td>
</tr>
<tr>
<td>Protein kinase, AMP-activated, alpha 2 catalytic subunit</td>
<td>PRKAA2</td>
<td>50.112</td>
<td>8.992E-06</td>
<td>1.579E-02</td>
<td>Energy-sensing enzyme involved in regulating biosynthesis of fatty acid and cholesterol</td>
</tr>
<tr>
<td>MIR2117 host gene</td>
<td>MIR2117HG</td>
<td>259.033</td>
<td>5.334E-26</td>
<td>3.237E-21</td>
<td>microRNA</td>
</tr>
<tr>
<td>Polo-like kinase 5</td>
<td>PLK5</td>
<td>30.148</td>
<td>2.242E-10</td>
<td>2.721E-06</td>
<td>Serine/threonine-protein kinase that plays a role in cell cycle progression</td>
</tr>
<tr>
<td>NLR family, pyrin domain containing 10</td>
<td>NLRP10</td>
<td>3.888</td>
<td>4.229E-10</td>
<td>4.276E-06</td>
<td>Likely regulatory mediator of the innate immune system</td>
</tr>
<tr>
<td>S100 calcium binding protein A9</td>
<td>S100A9</td>
<td>3.309</td>
<td>4.387E-08</td>
<td>2.700E-04</td>
<td>Regulator of inflammatory response, cell cycle, differentiation and adhesion</td>
</tr>
<tr>
<td>Family with sequence similarity 3, member D</td>
<td>FAM3D</td>
<td>16.794</td>
<td>4.449E-08</td>
<td>2.700E-04</td>
<td>Evidence of cytokine activity</td>
</tr>
<tr>
<td>Ankyrin repeat domain 35</td>
<td>ANKRD35</td>
<td>4.535</td>
<td>6.377E-08</td>
<td>3.517E-04</td>
<td>Possible regulator of apoptosis</td>
</tr>
<tr>
<td>S100 calcium binding protein A12</td>
<td>S100A12</td>
<td>14.335</td>
<td>1.184E-07</td>
<td>5.985E-04</td>
<td>Regulation of inflammatory processes and immune response involved in recruitment of leukocytes, promotion of cytokine and chemokine production, and regulation of leukocyte adhesion and migration</td>
</tr>
<tr>
<td>Small proline-rich protein 4</td>
<td>SPRR4</td>
<td>7.972</td>
<td>2.216E-07</td>
<td>1.013E-03</td>
<td>Cross-linked envelope protein of keratinocytes</td>
</tr>
<tr>
<td>Psoriasis susceptibility 1 candidate 2</td>
<td>PSORS1C2</td>
<td>12.346</td>
<td>2.464E-07</td>
<td>1.013E-03</td>
<td>Correlates with psoriasis susceptibility</td>
</tr>
<tr>
<td>Gene name</td>
<td>Gene symbol</td>
<td>Fold-change</td>
<td>p value</td>
<td>FDR</td>
<td>Gene function/Description</td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>------------</td>
<td>------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Upregulated in D vs WT without estrogen (DE- vs WTE-)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, H (pseudogene)</td>
<td>HLA-H</td>
<td>2.756</td>
<td>6.596E-12</td>
<td>1.099E-07</td>
<td>Pseudogene, possibly derived from HLA-A</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, F</td>
<td>HLA-F</td>
<td>3.875</td>
<td>7.244E-12</td>
<td>1.099E-07</td>
<td>Involved in the presentation of foreign antigens to the immune system</td>
</tr>
<tr>
<td>Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 gamma</td>
<td>PIK3C2G</td>
<td>4.553</td>
<td>6.800E-09</td>
<td>5.158E-05</td>
<td>Roles in signalling pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, A</td>
<td>HLA-A</td>
<td>2.456</td>
<td>1.076E-06</td>
<td>3.110E-03</td>
<td>Involved in the presentation of foreign antigens to the immune system</td>
</tr>
<tr>
<td>Actin related protein 2/3 complex, subunit 1B, 41kDa</td>
<td>ARPC1B</td>
<td>2.368</td>
<td>1.247E-06</td>
<td>3.440E-03</td>
<td>Regulation of actin polymerization</td>
</tr>
<tr>
<td>Spectrin repeat containing, nuclear envelope 1</td>
<td>SYNE1</td>
<td>4.085</td>
<td>1.377E-06</td>
<td>3.510E-03</td>
<td>Spectrin repeat that localizes to the nuclear membrane</td>
</tr>
<tr>
<td>Phospholipid transfer protein</td>
<td>PLTP</td>
<td>4.983</td>
<td>1.937E-06</td>
<td>3.881E-03</td>
<td>Lipid transfer proteins that may be involved in cholesterol metabolism</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I, G</td>
<td>HLA-G</td>
<td>3.830</td>
<td>3.336E-06</td>
<td>5.784E-03</td>
<td>Involved in the presentation of foreign antigens to the immune system</td>
</tr>
<tr>
<td>Family with sequence similarity 157, member A</td>
<td>FAM157A</td>
<td>2.491</td>
<td>3.992E-06</td>
<td>6.446E-03</td>
<td>Unknown</td>
</tr>
<tr>
<td>inhibitor of DNA binding 1, dominant negative helix-loop-helix protein</td>
<td>ID1</td>
<td>2.100</td>
<td>4.892E-06</td>
<td>7.240E-03</td>
<td>Transcripational regulator that may play a role in cell growth, senescence, and differentiation</td>
</tr>
</tbody>
</table>
6.4 Gene ontology analysis

For further analysis, less stringent filtering criteria were used to create larger data sets, generated by filtering on fold-change (≥±1.5) and statistical significance (P<0.05 only) (Figure 6.2). Again many more genes were differentially regulated based on genotype than by estrogen treatment.

There were 90 genes upregulated by estrogen in WT cells and 62 in disease cells, with almost no overlap in those gene groups (Figure 6.2a). 147 genes were downregulated by estrogen in WT cells and 265 in disease cells, with only 13 genes overlapping between both gene groups (Figure 6.2c). 838 genes were downregulated in disease cells compared to wild type cells under estrogen-positive conditions, whilst 893 were downregulated under estrogen-free conditions (Figure 6.2b). Of these genes, 167 were uniquely downregulated under estrogen-positive conditions and 222 uniquely downregulated in estrogen-free conditions. Finally, there were 537 genes upregulated in disease cells (downregulated in wild type cells) under estrogen-positive conditions and 773 under estrogen-free conditions (Figure 6.2d). Of these genes, 141 were only upregulated under estrogen positive conditions and 377 only under estrogen-free conditions.

The Gene Ontology Consortium PANTHER Overrepresentation Test software (Gene Ontology, 2015) was used to identify overrepresented groups of biologically-related genes based on annotation (gene ontology [GO]) term (Tables 6.2-6.7). The 5 most significant overrepresented terms for each group are displayed hierarchically with the most specific subclass displayed first, followed by parent terms. Again no statistically significant overrepresented gene ontology terms were identified for estrogen regulation. Firstly, genes regulated by genotype were assessed independent of estrogen treatment (i.e. D vs WT) (Tables 6.2-6.3) and then within estrogen status groups (i.e. D E+ vs WT E+ and D E- vs WT E-) (Tables 6.4-6.7).
Figure 6.2 – Induced and repressed genes by estrogen and across genotypes. Few but distinct groups of genes were upregulated (A) or downregulated (B) by estrogen (E) in wild type (WT) and venous ulcer SNP containing disease (D) cells. Larger sets of genes were upregulated (B) or downregulated (C) based on genotype, with a high degree of overlap between groups. (Genes ±1.5-fold change, p<0.05)
## Table 6.2 Overrepresented gene ontology (GO) groups in genes downregulated by disease cells

<table>
<thead>
<tr>
<th>GO biological term</th>
<th>Number of genes</th>
<th>Fold Enrichment</th>
<th>Corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>establishment of skin barrier</td>
<td>11</td>
<td>6.02</td>
<td>2.87E-02</td>
</tr>
<tr>
<td><strong>sk</strong> skin development</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>l</strong> animal organ development</td>
<td>455</td>
<td>1.51</td>
<td>2.59E-15</td>
</tr>
<tr>
<td><strong>l</strong> anatomical structure development</td>
<td>754</td>
<td>1.41</td>
<td>4.33E-22</td>
</tr>
<tr>
<td><strong>l</strong> developmental process</td>
<td>764</td>
<td>1.40</td>
<td>5.37E-21</td>
</tr>
<tr>
<td><strong>l</strong> system development</td>
<td>610</td>
<td>1.44</td>
<td>3.56E-18</td>
</tr>
<tr>
<td><strong>l</strong> multicellular organism development</td>
<td>692</td>
<td>1.42</td>
<td>4.24E-20</td>
</tr>
<tr>
<td><strong>l</strong> single multicellular organism process</td>
<td>811</td>
<td>1.42</td>
<td>1.02E-25</td>
</tr>
<tr>
<td><strong>l</strong> single-organism process</td>
<td>1563</td>
<td>1.18</td>
<td>7.30E-22</td>
</tr>
<tr>
<td><strong>l</strong> multicellular organismal process</td>
<td>882</td>
<td>1.29</td>
<td>2.27E-15</td>
</tr>
<tr>
<td><strong>l</strong> single-organism developmental process</td>
<td>738</td>
<td>1.38</td>
<td>6.16E-19</td>
</tr>
<tr>
<td><strong>l</strong> regulation of water loss via skin</td>
<td>12</td>
<td>5.88</td>
<td>1.34E-02</td>
</tr>
<tr>
<td><strong>l</strong> multicellular organismal water homeostasis</td>
<td>21</td>
<td>3.37</td>
<td>2.01E-02</td>
</tr>
<tr>
<td><strong>l</strong> regulation of biological quality</td>
<td>510</td>
<td>1.37</td>
<td>2.71E-10</td>
</tr>
<tr>
<td><strong>l</strong> biological regulation</td>
<td>1436</td>
<td>1.17</td>
<td>5.63E-15</td>
</tr>
<tr>
<td><strong>l</strong> homeostatic process</td>
<td>212</td>
<td>1.44</td>
<td>1.11E-03</td>
</tr>
<tr>
<td><strong>l</strong> water homeostasis</td>
<td>24</td>
<td>3.10</td>
<td>1.64E-02</td>
</tr>
<tr>
<td><strong>l</strong> chemical homeostasis</td>
<td>154</td>
<td>1.58</td>
<td>2.91E-04</td>
</tr>
<tr>
<td>cholesterol biosynthetic process</td>
<td>17</td>
<td>4.06</td>
<td>1.64E-02</td>
</tr>
<tr>
<td><strong>l</strong> secondary alcohol biosynthetic process</td>
<td>17</td>
<td>4.06</td>
<td>1.64E-02</td>
</tr>
<tr>
<td><strong>l</strong> alcohol biosynthetic process</td>
<td>27</td>
<td>3.59</td>
<td>2.32E-04</td>
</tr>
<tr>
<td><strong>l</strong> metabolic process</td>
<td>1159</td>
<td>1.14</td>
<td>2.17E-05</td>
</tr>
<tr>
<td><strong>l</strong> single-organism metabolic process</td>
<td>485</td>
<td>1.33</td>
<td>1.05E-07</td>
</tr>
<tr>
<td><strong>l</strong> alcohol metabolic process</td>
<td>65</td>
<td>2.37</td>
<td>4.35E-06</td>
</tr>
<tr>
<td><strong>l</strong> organic hydroxy compound metabolic process</td>
<td>79</td>
<td>2.00</td>
<td>1.10E-04</td>
</tr>
<tr>
<td><strong>l</strong> organic substance metabolic process</td>
<td>1116</td>
<td>1.14</td>
<td>1.59E-05</td>
</tr>
<tr>
<td><strong>l</strong> organic hydroxy compound biosynthetic process</td>
<td>36</td>
<td>2.79</td>
<td>7.00E-04</td>
</tr>
<tr>
<td><strong>l</strong> secondary alcohol metabolic process</td>
<td>36</td>
<td>2.91</td>
<td>2.52E-04</td>
</tr>
<tr>
<td><strong>l</strong> cholesterol metabolic process</td>
<td>35</td>
<td>2.91</td>
<td>4.05E-04</td>
</tr>
<tr>
<td><strong>l</strong> sterol metabolic process</td>
<td>36</td>
<td>2.66</td>
<td>2.20E-03</td>
</tr>
<tr>
<td><strong>l</strong> steroid metabolic process</td>
<td>57</td>
<td>2.34</td>
<td>8.13E-05</td>
</tr>
<tr>
<td><strong>l</strong> lipid metabolic process</td>
<td>221</td>
<td>1.76</td>
<td>8.09E-12</td>
</tr>
<tr>
<td><strong>l</strong> primary metabolic process</td>
<td>1057</td>
<td>1.14</td>
<td>2.07E-04</td>
</tr>
<tr>
<td><strong>l</strong> sterol biosynthetic process</td>
<td>19</td>
<td>4.02</td>
<td>4.86E-03</td>
</tr>
<tr>
<td><strong>l</strong> steroid biosynthetic process</td>
<td>35</td>
<td>2.99</td>
<td>2.13E-04</td>
</tr>
<tr>
<td><strong>l</strong> lipid biosynthetic process</td>
<td>100</td>
<td>1.80</td>
<td>2.91E-04</td>
</tr>
<tr>
<td>keratinization</td>
<td>21</td>
<td>3.91</td>
<td>1.99E-03</td>
</tr>
<tr>
<td><strong>l</strong> keratinocyte differentiation</td>
<td>35</td>
<td>3.23</td>
<td>3.34E-05</td>
</tr>
<tr>
<td><strong>l</strong> epidermal cell differentiation</td>
<td>42</td>
<td>2.70</td>
<td>1.62E-04</td>
</tr>
<tr>
<td><strong>l</strong> epithelial cell differentiation</td>
<td>100</td>
<td>1.90</td>
<td>1.82E-05</td>
</tr>
<tr>
<td><strong>l</strong> epithelium development</td>
<td>175</td>
<td>1.81</td>
<td>1.06E-09</td>
</tr>
<tr>
<td><strong>l</strong> tissue development</td>
<td>286</td>
<td>1.76</td>
<td>2.87E-16</td>
</tr>
<tr>
<td><strong>l</strong> cell differentiation</td>
<td>482</td>
<td>1.40</td>
<td>9.30E-11</td>
</tr>
<tr>
<td><strong>l</strong> cellular developmental process</td>
<td>502</td>
<td>1.36</td>
<td>1.37E-09</td>
</tr>
<tr>
<td><strong>l</strong> single-organism cellular process</td>
<td>1409</td>
<td>1.19</td>
<td>9.92E-19</td>
</tr>
<tr>
<td><strong>l</strong> cellular process</td>
<td>1703</td>
<td>1.10</td>
<td>1.35E-09</td>
</tr>
<tr>
<td><strong>l</strong> epidermis development</td>
<td>80</td>
<td>2.90</td>
<td>1.83E-12</td>
</tr>
<tr>
<td>positive regulation of epithelial cell migration</td>
<td>31</td>
<td>2.72</td>
<td>8.93E-03</td>
</tr>
<tr>
<td><strong>l</strong> positive regulation of cell migration</td>
<td>81</td>
<td>1.91</td>
<td>4.83E-04</td>
</tr>
<tr>
<td><strong>l</strong> positive regulation of cell motility</td>
<td>83</td>
<td>1.89</td>
<td>5.13E-04</td>
</tr>
<tr>
<td><strong>l</strong> regulation of cell motility</td>
<td>135</td>
<td>1.73</td>
<td>1.09E-05</td>
</tr>
<tr>
<td><strong>l</strong> regulation of cellular component movement</td>
<td>143</td>
<td>1.70</td>
<td>1.40E-05</td>
</tr>
<tr>
<td><strong>l</strong> regulation of localization</td>
<td>373</td>
<td>1.42</td>
<td>3.41E-08</td>
</tr>
<tr>
<td><strong>l</strong> regulation of biological process</td>
<td>1346</td>
<td>1.16</td>
<td>4.63E-11</td>
</tr>
<tr>
<td><strong>l</strong> regulation of cellular process</td>
<td>1292</td>
<td>1.17</td>
<td>2.51E-11</td>
</tr>
<tr>
<td><strong>l</strong> regulation of locomotion</td>
<td>138</td>
<td>1.70</td>
<td>2.39E-05</td>
</tr>
<tr>
<td><strong>l</strong> positive regulation of cellular component movement</td>
<td>86</td>
<td>1.91</td>
<td>2.26E-04</td>
</tr>
<tr>
<td><strong>l</strong> positive regulation of cellular process</td>
<td>684</td>
<td>1.38</td>
<td>2.82E-16</td>
</tr>
<tr>
<td><strong>l</strong> positive regulation of biological process</td>
<td>744</td>
<td>1.36</td>
<td>8.24E-17</td>
</tr>
<tr>
<td><strong>l</strong> positive regulation of locomotion</td>
<td>84</td>
<td>1.86</td>
<td>8.62E-04</td>
</tr>
<tr>
<td><strong>l</strong> regulation of cell migration</td>
<td>128</td>
<td>1.72</td>
<td>1.12E-05</td>
</tr>
<tr>
<td><strong>l</strong> positive regulation of multicellular organismal process</td>
<td>244</td>
<td>1.60</td>
<td>6.31E-09</td>
</tr>
<tr>
<td><strong>l</strong> regulation of multicellular organismal process</td>
<td>407</td>
<td>1.45</td>
<td>1.69E-10</td>
</tr>
<tr>
<td><strong>l</strong> regulation of epithelial cell migration</td>
<td>45</td>
<td>2.45</td>
<td>7.76E-04</td>
</tr>
<tr>
<td>sphingolipid metabolic process</td>
<td>38</td>
<td>2.42</td>
<td>9.56E-03</td>
</tr>
<tr>
<td><strong>l</strong> cellular lipid metabolic process</td>
<td>168</td>
<td>1.69</td>
<td>5.27E-07</td>
</tr>
</tbody>
</table>
### Table 6.3 Overrepresented gene ontology (GO) groups induced in disease cells (Disease v WT)

**Upregulated in D (D vs WT)**

<table>
<thead>
<tr>
<th>GO biological term</th>
<th>Number of genes</th>
<th>Fold Enrichment</th>
<th>Corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>xenobiotic glucuronidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 carboxylic acid metabolic process</td>
<td>120</td>
<td>2.01</td>
<td>1.32E-08</td>
</tr>
<tr>
<td>1 oxoacid metabolic process</td>
<td>120</td>
<td>1.99</td>
<td>2.01E-08</td>
</tr>
<tr>
<td>1 organic acid metabolic process</td>
<td>132</td>
<td>1.98</td>
<td>2.16E-09</td>
</tr>
<tr>
<td>1 organic substance metabolic process</td>
<td>869</td>
<td>1.20</td>
<td>3.35E-09</td>
</tr>
<tr>
<td>1 metabolic process</td>
<td>915</td>
<td>1.21</td>
<td>2.40E-11</td>
</tr>
<tr>
<td>1 single-organism cellular process</td>
<td>1115</td>
<td>1.14</td>
<td>4.25E-08</td>
</tr>
<tr>
<td>1 cellular process</td>
<td>1275</td>
<td>1.11</td>
<td>7.52E-09</td>
</tr>
<tr>
<td>1 small molecule metabolic process</td>
<td>235</td>
<td>1.79</td>
<td>6.94E-14</td>
</tr>
<tr>
<td>1 single-organism metabolic process</td>
<td>425</td>
<td>1.58</td>
<td>1.10E-18</td>
</tr>
<tr>
<td>1 cellular metabolic process</td>
<td>832</td>
<td>1.22</td>
<td>7.26E-10</td>
</tr>
<tr>
<td>negative regulation of cellular glucuronidation</td>
<td>8</td>
<td>12.57</td>
<td>3.05E-03</td>
</tr>
<tr>
<td>1 regulation of cellular glucuronidation</td>
<td>8</td>
<td>11.18</td>
<td>7.29E-03</td>
</tr>
<tr>
<td>negative regulation of glucuronosyltransferase activity</td>
<td>8</td>
<td>12.57</td>
<td>3.05E-03</td>
</tr>
<tr>
<td>1 regulation of glucuronosyltransferase activity</td>
<td>8</td>
<td>12.57</td>
<td>3.05E-03</td>
</tr>
<tr>
<td>protein folding in endoplasmic reticulum</td>
<td>8</td>
<td>9.14</td>
<td>3.16E-02</td>
</tr>
<tr>
<td>1 protein folding</td>
<td>46</td>
<td>2.58</td>
<td>1.18E-04</td>
</tr>
<tr>
<td>IRE1-mediated unfolded protein response</td>
<td>22</td>
<td>4.94</td>
<td>1.79E-05</td>
</tr>
<tr>
<td>1 endoplasmic reticulum unfolded protein response</td>
<td>36</td>
<td>4.04</td>
<td>4.88E-08</td>
</tr>
<tr>
<td>1 cellular response to unfolded protein</td>
<td>37</td>
<td>4.05</td>
<td>2.43E-08</td>
</tr>
<tr>
<td>1 response to unfolded protein</td>
<td>38</td>
<td>3.10</td>
<td>1.98E-05</td>
</tr>
<tr>
<td>1 response to topologically incorrect protein</td>
<td>39</td>
<td>2.97</td>
<td>3.89E-05</td>
</tr>
<tr>
<td>1 response to stress</td>
<td>354</td>
<td>1.35</td>
<td>1.74E-05</td>
</tr>
<tr>
<td>1 cellular response to topologically incorrect protein</td>
<td>38</td>
<td>3.85</td>
<td>5.06E-08</td>
</tr>
<tr>
<td>1 cellular response to stress</td>
<td>211</td>
<td>1.67</td>
<td>2.74E-09</td>
</tr>
<tr>
<td>1 response to endoplasmic reticulum stress</td>
<td>56</td>
<td>2.97</td>
<td>1.92E-08</td>
</tr>
</tbody>
</table>
Table 6.4 Overrepresented gene ontology (GO) groups in genes downregulated by disease cells treated with estrogen (D E+ vs WT E+)

<table>
<thead>
<tr>
<th>GO biological term</th>
<th>Number of genes</th>
<th>Fold Enrichment</th>
<th>Corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>establishment of skin barrier</td>
<td>9</td>
<td>10.71</td>
<td>2.15E-03</td>
</tr>
<tr>
<td>皮肤发展</td>
<td>41</td>
<td>3.91</td>
<td>4.01E-09</td>
</tr>
<tr>
<td>动物器官发展</td>
<td>215</td>
<td>1.55</td>
<td>4.16E-07</td>
</tr>
<tr>
<td>系统发展</td>
<td>357</td>
<td>1.45</td>
<td>3.24E-11</td>
</tr>
<tr>
<td>单一多细胞组织发展</td>
<td>360</td>
<td>1.43</td>
<td>2.72E-10</td>
</tr>
<tr>
<td>单一多细胞器官发展</td>
<td>288</td>
<td>1.48</td>
<td>9.36E-09</td>
</tr>
<tr>
<td>单一多细胞器官的发展</td>
<td>328</td>
<td>1.46</td>
<td>3.71E-10</td>
</tr>
<tr>
<td>单一多细胞器官的发育</td>
<td>381</td>
<td>1.45</td>
<td>1.82E-12</td>
</tr>
<tr>
<td>单一多细胞组织的发育</td>
<td>719</td>
<td>1.18</td>
<td>8.37E-09</td>
</tr>
<tr>
<td>单一多细胞组织的发育</td>
<td>410</td>
<td>1.3</td>
<td>1.34E-06</td>
</tr>
<tr>
<td>单一-多细胞器官的发育</td>
<td>344</td>
<td>1.4</td>
<td>2.02E-08</td>
</tr>
<tr>
<td>细胞多态化过程</td>
<td>381</td>
<td>1.18</td>
<td>4.49E-03</td>
</tr>
<tr>
<td>细胞发展过程</td>
<td>10</td>
<td>10.64</td>
<td>4.96E-04</td>
</tr>
<tr>
<td>水平衡的维持</td>
<td>14</td>
<td>4.88</td>
<td>1.58E-02</td>
</tr>
<tr>
<td>基因调控的生物质量</td>
<td>241</td>
<td>1.41</td>
<td>1.17E-04</td>
</tr>
<tr>
<td>单一-细胞发育过程</td>
<td>16</td>
<td>4.49</td>
<td>8.67E-03</td>
</tr>
<tr>
<td>胆固醇生物合成过程</td>
<td>12</td>
<td>6.22</td>
<td>7.33E-03</td>
</tr>
<tr>
<td>次级乙醇生物合成过程</td>
<td>12</td>
<td>6.22</td>
<td>7.33E-03</td>
</tr>
<tr>
<td>乙醇生物合成过程</td>
<td>17</td>
<td>4.91</td>
<td>1.20E-03</td>
</tr>
<tr>
<td>单一-单细胞代谢过程</td>
<td>235</td>
<td>1.4</td>
<td>2.39E-04</td>
</tr>
<tr>
<td>小分子代谢过程</td>
<td>126</td>
<td>1.54</td>
<td>9.90E-03</td>
</tr>
<tr>
<td>酒精代谢过程</td>
<td>36</td>
<td>2.86</td>
<td>3.57E-04</td>
</tr>
<tr>
<td>有机羟基化合物代谢过程</td>
<td>45</td>
<td>2.48</td>
<td>4.57E-04</td>
</tr>
<tr>
<td>有机羟基化合物的生物合成过程</td>
<td>23</td>
<td>3.88</td>
<td>5.90E-04</td>
</tr>
<tr>
<td>乙醇代谢过程</td>
<td>21</td>
<td>3.69</td>
<td>4.63E-03</td>
</tr>
<tr>
<td>单一-乙醇代谢过程</td>
<td>20</td>
<td>3.61</td>
<td>1.19E-02</td>
</tr>
<tr>
<td>单一-脂代谢过程</td>
<td>21</td>
<td>3.37</td>
<td>1.90E-02</td>
</tr>
<tr>
<td>乙酸盐代谢过程</td>
<td>20</td>
<td>3.37</td>
<td>1.90E-02</td>
</tr>
<tr>
<td>脂类代谢过程</td>
<td>108</td>
<td>1.87</td>
<td>5.14E-06</td>
</tr>
<tr>
<td>单一-脂类生物合成过程</td>
<td>14</td>
<td>6.43</td>
<td>6.20E-04</td>
</tr>
<tr>
<td>单一-脂类生物合成过程</td>
<td>20</td>
<td>3.71</td>
<td>7.93E-03</td>
</tr>
<tr>
<td>软化</td>
<td>14</td>
<td>5.66</td>
<td>2.83E-03</td>
</tr>
<tr>
<td>赫尔纳汀细胞分化过程</td>
<td>22</td>
<td>4.41</td>
<td>1.27E-04</td>
</tr>
<tr>
<td>表皮细胞分化过程</td>
<td>27</td>
<td>3.77</td>
<td>8.04E-05</td>
</tr>
<tr>
<td>表皮细胞分化过程</td>
<td>55</td>
<td>2.27</td>
<td>2.72E-04</td>
</tr>
<tr>
<td>细胞分化过程</td>
<td>88</td>
<td>1.98</td>
<td>1.78E-05</td>
</tr>
<tr>
<td>细胞分化过程</td>
<td>149</td>
<td>1.99</td>
<td>1.22E-11</td>
</tr>
<tr>
<td>细胞分化过程</td>
<td>222</td>
<td>1.4</td>
<td>9.33E-04</td>
</tr>
<tr>
<td>单一-细胞分化过程</td>
<td>227</td>
<td>1.34</td>
<td>1.66E-02</td>
</tr>
<tr>
<td>单一-单细胞分化过程</td>
<td>652</td>
<td>1.2</td>
<td>4.33E-08</td>
</tr>
<tr>
<td>皮脂代谢过程</td>
<td>46</td>
<td>3.62</td>
<td>2.20E-09</td>
</tr>
<tr>
<td>道道道过程</td>
<td>21</td>
<td>3.34</td>
<td>2.15E-02</td>
</tr>
<tr>
<td>单一-细胞脂类代谢过程</td>
<td>84</td>
<td>1.84</td>
<td>9.03E-04</td>
</tr>
<tr>
<td>环己烷脂类代谢过程</td>
<td>24</td>
<td>3.32</td>
<td>4.73E-03</td>
</tr>
</tbody>
</table>
Table 6.5 Overrepresented gene ontology (GO) groups induced in disease cells treated with estrogen (D E+ vs WT E+)

<table>
<thead>
<tr>
<th>GO biological term</th>
<th>Number of genes</th>
<th>Fold Enrichment</th>
<th>Corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent</td>
<td>7</td>
<td>32.95</td>
<td>2.52E-05</td>
</tr>
<tr>
<td>antigen processing and presentation of peptide antigen via MHC class I</td>
<td>13</td>
<td>5.80</td>
<td>5.36E-03</td>
</tr>
<tr>
<td>protein folding in endoplasmic reticulum</td>
<td>7</td>
<td>26.96</td>
<td>9.86E-05</td>
</tr>
<tr>
<td>protein folding</td>
<td>22</td>
<td>4.16</td>
<td>2.93E-04</td>
</tr>
<tr>
<td>IRE1-mediated unfolded protein response</td>
<td>11</td>
<td>8.32</td>
<td>1.21E-03</td>
</tr>
<tr>
<td>endoplasmic reticulum unfolded protein response</td>
<td>14</td>
<td>5.30</td>
<td>5.80E-03</td>
</tr>
<tr>
<td>response to stimulus</td>
<td>230</td>
<td>1.29</td>
<td>1.09E-02</td>
</tr>
<tr>
<td>single-organism cellular process</td>
<td>309</td>
<td>1.19</td>
<td>3.22E-02</td>
</tr>
<tr>
<td>cellular response to unfolded protein</td>
<td>14</td>
<td>5.16</td>
<td>7.87E-03</td>
</tr>
<tr>
<td>response to stress</td>
<td>117</td>
<td>1.5</td>
<td>2.61E-02</td>
</tr>
<tr>
<td>response to organic substance</td>
<td>96</td>
<td>1.57</td>
<td>3.93E-02</td>
</tr>
<tr>
<td>cellular response to topologically incorrect protein</td>
<td>14</td>
<td>4.78</td>
<td>1.87E-02</td>
</tr>
<tr>
<td>response to endoplasmic reticulum stress</td>
<td>21</td>
<td>3.75</td>
<td>3.16E-03</td>
</tr>
<tr>
<td>cell redox homeostasis</td>
<td>12</td>
<td>6.52</td>
<td>4.32E-03</td>
</tr>
<tr>
<td>regulation of biological quality</td>
<td>127</td>
<td>1.56</td>
<td>1.24E-03</td>
</tr>
<tr>
<td>regulation of neuron projection development</td>
<td>30</td>
<td>3.01</td>
<td>1.26E-03</td>
</tr>
<tr>
<td>regulation of neuron differentiation</td>
<td>35</td>
<td>2.60</td>
<td>3.74E-03</td>
</tr>
<tr>
<td>regulation of neurogenesis</td>
<td>40</td>
<td>2.47</td>
<td>1.98E-03</td>
</tr>
<tr>
<td>regulation of cell development</td>
<td>44</td>
<td>2.19</td>
<td>1.20E-02</td>
</tr>
<tr>
<td>regulation of cell differentiation</td>
<td>64</td>
<td>1.78</td>
<td>4.92E-02</td>
</tr>
<tr>
<td>regulation of nervous system development</td>
<td>43</td>
<td>2.35</td>
<td>2.59E-03</td>
</tr>
<tr>
<td>nervous system development</td>
<td>83</td>
<td>1.64</td>
<td>3.98E-02</td>
</tr>
<tr>
<td>system development</td>
<td>138</td>
<td>1.48</td>
<td>4.32E-03</td>
</tr>
<tr>
<td>anatomical structure development</td>
<td>161</td>
<td>1.37</td>
<td>3.99E-02</td>
</tr>
<tr>
<td>developmental process</td>
<td>170</td>
<td>1.41</td>
<td>2.63E-03</td>
</tr>
<tr>
<td>multicellular organism development</td>
<td>151</td>
<td>1.41</td>
<td>2.01E-02</td>
</tr>
<tr>
<td>single-organism developmental process</td>
<td>163</td>
<td>1.39</td>
<td>1.48E-02</td>
</tr>
<tr>
<td>regulation of cell projection organization</td>
<td>33</td>
<td>2.42</td>
<td>3.54E-02</td>
</tr>
</tbody>
</table>
### Table 6.6 Overrepresented gene ontology (GO) groups in genes downregulated by disease cells in the absence of estrogen (D E- vs WT E-)

**Downregulated in D without estrogen (D E- vs WT E-)**

<table>
<thead>
<tr>
<th>GO biological term</th>
<th>Number of genes</th>
<th>Fold Enrichment</th>
<th>Corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>establishment of skin barrier</td>
<td>11</td>
<td>12.07</td>
<td>3.06E-05</td>
</tr>
<tr>
<td>skin development</td>
<td>51</td>
<td>4.49</td>
<td>2.61E-14</td>
</tr>
<tr>
<td>animal organ development</td>
<td>254</td>
<td>1.69</td>
<td>3.02E-13</td>
</tr>
<tr>
<td>anatomical structure development</td>
<td>407</td>
<td>1.53</td>
<td>2.61E-17</td>
</tr>
<tr>
<td>developmental process</td>
<td>411</td>
<td>1.51</td>
<td>2.53E-16</td>
</tr>
<tr>
<td>system development</td>
<td>327</td>
<td>1.55</td>
<td>3.30E-13</td>
</tr>
<tr>
<td>multicellular organism development</td>
<td>371</td>
<td>1.53</td>
<td>6.28E-15</td>
</tr>
<tr>
<td>single-multicellular organism process</td>
<td>424</td>
<td>1.49</td>
<td>2.06E-16</td>
</tr>
<tr>
<td>single-organism process</td>
<td>807</td>
<td>1.22</td>
<td>4.51E-16</td>
</tr>
<tr>
<td>multicellular organismal process</td>
<td>460</td>
<td>1.35</td>
<td>2.39E-10</td>
</tr>
<tr>
<td>single-organism developmental process</td>
<td>394</td>
<td>1.48</td>
<td>4.38E-14</td>
</tr>
<tr>
<td>regulation of water loss via skin</td>
<td>12</td>
<td>11.78</td>
<td>7.89E-06</td>
</tr>
<tr>
<td>multicellular organismal water homeostasis</td>
<td>18</td>
<td>5.79</td>
<td>4.54E-05</td>
</tr>
<tr>
<td>regulation of biological quality</td>
<td>259</td>
<td>1.40</td>
<td>7.96E-05</td>
</tr>
<tr>
<td>biological regulation</td>
<td>710</td>
<td>1.16</td>
<td>2.58E-05</td>
</tr>
<tr>
<td>multicellular organismal homeostasis</td>
<td>37</td>
<td>2.50</td>
<td>5.68E-03</td>
</tr>
<tr>
<td>water homeostasis</td>
<td>21</td>
<td>5.44</td>
<td>7.43E-06</td>
</tr>
<tr>
<td>keratinization</td>
<td>18</td>
<td>6.72</td>
<td>4.67E-06</td>
</tr>
<tr>
<td>keratinocyte differentiation</td>
<td>28</td>
<td>5.17</td>
<td>4.03E-08</td>
</tr>
<tr>
<td>epidermal cell differentiation</td>
<td>33</td>
<td>4.25</td>
<td>9.43E-08</td>
</tr>
<tr>
<td>epithelial cell differentiation</td>
<td>66</td>
<td>2.52</td>
<td>2.04E-07</td>
</tr>
<tr>
<td>epithelium development</td>
<td>104</td>
<td>2.16</td>
<td>4.45E-09</td>
</tr>
<tr>
<td>tissue development</td>
<td>170</td>
<td>2.10</td>
<td>8.79E-16</td>
</tr>
<tr>
<td>cell differentiation</td>
<td>257</td>
<td>1.49</td>
<td>1.47E-07</td>
</tr>
<tr>
<td>cellular developmental process</td>
<td>265</td>
<td>1.44</td>
<td>2.27E-06</td>
</tr>
<tr>
<td>single-organism cellular process</td>
<td>733</td>
<td>1.25</td>
<td>1.55E-14</td>
</tr>
<tr>
<td>cellular process</td>
<td>854</td>
<td>1.11</td>
<td>2.42E-04</td>
</tr>
<tr>
<td>epidermis development</td>
<td>55</td>
<td>3.99</td>
<td>1.84E-13</td>
</tr>
<tr>
<td>cholesterol biosynthetic process</td>
<td>14</td>
<td>6.70</td>
<td>3.84E-04</td>
</tr>
<tr>
<td>secondary alcohol biosynthetic process</td>
<td>14</td>
<td>6.70</td>
<td>3.84E-04</td>
</tr>
<tr>
<td>alcohol biosynthetic process</td>
<td>19</td>
<td>5.06</td>
<td>1.42E-04</td>
</tr>
<tr>
<td>small molecule biosynthetic process</td>
<td>45</td>
<td>2.16</td>
<td>1.90E-02</td>
</tr>
<tr>
<td>single-organism metabolic process</td>
<td>258</td>
<td>1.42</td>
<td>1.56E-05</td>
</tr>
<tr>
<td>alcohol metabolic process</td>
<td>36</td>
<td>2.63</td>
<td>2.45E-03</td>
</tr>
<tr>
<td>organic hydroxy compound metabolic process</td>
<td>45</td>
<td>2.29</td>
<td>4.21E-03</td>
</tr>
<tr>
<td>organic hydroxy compound biosynthetic process</td>
<td>25</td>
<td>3.89</td>
<td>1.56E-04</td>
</tr>
<tr>
<td>secondary alcohol metabolic process</td>
<td>22</td>
<td>3.57</td>
<td>4.43E-03</td>
</tr>
<tr>
<td>cholesterol metabolic process</td>
<td>22</td>
<td>3.67</td>
<td>2.87E-03</td>
</tr>
<tr>
<td>sterol metabolic process</td>
<td>23</td>
<td>3.41</td>
<td>5.44E-03</td>
</tr>
<tr>
<td>steroid metabolic process</td>
<td>31</td>
<td>2.55</td>
<td>3.07E-02</td>
</tr>
<tr>
<td>lipid metabolic process</td>
<td>117</td>
<td>1.87</td>
<td>1.08E-06</td>
</tr>
<tr>
<td>sterol biosynthetic process</td>
<td>16</td>
<td>6.78</td>
<td>3.61E-05</td>
</tr>
<tr>
<td>steroid biosynthetic process</td>
<td>22</td>
<td>3.77</td>
<td>1.84E-03</td>
</tr>
<tr>
<td>lipid biosynthetic process</td>
<td>59</td>
<td>2.13</td>
<td>8.86E-04</td>
</tr>
<tr>
<td>negative regulation of inflammatory response</td>
<td>20</td>
<td>3.62</td>
<td>1.14E-02</td>
</tr>
<tr>
<td>negative regulation of response to external stimulus</td>
<td>36</td>
<td>2.60</td>
<td>3.21E-03</td>
</tr>
<tr>
<td>regulation of response to external stimulus</td>
<td>74</td>
<td>1.73</td>
<td>4.59E-02</td>
</tr>
<tr>
<td>regulation of response to stimulus</td>
<td>294</td>
<td>1.51</td>
<td>8.82E-10</td>
</tr>
<tr>
<td>regulation of biological process</td>
<td>664</td>
<td>1.14</td>
<td>2.50E-03</td>
</tr>
<tr>
<td>negative regulation of response to stimulus</td>
<td>129</td>
<td>1.75</td>
<td>6.91E-06</td>
</tr>
<tr>
<td>negative regulation of biological process</td>
<td>336</td>
<td>1.40</td>
<td>1.40E-07</td>
</tr>
<tr>
<td>negative regulation of defence response</td>
<td>25</td>
<td>3.15</td>
<td>7.26E-03</td>
</tr>
<tr>
<td>regulation of fat cell differentiation</td>
<td>21</td>
<td>3.50</td>
<td>1.08E-02</td>
</tr>
<tr>
<td>regulation of cell differentiation</td>
<td>133</td>
<td>1.63</td>
<td>2.24E-04</td>
</tr>
<tr>
<td>regulation of cellular process</td>
<td>632</td>
<td>1.14</td>
<td>8.62E-03</td>
</tr>
</tbody>
</table>
Table 6.7 Overrepresented gene ontology (GO) groups induced in disease cells in the absence of estrogen (D E- vs WT E-)

<table>
<thead>
<tr>
<th>GO biological term</th>
<th>Number of genes</th>
<th>Fold Enrichment</th>
<th>Corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent</td>
<td>7</td>
<td>23.10</td>
<td>2.83E-04</td>
</tr>
<tr>
<td>protein folding in endoplasmic reticulum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>upregulated in D without estrogen (D E- vs WT E-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO biological term</td>
<td>Number of genes</td>
<td>Fold Enrichment</td>
<td>Corrected p-value</td>
</tr>
<tr>
<td>protein folding in endoplasmic reticulum via MHC class I, TAP-independent</td>
<td>23</td>
<td>4.32</td>
<td>7.88E-05</td>
</tr>
<tr>
<td>homophilic cell adhesion via plasma membrane adhesion molecules</td>
<td>23</td>
<td>4.32</td>
<td>7.88E-05</td>
</tr>
<tr>
<td>cell redox homeostasis</td>
<td>16</td>
<td>6.09</td>
<td>1.51E-04</td>
</tr>
</tbody>
</table>

| GO biological term                                                                 | Number of genes | Fold Enrichment | Corrected p-value |
| IRE1-mediated unfolded protein response                                            |                 |                 |                   |
| endoplasmic reticulum unfolded protein response                                    |                 |                 |                   |
| single-organism cellular process                                                  |                 |                 |                   |
| cellular response to unfolded protein                                             |                 |                 |                   |
| response to unfolded protein                                                      |                 |                 |                   |
| response to topologically incorrect protein                                       |                 |                 |                   |
| response to stress                                                                |                 |                 |                   |
| cellular response to topologically incorrect protein                              |                 |                 |                   |
| response to topologically incorrect protein                                        |                 |                 |                   |
| response to endoplasm reticulum stress                                            |                 |                 |                   |
| response to endoplasm reticulum stress                                            |                 |                 |                   |
6.4.1 Overrepresented GO groups downregulated in disease cells include ‘cell migration’, ‘negative regulation of inflammatory response’ and ‘steroid hormone biosynthesis’

Gene ontology terms overrepresented in the downregulated gene group of disease cells versus WT cells included the establishment of skin barrier (11 genes, \( p=2.87 \times 10^{-3} \)) and keratinisation (21 genes, \( p=1.99 \times 10^{-3} \)) (Table 6.2). Formation of the skin barrier is essential to prevent water loss and pathogen ingress. Genes downregulated in these groups include filaggrin (\( FLG \)) (Fc=15.6, \( p=1.58 \times 10^{-4}, \) FDR=1.30 \( \times 10^{-2} \)), transglutaminase 1 (\( TGM1 \)) (Fc=4.16, \( p=1.99 \times 10^{-4}, \) FDR=1.80 \( \times 10^{-3} \)), claudin 4 (\( CLDN4 \)) (Fc=10.4, \( p=2.87 \times 10^{-7}, \) FDR=1.16 \( \times 10^{-4} \)) and keratin 10 (\( KRT10 \)) (Fc=9.00, \( p=7.36 \times 10^{-4}, \) FDR=3.80 \( \times 10^{-2} \)). Genes involved in sphingolipid metabolism (38 genes, \( p=9.56 \times 10^{-3} \)) were also downregulated in disease cells compared to wild type (Table 6.2). Sphingolipid metabolism is involved in the formation of ceramide and its precursors which contribute to the lipid lamellae of the epidermal barrier. Dysfunction in this pathway is associated with several dermatoses, including atopic dermatitis (Kleuser and Japtok, 2013). Enzymes in this pathway that are downregulated include sphingosine kinase I (\( SPHK1 \)) (Fc=1.60, \( p=1.60 \times 10^{-2}, \) FDR=2.94 \( \times 10^{-1} \)), elongation of very long chain fatty acids 4 (\( ELOVL4 \)) (Fc=7.06, \( p=3.90 \times 10^{-4}, \) FDR=2.40 \( \times 10^{-1} \)) and ceramide synthase 3 (\( CERS3 \)) (Fc=2.45, \( p=8.48 \times 10^{-3}, \) FDR=1.89 \( \times 10^{-1} \)).

The ‘negative regulation of inflammatory response’ (20 genes, \( p=1.14 \times 10^{-3} \)) GO term was overrepresented within the downregulated D vs WT comparison gene set under estrogen-free conditions (Table 6.6). Genes in this term, such as zinc finger protein 36 (\( ZFP36 \)) (Fc=2.53, \( p=4.26 \times 10^{-3}, \) FDR=0.340) exert anti-inflammatory effects via inhibition of pro-inflammatory cytokines (Joe et al., 2015). Underrepresentation in disease cells fits with the hyper-inflammatory response seen in chronic wounds.

The GO term ‘cholesterol biosynthetic process’ (17 genes, \( p=1.64 \times 10^{-2} \)) was overrepresented in the downregulated genes of disease cells (Table 6.2) regardless of estrogen treatment (Tables 6.4 and 6.6). Interestingly, the hierarchical parental term ‘steroid biosynthesis’ (35 genes, \( p=2.13 \times 10^{-3} \)), which includes the synthesis pathway for 17β-estradiol and its precursors, is also overrepresented. Numerous genes are downregulated in the cholesterol pathways (Figure 6.3) including the key enzymes 7-dehydrocholesterol reductase (\( DHCR7 \)) (Fc=1.75, \( p=2.40 \times 10^{-2}, \) FDR=0.350) and 24-dehydrocholesterol reductase 24 (\( DHCR24 \)) (Fc=2.16, \( p=3.86 \times 10^{-3}, \) FDR=0.114). Many genes are also downregulated in the steroid hormone synthesis pathway (Figure 6.3), including the enzymes directly involved in estrogenic ligand synthesis; hydroxysteroid (17-Beta) dehydrogenase 2 (\( HSD17B2 \)) (Fc=7.30, \( p=1.10 \times 10^{-4}, \) FDR=9.66 \( \times 10^{-3} \)), hydroxysteroid (17-Beta) dehydrogenase 7 (\( HSD17B7 \)) (Fc=2.72, \( p=5.67 \times 10^{-4}, \) FDR=3.14 \( \times 10^{-2} \)) and cytochrome P450 family 3 subfamily A member 4 (\( CYP3A4 \)) (Fc=8.25, \( p=1.33 \times 10^{-2}, \) FDR=0.253), but curiously not aromatase (\( CYP19A1 \)). This could potentially indicate that the capability of venous ulcer SNP harbouring
keratinocytes to synthesis estrogen ligand is reduced, resulting in less intracellular ligand available for signalling.
Figure 6.3  – Venous ulcer-associated SNP disease cells display less expression of genes involved in steroid hormone synthesis. (A) The cholesterol and subsequent steroid hormone synthesis pathway and (B) RNA-seq fold-change of genes downregulated in disease cells (Disease vs WT). Members of the cholesterol pathway that are downregulated are highlighted in purple and downregulated genes of the steroid synthesis pathway are highlighted in red.
Of particular relevance, overrepresentation was found in the ‘positive regulation of epithelial cell migration’ (31 genes, \( p=8.93 \times 10^{-3} \)) GO term for genes downregulated in disease vs WT (Table 6.2), with the broader term ‘positive signalling of cell migration’ (81 genes, \( p=4.83 \times 10^{-4} \)) being even more enriched in the dataset. This included various growth factors that promote wound healing, such as fibroblast growth factor 18 (\( \text{FGF18} \)) (\( \text{Fc}=11.68, p=1.17 \times 10^{-4}, \text{FDR}=1.20 \times 10^{-2} \)), the ECM constituent hyaluronic acid-binding cell migration-inducing and hyaluronan-binding protein (\( \text{CEMIP} \)) (\( \text{Fc}=19.08, p=1.15 \times 10^{-4}, \text{FDR}=1.00 \times 10^{-2} \)) and various transcription factors involved in wound healing such as the AP1 subunit jun proto-oncogene (\( \text{JUN} \)) (\( \text{Fc}=5.14, p=1.25 \times 10^{-4}, \text{FDR}=1.15 \times 10^{-2} \)) (Angel et al., 2001). Protein tyrosine kinase 2 (\( \text{PTK2} \)) (\( \text{Fc}=2.67, p=8.33 \times 10^{-6}, \text{FDR}=1.50 \times 10^{-3} \)), a focal adhesion kinase that is necessary for timely wound healing (Gates et al., 1994) (Wong et al., 2014), was also significantly downregulated in disease cells. This fits with the observed migratory deficiencies seen in disease keratinocyte and fibroblast scratch assays (Figures 3.10 and 3.11).

A key feature of this cell migration GO term is the mitogen-activated protein kinase signalling pathway (MAPK/ERK pathway) (51 genes, \( p=1.08 \times 10^{-7} \)). This pathway can phosphorylate and activate target proteins in response to external stimuli such as growth factors. The MAPK/ERK signalling pathway can regulate cell proliferation and differentiation (Schramek et al., 1997) and has been shown to be required for keratinocyte migration over collagen (Li et al., 2001). Ras signalling can cause the rearrangement of the actin cytoskeleton that creates protrusions and ruffling that are important for cell migration (Tscharntke et al., 2005), and can directly promote wound healing in keratinocytes and fibroblasts through the activation of p38 MAPK (Charvat et al., 1999) (Bulat et al., 2009). Many components of the Ras pathway are downregulated in disease cells versus wild type (illustrated in Figure 6.4). These include upstream mediators; colony stimulating factor 1 (\( \text{CSF1} \)) (\( \text{Fc}=5.72, p=2.43 \times 10^{-5}, \text{FDR}=3.26 \times 10^{-3} \)), RAS protein activator like 1 (rasGAP) (\( \text{RASL1} \)) (\( \text{Fc}=18.18, p=1.56 \times 10^{-6}, \text{FDR}=2.43 \times 10^{-3} \)) and insulin-like growth factor 2 (\( \text{IGF2} \)) (\( \text{Fc}=12.30, p=5.50 \times 10^{-5}, \text{FDR}=5.92 \times 10^{-3} \)) and downstream factors; including Phosphoinositide-3-kinase regulatory subunit 1 (\( \text{PI3K}(\text{PIK3R1}) \)) (\( \text{Fc}=3.04, p=6.99 \times 10^{-4}, \text{FDR}=3.62 \times 10^{-3} \)), Nuclear factor kappa B (\( \text{NFkB1} \)) (\( \text{Fc}=1.75, p=4.58 \times 10^{-5}, \text{FDR}=1.27 \times 10^{-1} \)), Mitogen-activated protein kinase (\( \text{ERK/MAPK3} \)) (\( \text{Fc}=2.16, p=1.82 \times 10^{-2}, \text{FDR}=3.09 \times 10^{-1} \)) and AKT serine/threonine kinase 3 (\( \text{AKT3} \)) (\( \text{Fc}=3.44, p=3.11 \times 10^{-2}, \text{FDR}=4.19 \times 10^{-1} \)).
Figure 6.4 - Venous ulcer-associated SNP disease cells display less expression of genes involved in the Ras signalling pathway. (A) The Ras signalling pathway with genes downregulated by disease cells (Disease vs WT) and genes associated with upstream growth factor signalling highlighted in red. Downstream effects of aspects of the pathway are highlighted in green. (B) RNA-seq fold-change of genes downregulated in disease cells (Disease vs WT) in this pathway.
Similarly, Rap1 signalling is upregulated in the leading wound edge (Fujita et al., 2005) and regulates cell-cell adhesion and adhesion to the extracellular matrix (Boettner and Van Aelst, 2009). Genes downregulated by disease cells upstream to this pathway include protein kinase D2 (PKD) (PRKD2) (Fc=1.83, p=2.71x10\(^{-3}\), FDR=8.91x10\(^{-2}\)) and Rap guanine nucleotide exchange factor 5 (repac) (RAPGEF5) (Fc=5.16, p=2.10x10\(^{-4}\), FDR=1.57x10\(^{-2}\)). Downstream mediators include mitogen-activated protein kinase 14 (MAPK14) (p38MAPK) (Fc=2.15, p=2.41x10\(^{-4}\), FDR=1.72x10\(^{-2}\)), protein kinase C iota (PRKCi) (Fc=1.70, p=4.26x10\(^{-3}\), FDR=1.21x10\(^{-1}\)) and integrin beta 6 (ITGB6) (Fc=3.39, p=1.39 x10\(^{-2}\), FDR=2.26x10\(^{-2}\)) (illustrated by Figure 6.5).
Figure 6.5 - Venous ulcer-associated SNP disease cells display lower expression of genes involved in the Rap1 signalling pathway. (A) RNA-seq fold-change of genes downregulated in disease cells (Disease vs WT) in the Rap1 pathway and downstream effects of aspects of the pathway highlighted in green. (B) The Rap1 signalling pathway with genes downregulated by disease cells (Disease vs WT) highlighted in red.
6.4.2 Overrepresented GO groups upregulated in disease cells include ‘MHC Class I processing and presentation’ and ‘homophilic cell adhesion molecules’

Several GO terms of interest were found for overrepresented genes upregulated in disease cells (Tables 6.3, 6.5 and 6.7). The ‘xenobiotic glucuronidation’ (9 genes, p=5.73x10^{-4}) GO term was found to be enriched in genes upregulated in disease cells (Table 6.3), as was ‘protein folding’ (8 genes, p=3.16x10^{-2}). More applicable to chronic wounds, the ‘antigen processing and presentation of exogenous peptide antigen via MHC class I’ GO term (13 genes, p=5.36x10^{-3}) was found overrepresented with disease upregulated genes under both estrogen positive (Table 6.5) and negative (Table 6.7) conditions. Found only in the absence of estrogen (Table 6.7) was an enrichment in cell adhesion genes (23 genes, p=7.88x10^{-5}) in disease cells, which could also be significant towards wound healing.

A large number of genes in the MHC class I antigen processing and presentation pathway are upregulated in disease cells compared to WT cells (Figure 6.6). MHC class I is involved in presenting endogenously produced antigens at the cell surface for recognition by CD8+ cytotoxic T-cells, whilst MHC class II mainly presents antigen to CD4+ T-helper cells. These T-cells secrete regulatory and inflammatory cytokines in the wound that can impact healing (Chen et al., 2014b). Excessive antigen presentation by MHC class I could cause an increase in wound CD8+ T-cells which in turn would alter the balance of inflammatory regulation at the wound site. MHC class I overexpression can be associated with various inflammatory and autoimmune pathologies (Li et al., 2009) (Kay et al., 1991) and MHC class II deficiency has been shown to impair wound healing (Braunstein et al., 1991), whilst the CD4+/CD8+ ratio in chronic wounds is significantly lower compared to acute wounds (Loots et al., 1998). Genes at all sections of the MHC class I antigen presentation pathway are found to be upregulated in disease keratinocytes (Figure 6.6) including major histocompatibility complex class I F (HLA-F) (Fc=3.72, p=2.10x10^{-11}, FDR=6.08x10^{-7}), beta-2-microglobulin (B2M) (Fc=1.71, p=2.32x10^{-3}, FDR=3.46x10^{-1}), transporter 1, ATP binding cassette subfamily B member (TAP1) (Fc=1.50, p=2.81x10^{-3}, FDR=3.71x10^{-1}) and SEC24 homolog D COPII coat complex component (SEC24D) (Fc=2.14, p=1.32x10^{-3}, FDR=2.81x10^{-1}).

Disease cells also expressed genes associated with cell adhesion to a greater extent than WT cells (Figure 6.7), such as cadherin 3 (P-Cadherin) (CDH3) (Fc=1.93, p=1.35x10^{-2}, FDR=7.16x10^{-1}) and cell adhesion molecule 1 (CADM1) (Fc=3.97, p=7.86 x10^{-6}, FDR=1.44x10^{-2}). Cell-cell adhesions are essential for timely wound healing (Nagaoka et al., 2000), but need to be downregulated and deconstructed to allow for migration in the initial stages of wounding (Krawczyk and Wilgram, 1973) (Wallis et al., 2000) and dysregulation could lead to chronic wounds (Thomason et al., 2012). Evidence shows that E-cadherin and P-cadherin are downregulated upon wounding and that an increase in this expression could participate in a delay of terminal differentiation of keratinocytes for supply towards the wound (Koizumi et al., 2005). The further inhibitory action of
overexpression of P-cadherin is demonstrated by the reduction of invasiveness and migration of fibroblasts-like breast cancer cells (Sarrio et al., 2009). An age-associated increase in cell adhesion molecules in endothelial cells is also thought to play a role in wound inflammation and delayed wound healing through the regulation of leukocyte ingress to the wound site (Ashcroft et al., 1998).
Figure 6.6 – Venous ulcer-associated SNP disease cells display greater expression of genes involved in the MHC Class I antigen processing and presentation. (A) The MHC Class I antigen processing and presentation pathway with genes upregulated by disease (D) cells (D vs WT) highlighted in red. (B) RNA-seq fold-change of genes upregulated in disease cells (D vs WT).
Figure 6.7 - Venous ulcer-associated SNP disease cells display greater expression of genes involved in cell-cell adhesion. (A) Cell-cell adhesion factors and their partners with genes upregulated by disease (D) cells (D vs WT) highlighted in red. (B) RNA-seq fold-change of genes upregulated in disease cells (D vs WT).
6.5 Discussion

Over eight years ago Ashworth et al., 2008 demonstrated clear association between mutations in the human ERβ gene and susceptibility to chronic wounds (Ashworth et al., 2008). By transcriptionally profiling keratinocytes harbouring these chronic wound associated ERβ SNPs, we now provide new mechanistic insight into the causative changes underlying poor healing.

The timepoint of 4 hours post-wounding was chosen to maximise the level of wound related gene expression. This was following from the previous scratch assays that identified peak gene expression between 2 and 6 hours post-wounding (Figure 5.1 and 5.3). This could however impact on the results as different genes are important for different stages of scratch closure and so some important genes could be overlooked in this experimental setup. Comparing estrogen treated cells to untreated cells, whether as an overall group or grouped by genotype, revealed few genes that were induced or suppressed by estrogen treatment (Table 6.1 and Figure 6.2). This is somewhat surprising because whilst in vitro keratinocyte migration did not appear to be greatly affected by estrogen treatment (Figure 6.6), keratinocyte proliferation was significantly affected (Figure 6.5a), which would require an upregulation of numerous genes associated with the cell cycle and proliferation. However, in line with previous findings, there were numerous differences in gene expression when comparing across genotype (Table 6.1 and Figure 6.2), hinting that the cells have an innate wound healing deficiencies rather than estrogen signalling-dependent deficiencies. Genes downregulated in disease cells could be broadly characterised as pro-healing and anti-inflammatory (Tables 6.2, 6.4 and 6.6), whilst disease cells present with upregulated genes associated with delayed healing and with a capacity for increased immunogenicity (Tables 6.3, 6.5 and 6.7).

Genes downregulated in disease cells were overrepresented in GO terms associated with the establishment of the skin barrier, keratinisation and sphingolipid metabolism (Tables 6.2, 6.4 and 6.6). These genes are associated with keratinocyte differentiation and so could indicate that the disease cells are merely less differentiated than the WT cell counterparts, however the experimental design ensured that all cells were seeded at the same density and grown under the same conditions and furthermore, this pattern of gene expression was seen across all biological replicates. Deficiency in successful skin barrier restoration can lead to chronic wounds. This lack of barrier function increases the susceptibility to bacterial infection, which initiates the immune response leading to prolonged inflammation and delayed wound healing (Stojadinovic et al., 2008). Although the primary function of these genes is to contribute to the skin barrier, they can also contribute to wound healing. Many transglutaminases are upregulated in WT cells, including Tgase I (TGM1) which when knocked-out causes delayed healing (Inada et al., 2000).
enzymes catalyse the cross-linking of proteins such as involucrin and loricrin. Tgase I has a similar regulatory mechanism (by the jun transcription factor) to keratin 6 and during wound healing, they have a very similar expression and localisation to the plasma membrane at the leading edge of migrating keratinocytes. K6 and partner K16 are necessary for the retraction of tonofilaments and the migration of keratinocytes (Takahashi et al., 1994). Tgase I also colocalises with involucrin in the migrating keratinocyte and is necessary for the rapid remodelling of the stratum corneum. It is thought that the action of Tgase I and the formation of premature cornified envelope constituents could provide the mechanical strength needed for keratinocyte migration and thus enhancing wound healing (Inada et al., 2000). Sphingolipid metabolism is downregulated in disease cells. The key enzyme sphingosine kinase I (SPHK1), which has been shown to decrease wound healing time in a diabetic rat model (Yu et al., 2014), is downregulated and treatment with the sphingolipid metabolite sphingosylphosphorylcholine (SPC) significantly improves wound healing in diabetic mice (Sun et al., 1996). Estrogen ligands can interact with sphingolipids which are thought to have a role in the cytoplasmic signalling of estrogen (reviewed (Sukocheva et al., 2009)), with sphingosine kinase I playing an important role in the mediation of estrogen action (Nava et al., 2002) (Lebman and Spiegel, 2008). This again suggests that WT cells are better equipped to transduce estrogen signalling and bring about the beneficial effects on migration and proliferation than disease cells. These mechanisms would be particularly relevant in the low estrogen environment of a venous ulcer. Cells harbouring these SNPs would be less capable of deploying sphingolipids to mediate estrogen signalling and would not have the mechanical strength to reepithelialise the wound in a timely manner.

Of importance, the ‘positive regulation of epithelial cell migration’ overrepresented gene ontology term was found in downregulated genes of disease cells compared to wild type (Table 6.2). Growth factors associated with keratinocytes migration and wound healing were downregulated in disease cells, as was the MAPK/ERK signalling pathway, with the Ras and Rap1 pathways being particularly enriched in under-expressed genes. There is a well-established body of evidence demonstrating the importance of the MAPK signalling pathway in proliferation and migration. Mitogen-activated protein kinase kinase kinase 1 (MAP3K1), a component of the protein kinase signal transduction cascade, inhibition reduces wound healing, whilst overexpression increases wound healing, thought to be through the action of c-Jun NH2-terminal kinases (JNK) (Deng et al., 2006). β-integrin act with growth factors such as EGF to regulate keratinocyte growth via PI3K and MAP/ERK (both seen downregulated in disease cells) (Watson et al., 2009). Rap1 can regulate actin rearrangement and cell adhesion molecules such as cadherins and integrins (Retta et al., 2006), which reduction of has been associated with increased migration (Lynch et al., 2005). Of note here is that cell adhesion molecules are upregulated in disease cells in the absence of estrogen (Table 6.7). Growth factor activated Ras mediates changes in keratinocyte shape priming them for migration (Tscharntke et al., 2005), whilst nerve growth factor (NGF) induces migration
with the increase of PI3K/Akt, JNK, ERK and Rac1, of which the inhibition of any one delayed wound healing (Chen et al., 2014a). p38MAPK has been shown to induce epithelial migration while ERK1/2 activation induces proliferation and corneal healing (Sharma et al., 2003). Interestingly, estrogen signalling has an influence on this pathway. Estradiol treatment increases the proliferation of keratinocytes along with the increased phosphorylation of ERK1/2, increased expression of c-fos, c-jun (which is seen upregulated in WT keratinocytes) and cyclin D (Verdier-Sevrain et al., 2004). It has further been shown that the proliferative effects of estrogen signalling in keratinocytes acts through ERK and downstream through Akt (Zhou et al., 2016). It is understood that membrane-bound estrogen receptors associated with G-protein coupled receptors can signal through PI3K, Akt and MAPK leading to the activation of Ras, Raf, Erk, p38 and JNKs (Levin, 2009). As such, it is likely that the effects of estrogen on keratinocytes are mediated in part by activation of the non-genomic, membrane associated estrogen receptors through the MAPK signalling pathway and in part by canonical nuclear estrogen receptor genomic signalling. Many elements involved in estrogen signalling through membrane receptors are downregulated in disease cells (illustrated in Figure 6.8), which would allow for lesser propagation of the signal and a reduced response.
Figure 6.8 – Estrogen signalling pathway components are downregulated in disease cells. The estrogen signalling pathways with elements downregulated by disease cells (Disease vs WT) coloured red. Many elements of the membrane-associated estrogen receptor pathway are downregulated indicating that the beneficial effects of estrogen in early wound healing are likely via membrane-associated receptors. (blue P= phosphorylation)
Wild type cells also had an enrichment of downregulated genes in the GO terms for cholesterol and steroid hormone biosynthesis. It appears that WT cells have a greater potential to synthesise steroid hormones. It has been estimated that peripheral tissues provide 75% of the intracrine formation of estrogen in women and almost 100% in post-menopausal women (Labrie et al., 2000) and it has been demonstrated that mechanical wounding can cause an increase in steroid hormone synthesis in keratinocytes, amplifying the bioavailability of intracellular estrogen (Pomari et al., 2015). The data here indicates that cells with venous ulcer associated ERβ SNPs have less local hormone synthesis. This may contribute to the faster scratch assay closure seen by WT cells compared to disease cells (Figures 3.6-3.9) and may be representative of broader implications upon venous ulcers. The mechanism may in part be that the SNPs cause less local estrogen signalling. Due to the anti-inflammatory and pro-healing actions of estrogen, WT cells innate ability to synthesise estrogen to a higher degree would have a protective effect against the formation of chronic wounds (Margolis et al., 2002).

In the absence of estrogen, disease-associated keratinocytes showed a higher expression of genes involved in the ‘homophilic cell adhesion via plasma membrane adhesion molecules’ GO term (Table 6.7). Cell-cell adherens junctions are formed of cadherins, α/β-catenin and other cell adhesion molecules, linked to the actin cytoskeleton through vinculin. There is contradictory evidence relating to the importance of these junctions in migration. These adhesions need to be downregulated and deconstructed during the initial stages of reepithelialisation to allow for keratinocyte migration (Krawczyk and Wilgram, 1973) (Wallis et al., 2000), their downregulation allows for migration and invasion of cancer cells (Hazan et al., 2000) and dysregulation of this mechanism could lead to chronic wounds (Thomason et al., 2012). Further evidence suggests that cadherins, such as P-cadherin that is upregulated in disease-associated keratinocytes, are downregulated upon wounding and that overexpression could contribute towards arresting keratinocyte terminal differentiation so they may be supplied towards repopulating the wound (Koizumi et al., 2005). However a recent study melding mathematical modelling and HaCaT scratch assay observations showed that models representing ‘positive’ and ‘negative’ roles for cell-cell adhesion in collective migration both fitted with experimental observations, but that the ‘positive’ model fitted more robustly and that improved wound healing was likely a result of a concerted, collective, forward migrational force maintained by the firm cell-cell adhesions (Nardini et al., 2016). Overall the literature suggests that cell-cell adhesion molecules are important for timely wound healing and that whilst downregulation can increase cell migration, collective cell migration depends upon at least some transfer of force between cells, facilitated through cell-cell adhesion molecules. A balance of expression would therefore be necessary for timely wound healing, which may be compromised in disease-associated keratinocytes. It appears that in the absence of estrogen, this balance is disrupted in disease-associated keratinocytes, which likely leads to dysfunctional wound repair, as seen in venous ulcers. Going forward, it
would be possible to physically test if adhesion is altered in disease cells and what effects this has on migration and wound healing.

Disease-associated keratinocytes appear to upregulate genes involved in the immune response, with the GO term ‘MHC antigen processing and presentation’ being overrepresented (Tables 6.5 and 6.7). This is in direct contrast to wild type cells which have a greater number of highly expressed genes involved in ‘negative regulation of the immune response’, especially under low estrogen conditions (Table 6.6). MHC class I is involved in presenting antigen to cytotoxic CD8+ T cells, which then produce inflammatory cytokines and orchestrate the immune response (Chen et al., 2014b). MHC class I expression is implicated in various autoimmune and inflammatory pathologies such as skeletal and dermal muscle myositis (Li et al., 2009) and psoriasis (Knight et al., 2012). Human umbilical cord cells (Cai et al., 2014) and foetal skin cells (Zuliani et al., 2013) have very low MHC class I expression, which aids in scarless healing through the lack of an inflammatory response. MHC class II deficiency has been demonstrated to impair wound healing (Braunstein et al., 1991) and a decrease in the ratio of CD4+ to CD8+ ratio is significantly associated with chronic wounds (Loots et al., 1998). An increase in MHC class I activity and interaction with CD8+ could have an impact upon this ratio, making chronic wounds more likely.

RNAseq analysis of venous ulcer-associated SNP harbouring keratinocytes against wild type keratinocytes has revealed major changes in innate gene expression based on ERβ genotype. Venous ulcer-associated SNPs of the ERβ gene in keratinocytes cause a reduction in gene expression of genes involved in promoting cell migration. Pathways particularly restricted include the MAPK signalling cascade and the ras and rap signalling pathways, which have an interplay with membrane-bound estrogen receptor signalling. It was previously demonstrated that wild type cells express more ERβ than disease cells (Figure 5.2). This could imply that genomic estrogen activity through nuclear receptors can regulate genes involved in the non-genomic signalling pathway. The reduction in these pathway mediators would cripple the cells capacity to facilitate membrane-bound estrogen receptor signalling. Key enzymes of estrogen biosynthesis are also downregulated in disease cells, potentially leading to a lack of intracellular ligand that becomes exacerbated when systemic estrogen levels decrease with advancing age. SNPs of the ERβ gene cause an increase in the expression of genes involved in the inflammatory response, such as an increase MHC class I presentation, fail to upregulate genes that negatively regulate inflammation and increase the expression of cell-cell adhesion molecules, all of which are synonymous to venous ulcers. Data here provides a functional link between venous ulcer-associated ERβ SNPs and the regulation of genes and pathways that are necessary for timely wound healing. Addressing the dysregulation of these pathways could provide therapeutic targets for improved healing in venous ulcer patients.
7. Conclusions and Future Work

Estrogen is an important regulator of acute wound healing. Indeed, reduced estrogen is responsible for age-related healing impairment and a major contributing factor in chronic wound development (Ashcroft et al., 1999) (Ashcroft et al., 2003). Estrogen treatment, by either systemic or topical route, can accelerate wound healing in the elderly and protect against the formation of chronic wounds (Ashcroft et al., 1997a) (Ashcroft et al., 1999) (Margolis et al., 2002). Estrogen modulates aspects of the inflammatory response and cytokine expression, accelerates re-epithelisation and cell proliferation, stimulates angiogenesis and wound contraction and regulates proteolysis and matrix deposition (reviewed by (Ashcroft and Ashworth 2003)). The estrogen precursor DHEA can also protect against chronic wounds and venous ulcer patients present with significantly decreased serum DHEA levels (Mills et al., 2005) (Margolis et al., 2002). Venous ulcers exhibit a reduction in ERβ expression in keratinocytes and fibroblasts, but not ERα (Strudwick, 2006). Two previous studies from our lab have identified mutations of ERβ, but not ERα, to be in association with predisposition to venous ulcers. Specifically a haplotype of SNPs in the ON promoter region of the ERβ gene (ESR2) (rs2978381, rs2987983, rs1887994 and rs3783736) (Ashworth et al., 2005) (Ashworth et al., 2008).

Venous ulcers are non-healing chronic wounds found most commonly in the elderly with complex aetiology (Bergqvist et al., 1999). In general they are characterised by a lack of reepithelialisation, poor quality granulation tissue and a state of hyper-inflammation. It is estimated that between 0.2% and 1% of people will develop a venous ulcer in their lifetime (Grey et al., 2006b) (Fowkes et al., 2001). The National Health Service spends approximately £5 billion per year on chronic wounds, yet current treatments are ineffective (Guest et al., 2016). The principle driving factor in venous ulcer formation is venous insufficiency, although not all cases of venous insufficiency lead to an ulcer (reviewed here (Grey et al., 2006b)). In venous insufficiency dysfunctional venous valves and calf muscular pump function lead to reflux of the blood supply and localised hypertension. This pressure is conveyed to the fragile cutaneous capillary bed where endothelial hypertrophy and widening of the intraepithelial pores is observed (Chatterjee, 2012). As a consequence, macromolecules such as fibrinogen can escape into the dermis causing ischemia, hypoxia, reduce nutrient supply and necrosis (Browse and Burnand, 1982). Circulating leukocytes are recruited and become trapped in the microcirculation driving a pro-inflammatory local environment (Smith, 2001) (Bullen et al., 1995).
The effects of estrogen are transduced via the estrogen receptors ERα and ERβ. Although similar in structure and function, they are transcribed from different genes and have both unique and overlapping tissue distributions and downstream target genes (Stygar et al., 2007) (Welboren et al., 2007) (Bottner et al., 2014) (Brandenberger et al., 1997). Estrogen receptors can exist in two forms; as intracellular nuclear receptors, or as membrane-bound G-protein associated receptors. It is understood that these receptors have a common genetic origin (Razandi et al., 2004). Once activated by estrogen, the intracellular nuclear receptors dimerise (as hetero- or homo- dimers) and translocate to the nucleus where they bind to DNA at estrogen response elements (ERE) in the promoter regions of target genes and act as transcription factors. They can also induce gene transcription by interacting with other transcription factors in an ERE-independent manner. Membrane bound estrogen receptors initiate rapid signalling effects through the modulation of intracellular signalling cascades in response to estrogen activation (Levin, 2009). The activation of ERα or ERβ by estradiol or other estrogenic ligands, such as selective estrogen receptor modulators (SERMs), can lead to different signalling outcomes due to their different gene target sets (Leitman et al., 2010). Cellular receptor distribution within a tissue is therefore important for estrogen signalling outcome. This is further complicated by the formation of dimers, where ERβ has been demonstrated to repress the transcriptional activity of ERα when dimerised (Hall and McDonnell, 1999). In the context of wound healing keratinocytes, fibroblasts and leukocytes all contain both ERα and ERβ. Prior studies have shown that signalling through ERβ has beneficial effects on wound healing, whereas ERα signalling is detrimental (Campbell et al., 2010). Any reduction in the level or activity of ERβ would therefore have a disproportionately large influence on wound healing.

Clinical data demonstrate that four single nucleotide polymorphisms (SNPs), in close linkage disequilibrium with one another, spanning the 0N promoter region of the ERβ gene are significantly associated with venous ulceration (Ashworth et al., 2008). Further, a major susceptibility haplotype formed from these SNPs was significantly associated with elevated serum levels of TNFα, a marker of inflammation. Combined with the knowledge that estrogen is beneficial to wound healing (Ashcroft et al., 1999), that estrogen treatment protects against the formation of chronic wounds (Margolis et al., 2002), that ERβ signalling promotes healing (Campbell et al., 2010) and that ERβ expression appears to be reduced in chronic wounds (Strudwick, 2006), it is reasonable to hypothesise that these SNPs are functionally influencing the estrogen signalling axis.
Experiments presented early in this thesis provide confirmation that venous ulcer-associated ERβ SNPs do directly influence primary human skin cells, modifying expression and cellular function (Chapter 3). In order to examine the functional effects of the SNPs on wound related processes, both keratinocyte and fibroblasts harbouring either the venous ulcer associated SNP variants (Disease) or healthy SNP variants (Wild Type) were selected from the IMB Skin Cell Bank. These cells were then used in assays to characterise ERβ expression, in vitro proliferation and in vitro scratch wound closure both in isolation and in co-culture. Intriguingly, ERβ expression was reduced in cells carrying the Disease SNPs, at both the transcriptional and translational level. Whilst the effect of these SNPs on ERβ expression has not been previously tested, data suggest that ERβ expression, but not ERα, could be downregulated in venous ulcers (Strudwick, 2006). In terms of cell function, both keratinocytes and fibroblasts proliferate and migrate in a SNP-dependant manner. WT keratinocytes and fibroblasts proliferate at a higher rate than their venous ulcer SNP-containing counterparts. Moreover, keratinocytes were sensitive to estradiol treatment and both receptor agonists, which increased the rate of proliferation in consensus with other published studies in human keratinocyte cell lines (Merlo et al., 2009).

Migration assays also revealed that WT fibroblasts and keratinocytes close scratch wounds at a higher rate than venous ulcer SNP-containing cells. Uncoupling the contribution of proliferation via Mitomycin C revealed that the migration of keratinocytes was not affected by any of the estradiol or ER-specific agonists. Previous work from our lab indicates that murine in vivo and in vitro wound reepithelialisation is improved with estradiol and ERβ-specific agonist treatment, but delayed by ERα-specific agonist treatment (Campbell et al., 2010). Moreover, in vitro scratch assays using a human keratinocyte cell line (NCTC 2544) revealed estradiol, ERβ-specific agonist and ERα-specific agonist treatment all improve wound closure (Merlo et al., 2009), with the caveat that cells were not treated with Mitomycin C in these assays. It has previously been demonstrated that human fibroblast scratch assay closure times are improved by treatment with estradiol and ERα-specific agonist (Stevenson et al., 2009), whilst murine in vitro assays revealed improvement in relation to estradiol and ERβ-specific agonist (Campbell et al., 2010) (both with Mitomycin C treatment). Here, only estradiol treatment to WT fibroblasts significantly improved wound closure on a background of Mitomycin C treatment. Clearly, there are differences between our data and published studies, that are most likely the result of experimental design and the use of primary cells.

Perhaps the most interesting result was obtained from co-culture studies. Here, scratch wound healing of keratinocytes was altered by fibroblast conditioned media; media derived from Wild
type fibroblasts was beneficial to keratinocyte wound closure, whilst media derived from disease fibroblasts was detrimental to keratinocyte wound closure. This experiment begins to address the potential of venous ulcer SNPs to alter the growth factor paracrine signalling profile of fibroblasts, which can have an inhibitory effect on keratinocyte migration. Indeed, it has been demonstrated that estrogen signalling can regulate growth factor release by cultured fibroblasts, specifically TGFβ (Stevenson et al., 2008). Future studies to address the constituent profile of the conditioned media would be fascinating.

In chapter 4 allele specific expression (ASE) assays, bioinformatic investigation and electrophoretic mobility shift assays were employed to determine the effect of venous ulcer SNPs on ERβ transcription. Allele specific expression assays use heterozygous cells, which have both nucleotide variants of the SNP in question, to measure the relative expression of each transcript, thereby determining the effect of the nucleotide change on expression. The results of ASE assays initially appear contradictory, with rs3783736 reporting 2.77 fold greater expression for the wild type variant, rs2987983 reporting an equal expression between variants and rs2978381 reporting 4.75 fold greater expression for the disease variant. However, due to its location downstream of the ON promoter region, rs3783736 likely reports on the functional effect of another SNP, upstream in the ON promoter region (i.e. the other venous ulcer-associated SNPs), that is in linkage disequilibrium. The remaining SNPs are located upstream of the ON region, but downstream of the OK promoter and so these ASE assays likely report on the function of the OK promoter.

Examining the regulatory landscape at each SNP site revealed the rs2987983 SNP to be located in an important regulatory hub, whilst the remaining SNPs were not (Kent et al., 2002). It is located immediately upstream of the ON transcriptional initiation site in an area of DNase1 hypersensitivity, in a region of high histone markers (Ernst et al., 2011) and at the location of numerous ChIPseq validated transcription factor binding sites (Wang et al., 2012). Furthermore, it is associated with prostate and breast cancer progression (Treeck et al., 2009) (Thellenberg-Karlsson et al., 2006), methylation of the immediate area inactivates ERβ expression (Zhu et al., 2004) and a nearby SNP has been demonstrated to have the functional ability to reduce ERβ transcription (Philips et al., 2012). Transcription factor binding prediction algorithms suggested a difference in transcription factor binding due to this SNP and electrophoretic mobility shift assays confirmed in vitro changes in the nucleotide sequence at the SNP site altered transcription factor binding. This consortium of evidence strongly suggests that rs2987983 functionally affects ERβ expression by altering transcription factor binding and subsequently impeding transcription.
The next step was to establish how expression of ERβ and other wound relevant genes changed longitudinally (Chapter 5). ERβ expression has not previously been measured over the course of wound closure. Analysing ERβ mRNA expression during scratch wound closure revealed a sharp increase in expression at 2 hours post-wounding. Data suggest that ERβ is synthesised in response to wounding and that disease cells have a reduced capability to upregulate this expression. Examining the protein expression of ERβ throughout keratinocyte scratch wound closure revealed that cells harbouring the venous ulcer SNPs express ERβ to a lesser extent than the wild type. Of note, wild type cells also appeared to express ERβ with a larger molecular weight than expected, indicative of post-translational modifications or splice variation, which correlates with improved migration. Further studies to characterise this higher molecular weight form are certainly justified.

Analysing the expression of wound relevant genes through keratinocyte wound closure revealed a different growth factor and cytokine expressional profile dependent on the venous ulcer SNPs. This was also found in fibroblasts, although estradiol treatment had a significant effect on expression in wild type cells, but disease cells were not sensitive to estradiol treatment. Literature reports that wound expression of inflammatory mediators, such as IL-6 (Rachon et al., 2002) and TGFβ (Ashcroft et al., 1997a), are affected by estrogen treatment and that some of these effects can be seen in keratinocytes (Moeinpour et al., 2008). In contrast, we report here that neither wild type nor disease keratinocyte respond to estrogen treatment in terms of growth factors and cytokines expression. Wild type fibroblasts are responsive to estradiol treatment, in line with the literature (Stevenson et al., 2008), but disease cells have lost this sensitivity.

There is evidence that fibroblasts can induce a particular phenotype in monocytes based on their location or disease state (Silzle et al., 2003). Fibroblasts have close signalling relationships with monocytes in the granulation tissue of the wound and it can therefore be expected that differences in the fibroblasts would have differing effects on monocytes. Here, the co-culture of fibroblasts with monocytes revealed drastic differences between wild type and disease cells. Wild type fibroblasts expressed cytokines and proteases to a lesser degree and markers for estrogen receptor signalling suggested a lack of receptor activity in disease fibroblasts. Wounded fibroblasts harbouring venous ulcer SNPs induced a pro-inflammatory M1 polarisation in the monocytes, whilst wild type cells induced a pro-healing M2 polarisation. It has been demonstrated that venous ulcers have high numbers of M1 polarised macrophages, which are thought to be counterproductive to wound healing due to the damage associated with excessive inflammation (Sindrilaru et al., 2011) (MacLeod and Mansbridge, 2016).
Finally, in order to establish the broader impact of venous ulcer ERβ SNPs on gene expression during healing RNAseq was employed for transcriptome profiling (Chapter 6). Whilst this technique is limited to gene expression and does not necessarily report on protein expression, it does give a strong indication on what might be causing phenotypic differences. RNaseq analysis of wounded SNP-containing keratinocytes treated with and without estradiol was compared to wild type counterparts. Keratinocyte gene expression did not appear to be differentially regulated by estradiol under these experimental conditions. However, many genes were differentially regulated based on ERβ SNP genotype. Cells containing these SNPs had downregulated genes involved in migration, regulation of the inflammatory response and hormone synthesis, whilst upregulated genes were involved in cell adhesion and MHC class I presentation. Many components of the pathways involved in cell migration are repressed, as are key elements of the membrane-bound estrogen receptor non-genomic signalling cascade, which can have rapid effects on cell migration (Zhou et al., 2016). The overall gene expression profile of keratinocytes with venous ulcer ERβ SNPs is one of repressed migration and pro-inflammation. The hormone synthesis pathway also had genes for important enzymes downregulated in ERβ SNP keratinocytes. This was suggestive of a reduction in estradiol synthesis which would result in reduced autocrine signalling that, when coupled with an age-related reduction in systemic estradiol synthesis, would further exacerbate the healing deficiencies. It is likely that the dysfunctional gene regulation identified here is, at least in part, responsible for the reduced healing seen with these wounds.

The data presented across these chapters combine to provide important insight into ERβ-SNP mediated effects on wound healing. We demonstrate that migration and proliferation of keratinocytes and fibroblasts is downregulated and that cells produce a more pro-inflammatory cytokine and growth factor expression profile, with the presence of venous ulcer associated ERβ SNPs. Further, the SNP-induced inflammatory profile in fibroblasts can prime co-cultured monocytes towards a more pro-inflammatory phenotype. RNAseq analysis has identified the genes and pathways that appear to be responsible for these observed phenotypes, although further functional validation is required. Key elements of the MAPK signalling cascade and related ras and rap1 signalling pathways, which are involved in cell migration and proliferation, are downregulated and genes involved in inflammation are dysregulated in cells with ERβ SNPs. These effects are likely to be caused by an alteration in receptor expression. Analysis of ERβ expression at the RNA and protein level has revealed deficiencies in receptor expression in cells with ERβ SNPs. The rs2987983 has been identified to be the most likely SNP to directly functionally effect ERβ expression. This is based on evidence from allele specific expression assays, the determination that it lays in a major regulatory hub of the 0N promoter and that electrophoretic
mobility shift assays, combined with bioinformatic predictive software, have indicated that this reduction in expression is caused by a SNP-dependent disruption in transcription factor binding.

The phenotypes that have been identified in vitro are consistent to those observed in venous ulcers. Venous ulcers are characterised by a lack of reepithelialisation, a poor-quality granulation tissue and an excessive inflammatory response (Grey et al., 2006b). A direct link can now be established between the lack or cell migration/reepithelialisation and the excessive inflammatory response in venous ulcers with specific ERβ SNPs and that this is likely caused by a SNP-induced downregulation in ERβ and subsequent change gene expression profile. Understanding the exact pathways that underline the involvement of ERβ SNPs in venous ulcers reveals potential therapeutic targets. Estrogen can clearly improve wound healing and protect against the formation of chronic wounds (Ashcroft and Ashworth, 2003) (Margolis et al., 2002), but until now it has been unclear precisely how this occurs.

This study has identified that numerous genes involved in cell migration are downregulated in cells with ERβ SNPs, including members of the MAPK signalling pathway. Therapeutic stimulation of this pathway in venous ulcers could therefore be a potential option. There are agonists available for MAPK stimulation, many of which are growth factors that can also signal through membrane-bound estrogen receptors, such as EGF (Ostrowski et al., 2000) (Egloff et al., 2009). However, stimulation of MAPK signalling is unideal due to the well-established role of this pathway in cancer progression (Cheng et al., 2013) (Dhillon et al., 2007). Though, any treatment of chronic wounds is likely to possess potentially carcinogenic properties due to the interrelatedness of wound healing and cancer (Antonio et al., 2015) (Schafer and Werner, 2008a).

Due to the inflammatory phenotype stimulated by the SNPs and the excessive inflammation accompanying venous ulcers, targeting inflammation could be a therapeutic option for venous ulcer treatments. There are a variety of nonsteroidal anti-inflammatory drugs (NSAIDs) that are used in the treatment of inflammatory conditions, such as arthritis (Crofford, 2013), where they act as inhibitors of cyclooxygenase (COX) enzymes. Indeed, NSAIDs are given to venous ulcer patients with the aim of reducing pain (Salavastru et al., 2012), but no substantial clinical data suggesting that they are beneficial for healing is available (Langer, 2014). Corticosteroids have been used topically to treat chronic wounds and have been reported to have pro-healing effects in certain cases (Hofman et al., 2007) (Bosanquet et al., 2013). However, it is well characterised that these steroids can reduce cellular wound function, reducing proliferation, granulation tissue
formation and wound contraction (reviewed (Guo and Dipietro, 2010)). Further, it may be counterproductive to inhibit the immune response and risk infection in chronic wounds, which often have a large microorganism burden.

Due to its position as a regulator of both inflammation and cell migration, through MAPK and other genomic pathways, estrogen treatment is an ideal candidate for venous ulcer therapy. Indeed, estrogen supplementation has been shown to protect against the formation of chronic wounds (Margolis et al., 2002) as it appears that the age-related delay in wound healing is almost entirely due to estrogen regulation (Hardman and Ashcroft, 2008). Replacing this systemic reduction in estrogen may be beneficial to wound healing and may act prophylactically to prevent the initial formation of an ulcer. However, treating with estrogen could have drawbacks and may not have the same effect in every patient. We have demonstrated that cells harbouring venous ulcer ERβ SNPs have a reduced expression of receptor and it has been suggested that venous ulcers have a reduction in ERβ expression, but not ERα. Estrogen stimulation of ERα in isolation of ERβ has been demonstrated to have inhibitory effects on wound healing (Gilliver et al., 2010) (Campbell et al., 2010). If estrogen treatment were to be given to these patients with a lack of ERβ, the effects on healing would likely be detrimental, whilst patients with a normal ER distribution may benefit. Furthermore, estrogen treatment can act as a carcinogen and increase the risk of developing breast and uterine cancer (Rossouw et al., 2002; Yager and Davidson, 2006).

Selective estrogen receptor modulators (SERMs) can have agonistic or antagonistic effects in a tissue-specific manner, with numerous effects observed in the skin (Stevenson and Thornton, 2007). Tamoxifen is a SERM breast cancer treatment that blocks the action of estrogen in breast cancer cells (Early Breast Cancer Trialists’ Collaborative et al., 2011), as does raloxifene, which also has a reduced effect on uterine cells and minimises the risk of endometrial cancer (Stygar et al., 2003) and both are approved by the Food and Drug Administration, making translation application more straightforward. Fittingly, both tamoxifen and raloxifene significantly accelerate wound healing in ovariectomised mice with in vitro anti-inflammatory effects comparable to 17β-estradiol (Hardman et al., 2008). Importantly, this study also observed that ERβ gene and protein expression was induced by raloxifene treatment in the wound and that there was a reduction in ERα expression in keratinocytes. Raloxifene has also shown potential as an estrogen-receptor-beta-targeted therapy in prostate cancer (Shazer et al., 2006). By targeting and inducing ERβ expression but not ERα, the potential for detrimental effects of signalling through ERα would be removed in venous ulcer patients with ERβ SNPs. By delivering as a topical treatment as opposed to a systemic treatment would minimise the off-site side-effects and limit any cancer-associated
risk to the treatment. However, there is a case to be made for systemic treatment. SNPs of the ERβ gene are associated with a number of disorders (including the same venous ulcer associated SNPs), such as Alzheimer’s disease (Pirskanen, Hiltunen et al. 2005), prostate cancer (Thellenberg-Karlsson, Lindstrom et al. 2006), breast cancer (Treeck, Elemenler et al. 2009) and a loss in bone mineral density (Ichikawa, Koller et al. 2005). If these disorders are also affected by a SNP-induced loss of ERβ expression, the treatment may also have a prophylactic effect on their prognosis.

Future Directions

Although data presented in this thesis has demonstrated the first direct link between ERβ SNPs and dysfunctional wound healing, there are still many unanswered questions. Although there was evidence from electrophoretic mobility shift assay that ERβ SNPs alter transcription factor binding, the identities were not determined. To identify these transcription factor(s), specific antibodies could be incorporated into the assay to create a ‘supershift’ when the protein is bound. Otherwise, the band could be excised from the gel and analysed via mass spectrometry. Knowing the mechanism through which ERβ is downregulated would open up new therapeutic opportunities. Understanding of the exact role of rs2987983 in ERβ expression would also be desirable. Whilst allele specific expression assays can report on upstream SNPs of promoter regions in linkage disequilibrium, they cannot report directly on a specific SNP. In order to measure the expressional effect of the SNPs on the promoter region, luciferase reporter assays could be designed. This would enable the incorporation and direct comparison of both nucleotide variants on the promoter expression. Due to the unavailability of primary samples, this study could not directly test the effect of ERβ SNPs in inflammatory cells, but rather used interactions with skin cells. Obtaining primary inflammatory cells would require identifying volunteers with the desired genotype and collecting monocytes from the blood. It would be fascinating to determine whether ERβ SNPs had cell intrinsic effects in inflammatory cells and how this would alter their phenotype in a wound setting.

Whilst the RNAseq experiment provided interesting and novel understanding of the role of venous ulcer-associated ERβ SNPs, there were limitations to the experimental arrangement. The cell cohort had limitations in collection method, ethnicity and body site. The cells tested were cultured from healthy skin of patients with the relevant SNPs. The isolation of skin cells and in vitro culture can change cell expression profiles which may not be indicative of in vivo expression. Furthermore, the cells chosen were from a homogenous population (Hun Chinese) whereas the original association study was from a Caucasian population. Furthermore, the cells were from the
torso of facial region, whereas venous ulcers occur on the lower limb. Ideally, cells would be harvested from wound margin skin of ERβ SNP patients of various ethnicities suffering from venous ulcers. This would give a more relevant picture of dysfunctional gene expression. It would also be beneficial to repeat RNAseq analysis in fibroblasts to further define the effect of these SNPs in wound-related cell function and to determine the underlying differences between keratinocytes and fibroblasts. Another limitation of the current experiment is that gene expression was not validated by qPCR. Although RNAseq is a very robust method of measuring gene expression, it is necessary to validate through alternative methods. Further experimentation should also be done to further explore the relevance of the identified signalling pathways in skin cell migration, hormone production, adhesion and inflammatory mediation. This has been shown to some extent in the in vitro proliferation and scratch assays, but could be broadened to include adhesion assays and direct measurement of hormone and cytokine production through ELISA or membrane cytokine array. Knock-down or antagonist treatments could also be exploited to further investigate the effects of ERβ SNPs (through the downregulation of receptor expression and signalling) on the biological pathways identified in the RNAseq experiment.

During this study, preliminary investigation of the effects of ERβ SNPs on wound healing were carried out using three-dimensional organotypic models (Supplementary Figure S.3). These in vitro models combine a keratinocyte epidermis overlying a collagen dermis seeded with fibroblasts, to form a more physiologically accurate assay model. Using this system, the interactions of SNP harbouring keratinocytes and fibroblasts could be explored in detail along with the effects of different estrogen treatments. Preliminary data reveal that these three-dimensional models can be developed under estrogen-free-conditions, where estrogen replacement promotes development of the epidermis in these models (Supplementary Figure S.3a). Future work here would include building upon the wound model (Supplementary Figure S.3b) and incorporating cells harbouring ERβ SNPs to report on wound healing affects in a more physiologically relevant environment. RNAseq analysis of keratinocytes revealed a number of interesting, wound relevant dysregulated pathways in ERβ SNP-containing cells. These targets could be directly activated/inhibited in the skin equivalent model.

In conclusion, this thesis provides a range of experimental evidence to demonstrate that venous ulcer-associated ERβ SNPs directly alter cell transcriptional programmes and alter wound-relevant cellular functions. These findings should now be used to develop a therapy, particularly in patients known to have the venous ulcer-associated genotype, which positively stimulates ERβ to prophylactically or actively treat venous ulcers.
8. Supplementary Material

Supplementary Table ST1- Primary cell patient and sample information. Information on primary keratinocyte and fibroblast source, provided by the Institute of Medical Biology, Skin Cell Bank - A*Star Singapore.

<table>
<thead>
<tr>
<th>Cell Bank Identifier</th>
<th>Gender</th>
<th>Body site</th>
<th>Ethnicity</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-S-(K/F)-9</td>
<td>Female</td>
<td>Abdomen</td>
<td>Chinese</td>
<td>46</td>
</tr>
<tr>
<td>12-S-(K/F)-11</td>
<td>Female</td>
<td>Abdomen</td>
<td>Chinese</td>
<td>45</td>
</tr>
<tr>
<td>EBL(K/F)029</td>
<td>Female</td>
<td>Left breast</td>
<td>Malay</td>
<td>39</td>
</tr>
<tr>
<td>Disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-S-(K/F)-13</td>
<td>Female</td>
<td>Abdomen</td>
<td>Caucasian</td>
<td>44</td>
</tr>
<tr>
<td>EBL(K/F)013</td>
<td>Female</td>
<td>Abdomen</td>
<td>No details</td>
<td>No details</td>
</tr>
<tr>
<td>EBL(K/F)027</td>
<td>Female</td>
<td>Face</td>
<td>No details</td>
<td>No details</td>
</tr>
</tbody>
</table>

Supplementary Figure S.1 – Venous ulcer SNP linkage disequilibrium. Linkage disequilibrium values of r-squared and D-prime for venous ulcer-associated ERβ SNPs, based on results from LDlink (Machiela and Chanock, 2015).
Supplementary Figure S.2 - Titration of estradiol treatment for scratch wound closure with keratinocytes. Wound closure profile of scratch assays performed on (A) disease keratinocytes (harbouring venous ulcer-associated ERβ SNPs) and (B) wild type keratinocytes. Keratinocytes were treated with increasing concentrations of 17β-estradiol (physiological levels =0-150nM) to confirm signalling activity and to identify the most appropriate concentration for further assays.
Supplementary Figure S.3 - Organotypic skin-equivalent three-dimensional models of wild-type cells. Haematoxylin and eosin stained wax section of organotypic skin models. Wild type fibroblasts were seeded in collagen I to form a dermis with wild type keratinocytes seeded on top to form an epidermis. (A) Organotypic models were grown for 3 weeks in estrogen-free or 100nM estradiol treated media, leading to different epidermal characteristics. Organotypic models presented with a thicker epidermis under estradiol treatment, whilst without estradiol, the epidermis was thin but seemingly more cornified. (B) After 3 weeks, organotypic models were wounded with a 2mm punch biopsy (wound margins indicated by red arrows).
9. References


enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol. 13:1672-1685.


