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THE ROLES OF HEPATOCYTE GROWTH FACTOR FAMILY MEMBERS IN ANDROGEN-REGULATION OF HUMAN HAIR GROWTH

S. A. AL-WALEEDI

PhD

2010
THE ROLES OF HEPATOCYTE GROWTH FACTOR FAMILY MEMBERS IN ANDROGEN-REGULATION OF HUMAN HAIR GROWTH

A comparison of the expression of hepatocyte growth factor family members, HGF and MSP, and their receptors, c-Met and RON, in isolated hair follicles from normal and androgenetic alopecia (balding) scalp

Saeed A. AL-WALEEDI

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Department of Biomedical Sciences

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2010
Abstract

The roles of hepatocyte growth factor family members in androgen-regulation of human hair growth

Saeed A. Al-Waleedi

Keywords: HGF, MSP, c-Met, RON, hair follicles, androgen, androgenetic alopecia, PCR, gene microarray, balding, immunohistochemistry.

Androgens are the main regulators of human hair growth stimulating larger, terminal hair development e.g. beard and causing scalp balding, androgenetic alopecia. Hair disorders cause psychological distress but are poorly controlled. Androgens probably act by altering regulatory paracrine factors produced by the mesenchyme-derived dermal papilla. This study aimed to investigate paracrine factors involved in androgen-regulated alopecia, particularly hepatocyte growth factor (HGF) family members, by investigating their in vivo status.

Balding and non-balding scalp hair follicles and their component tissues were isolated and analysed by molecular biological methods (reverse transcriptase-polymerase chain reaction (RT-PCR), quantitative PCR and DNA microarray analysis), cell culture and immunohistochemistry. Scalp follicles expressed a range of paracrine messenger genes. The dermal papilla, cultured dermal papilla cells and dermal sheath expressed several HGF family genes, while matrix cells only produced the receptor RON suggesting autocrine roles for HGF and MSP, but a paracrine route only for MSP.

Comparing balding and non-balding follicles from the same individuals revealed the expected reduction in several keratin and keratin-related protein genes supporting this approach’s validity. There were also significant differences in paracrine factors previously implicated in androgen action by in vitro studies. Several factors believed to increase during androgen stimulation of larger, darker follicles, e.g. IGF-I and SCF, were lowered in balding follicles, while putative inhibitory factors, e.g. TGFß-1, were increased. HGF and MSP and their receptors, c-Met and RON, were significantly reduced. These results increase our understanding of androgen action in human hair follicles; this could lead to better treatments for hair disorders.
Acknowledgements

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Special thanks must go to the Saudi government, who sponsored this project, for giving me the opportunity to do this project and for their funding throughout the research work. Without their financial support, this work would not have been possible.

Finally, I am very grateful to my family and friends. I cannot thank them enough. I would not have made it through to this stage without their love and encouragement.
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVM</td>
<td>Avian Myeloblastosis Virus</td>
</tr>
<tr>
<td>APE1</td>
<td>Apurinic/apyrimidinic endonuclease</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDS</td>
<td>cDNA synthesis</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyproterone acetate</td>
</tr>
<tr>
<td>c-Met</td>
<td>Met proto-oncogene (HGF receptor)</td>
</tr>
<tr>
<td>CO</td>
<td>Cortex</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary ribonucleic acid</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CTS</td>
<td>Connective tissue sheath</td>
</tr>
<tr>
<td>D</td>
<td>Dermis</td>
</tr>
<tr>
<td>DHT</td>
<td>5α-dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DP</td>
<td>Dermal papilla</td>
</tr>
<tr>
<td>DS</td>
<td>Dermal sheath</td>
</tr>
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<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>Epidermis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFs</td>
<td>Epidermal growth factors</td>
</tr>
<tr>
<td>FGFs</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>FLU</td>
<td>Flutamide</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HF</td>
<td>Hair fibre</td>
</tr>
<tr>
<td>HF</td>
<td>Hair follicle</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HGFL</td>
<td>Hepatocyte growth factor like</td>
</tr>
<tr>
<td>HGFR</td>
<td>Hepatocyte growth factor receptor</td>
</tr>
<tr>
<td>HL</td>
<td>Hairpin loop</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HS</td>
<td>Hair shaft</td>
</tr>
<tr>
<td>IGFs</td>
<td>Insulin like growth factors</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin like growth factor-I</td>
</tr>
<tr>
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</tr>
<tr>
<td>IL-1α</td>
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</tr>
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<td>Interleukin-6</td>
</tr>
<tr>
<td>IRS</td>
<td>Inner root sheath</td>
</tr>
<tr>
<td>IVT</td>
<td><em>In vitro</em> transcription</td>
</tr>
<tr>
<td>K</td>
<td>Kringle</td>
</tr>
<tr>
<td>KRT</td>
<td>Keratin</td>
</tr>
<tr>
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<td>Keratin associated protein</td>
</tr>
<tr>
<td>L</td>
<td>Ladder</td>
</tr>
<tr>
<td>MD</td>
<td>Medulla</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSH</td>
<td>Melanocyte stimulating hormone</td>
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<td>MSP</td>
<td>Macrophage stimulating protein</td>
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<td>MST1</td>
<td>Macrophage stimulating 1</td>
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<tr>
<td>MST1R</td>
<td>Macrophage stimulating 1 receptor</td>
</tr>
<tr>
<td>MX</td>
<td>Matrix</td>
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<tr>
<td>N</td>
<td>Negative control</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>OCT</td>
<td>Optimal cutting temperature compound</td>
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<td>ORS</td>
<td>Outer root sheath</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
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<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic ovary syndrome</td>
</tr>
<tr>
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</tr>
<tr>
<td>PDGFs</td>
<td>Platelet-derived growth factors</td>
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<td>PRGFs</td>
<td>Plasminogen-related growth factors</td>
</tr>
<tr>
<td>RMA</td>
<td>Robust multi-array analysis</td>
</tr>
<tr>
<td>RNA</td>
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<td>RON</td>
<td>Recepture d’origin Nantaise</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
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<tr>
<td>RTKs</td>
<td>Protein kinases</td>
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<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
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<tr>
<td>S</td>
<td>Serine</td>
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<td>SAPE</td>
<td>Streptavidin phycoerythrin</td>
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<td>SCF</td>
<td>Stem cell factor</td>
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<td>SF</td>
<td>Subcutaneous fat</td>
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<td>SG</td>
<td>Sebaceous gland</td>
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<td>SHBG</td>
<td>Sex-hormone binding globulin</td>
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<td>SP</td>
<td>Signal peptide</td>
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<td>Tris-acetate-EDTA</td>
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<td>Transforming growth factors</td>
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<td>TGF-β2</td>
<td>Transforming growth factor-β2</td>
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<td>TNF</td>
<td>Tumor necroses factor</td>
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<tr>
<td>TNFR</td>
<td>Tumor necroses factor receptor</td>
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<tr>
<td>UDG</td>
<td>Uracil-DNA glycosylase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
# Contents

Abstract 03  
Acknowledgment 04  
Glossary 05  
Contents 07  

1- Introduction:  
1.1 Hair functions & its importance  
1.2 Hair structure  
1.3 Hair Growth Cycle  
1.4 Hair follicle embryogenesis  
1.5 The role of dermal papilla in hair follicle growth  
1.6 The involvement of androgen in human hair growth  
1.7 Hair disorders  
1.8 Mechanism of androgen action in hair follicle  
1.9 Current hypothesis of androgen action in hair follicle  
1.10 Paracrine factors in the hair follicles  
1.11 HGF and MSP as Possible paracrine factors produced by hair follicle  
1.11.1 Structure of HGF and MSP  
1.11.2 Receptors for HGF and MSP  
1.11.3 Hepatocyte growth factor (HGF) and hair growth  
1.11.4 Macrophage stimulating protein and hair growth  
1.12 Aims and experimental design  

2- Materials and methods:  

2.1 Identification of HGF family members genes and their receptors in human hair follicles and their components using RT-PCR  

2.1.1 The Reverse Transcription-Polymerase Chain Reaction  
2.1.1.1 Biological materials  
2.1.1.2 Microdissections and tissues preparation  
2.1.1.2.1 Microdissections of whole anagen follicles  
2.1.1.2.2 Microdissections of hair bulb components  
2.1.1.3 Total RNA isolation for RT-PCR  
2.1.1.3.1 Checking the quality of total RNA  
2.1.1.3.1.1 Agarose gel preparation  
2.1.1.3.1.2 Agarose gel electrophoresis  
2.1.1.4 Poly (A) RNA isolation  
2.1.1.5 DNase treatment of poly (A) RNA samples  
2.1.1.6 cDNA synthesis by reverse transcription  
2.1.1.7 Polymerase Chain Reaction (PCR)  
2.1.1.8 DNA sequencing of PCR products
2.1.2  Histological examination of skin samples & localization of the HGF receptor, c-Met, by immunohistochemistry

2.1.2.1 Tissue samples
2.1.2.1.1 Human skin samples
2.1.2.1.2 Deer skin samples
2.1.2.2 Preparation of slides
2.1.2.3 Preparation of frozen tissue sections
2.1.2.4 General histology
2.1.2.4.1 haematoxylin & eosin staining
2.1.2.4.2 Saposic staining
2.1.2.5 Immunohistochemistry
2.1.2.5.1 Detection of cytokeratin 6 expression by immunohistochemistry
2.1.2.5.2 Detection of HGF receptor, c-Met, by immunohistochemistry
2.1.2.6 Visualising the staining

2.1.3  Investigation of the expression of gene for specific molecules in human cultured dermal papilla cells using RT-PCR

2.1.3.1 Dermal papilla cell lines
2.1.3.2 Cell culture procedure
2.1.3.2.1 Maintenance of Cell cultures
2.1.3.2.2 Visualising cell cultures
2.1.3.2.3 Passaging of cell cultures
2.1.3.2.4 Collection and storage of cultured cells

2.2  Comparison of the gene expression of normal and balding scalp hair follicles from men with androgenetic alopecia

2.2.1 Biological materials
2.2.2 Total RNA isolation for microarray and real-time PCR
2.2.2.1 Assessing yield and purity of total RNA

2.2.3  Comparing gene expression in isolated hair follicles in balding and non-balding individuals using DNA microarray:

2.2.3.1 DNA microarray procedure
2.2.3.1.1 First-strand cDNA synthesis
2.2.3.1.2 Second-strand cDNA synthesis
2.2.3.1.3 cRNA synthesis by in vitro transcription
2.2.3.1.4 2nd cycle cDNA synthesis
2.2.3.2 Fragmentation & labeling of single-strand DNA
2.2.3.3 Hybridization
2.2.3.4 Washing, staining & scanning
2.2.3.5 DNA microarray data analysis

2.2.4  Comparison of the expression of specific genes in non-balding and balding hair follicles using real-time PCR

2.2.4.1 RNA amplification
2.2.4.1.1 First-strand cDNA synthesis
2.2.4.1.2 Second-strand cDNA synthesis
2.2.4.1.3 Purification of double-strand cDNA
2.2.4.1.4 Synthesis of cRNA (in vitro transcription)
2.2.4.1.5 Purification of cRNA
2.2.4.1.6 Checking the quality of purified cRNA
2.2.4.2 Primer design used for relative quantitative real-time PCR
2.2.4.3 The relative quantitative real-time PCR procedure
2.2.4.4 Data analysis

3- Results:

3.1 Histological investigation of human and red deer hair follicles:

3.1.1 Histology of red deer skin using Sacpic staining
3.1.2 Histology of human skin using Sacpic staining and H&E

3.2 RT-PCR analysis of some specific genes in human scalp hair follicles:

3.2.1 Isolation of whole lower anagen hair follicles by microdissection
3.2.2 Checking the quality of total RNA
3.2.3 Configuration of cDNA quality using β-actin in human hair follicle
3.2.4 Human hair follicles express the genes for HGF, MSP and their receptors:
3.2.4.1 Human scalp hair follicles express the gene for HGF
3.2.4.2 Human scalp hair follicles express the gene for MSP
3.2.4.3 Human scalp hair follicles express the gene for RON
3.2.4.4 Human scalp hair follicles express the gene for c-Met

3.3 RT-PCR analysis of some specific genes in human hair bulb components:

3.3.1 Isolation of human scalp hair bulb components by microdissection
3.3.2 Configuration of cDNA quality using β-actin in human hair bulb components
3.3.3 Localisation of RON within hair bulb components
3.3.4 Localisation of HGF within hair bulb components
3.3.5 Localisation of c-Met within hair bulb components

3.4 Immunohistochemistry

3.4.1 Detection of cytokeratin 6 expression in human hair follicle.
3.4.2 Detection of c-Met expression in human hair follicle

3.5 RT-PCR analysis of some specific genes in human cultured DP cells:

3.5.1 Checking the quality of cultured DP cells cDNA using β-actin
3.5.2 Human cultured dermal papilla cells express the gene for HGF
3.5.3 Human cultured dermal papilla cells express the gene for MSP
3.5.4 Human cultured dermal papilla cells express the gene for RON
3.5.6 Human cultured dermal papilla cells express the gene for c-Met
3.6 Comparison of the gene expression of normal and balding scalp hair follicles from men with androgenetic alopecia using DNA microarray:

3.6.1 Checking the quality of total RNA
3.6.2 Microarray data analysis and data quality assessment
3.6.2.1 Data normalization and transformation
3.6.2.2 Correlation between grouped data
3.6.2.3 Principal Component Analysis
3.6.3 Comparison of overall differences in gene expression between balding and non-balding follicles in androgenetic alopecia
3.6.3.1 Sample clustering
3.6.3.2 Statistical analysis
3.6.3.3 Fold change analysis
3.6.4 Comparison of the expression of keratin and keratin associated protein genes in balding and non-balding scalp hair follicles in men with androgenetic alopecia
3.6.5 Comparison of the expression of androgen receptor gene in balding and non-balding follicles in men with androgenetic alopecia
3.6.6 Comparison of the expression levels of some potential paracrine factor genes in balding and non-balding scalp hair follicles in men with androgenetic alopecia
3.6.7 Comparison of the expression levels of HGF family genes in balding and non-balding scalp hair follicles in men with androgenetic alopecia

3.7 Comparison of the expression of the genes for HGF, MSP and their receptors in non-balding and balding scalp hair follicles from men with androgenetic alopecia using real-time PCR analysis:

3.7.1 Annealing temperature optimization for each set of primers
3.7.2 The relative expression levels for HGF and c-Met in non-balding follicles
3.7.3 The relative expression levels for MSP and RON in non-balding follicles
3.7.4 Comparison of the expression levels of HGF, MSP and their receptors in balding scalp follicles with non-balding follicles in men with androgenetic alopecia
3.7.4.1 Checking the quality of amplified RNA
3.7.4.2 The relative expression levels of HGF in balding and non-balding follicles
3.7.4.3 The relative expression levels of c-Met in balding and non-balding follicles
3.7.4.4 The relative expression levels of MSP in balding and non-balding follicles
3.7.4.5 The relative expression levels of RON in balding and non-balding follicles

4- Discussion

4.1 Identification of HGF family members in isolated non-balding hair follicles
4.1.1 Investigating the expression of HGF and c-Met in non-balding scalp follicles
4.1.2 Investigating the expression of MSP and RON in non-balding scalp follicles
4.2 Comparison of gene expression in balding vs non-balding follicles

References

Appendices
## List of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic diagram showing the hair follicle and associated structures</td>
<td>18</td>
</tr>
<tr>
<td>1.2</td>
<td>Scanning electron micrograph of the hair shaft</td>
<td>20</td>
</tr>
<tr>
<td>1.3</td>
<td>The structure of the hair root</td>
<td>22</td>
</tr>
<tr>
<td>1.4</td>
<td>The successive phases of human scalp hair growth cycle</td>
<td>25</td>
</tr>
<tr>
<td>1.5</td>
<td>The different stages of human scalp hair growth cycle</td>
<td>27</td>
</tr>
<tr>
<td>1.6</td>
<td>Development of hair follicle</td>
<td>30</td>
</tr>
<tr>
<td>1.7</td>
<td>The paradoxical effects of androgens on human hair follicles</td>
<td>37</td>
</tr>
<tr>
<td>1.8</td>
<td>The pattern of hair loss in androgenetic alopecia</td>
<td>39</td>
</tr>
<tr>
<td>1.9</td>
<td>The mechanism of action of androgens</td>
<td>45</td>
</tr>
<tr>
<td>1.10</td>
<td>Human hair patterns under various endocrine conditions</td>
<td>47</td>
</tr>
<tr>
<td>1.11</td>
<td>The hypothesis of androgen action in the hair follicle</td>
<td>49</td>
</tr>
<tr>
<td>1.12</td>
<td>The structure of HGF and MSP compared with plasminogen</td>
<td>58</td>
</tr>
<tr>
<td>1.13</td>
<td>The structure of hepatocyte growth factor receptor (c-Met)</td>
<td>60</td>
</tr>
<tr>
<td>1.14</td>
<td>The structure of hepatocyte growth factor</td>
<td>62</td>
</tr>
<tr>
<td>2.1</td>
<td>Microdissection steps of human hair follicle</td>
<td>73</td>
</tr>
<tr>
<td>2.2</td>
<td>Microdissection steps of human hair bulb components</td>
<td>76</td>
</tr>
<tr>
<td>2.3</td>
<td>Cell culture procedure</td>
<td>96</td>
</tr>
<tr>
<td>2.4</td>
<td>GeneChip® probe array and its parts</td>
<td>107</td>
</tr>
<tr>
<td>2.5</td>
<td>Preparation of microarray and microarray procedure</td>
<td>109</td>
</tr>
<tr>
<td>2.6</td>
<td>NanoDrop 2000 instrument and checking the quality of RNA</td>
<td>116</td>
</tr>
<tr>
<td>2.7</td>
<td>The different positions of reaction mix in 96-well plate</td>
<td>120</td>
</tr>
<tr>
<td>3.1</td>
<td>Histology of deer hair follicle using Sacpic staining</td>
<td>125</td>
</tr>
<tr>
<td>3.2</td>
<td>Longitudinal section of human scalp skin stained with Sacpic staining</td>
<td>127</td>
</tr>
<tr>
<td>3.3</td>
<td>Longitudinal section of human scalp hair follicle</td>
<td>128</td>
</tr>
<tr>
<td>3.4</td>
<td>Cross section of huma scalp skin stained with Sacpic staining</td>
<td>129</td>
</tr>
<tr>
<td>3.5</td>
<td>Cross section of human hair follicle using H &amp; E</td>
<td>130</td>
</tr>
<tr>
<td>3.6</td>
<td>Surface view of the lower part of human scalp skin</td>
<td>132</td>
</tr>
<tr>
<td>3.7</td>
<td>Human scalp anagen hair follicles within the skin</td>
<td>132</td>
</tr>
<tr>
<td>3.8</td>
<td>Isolated human scalp hair follicle and its bullb</td>
<td>133</td>
</tr>
<tr>
<td>3.9</td>
<td>Gel electrophoresis of total RNA</td>
<td>134</td>
</tr>
<tr>
<td>3.10</td>
<td>Expression of β-actin in non-balding scalp hair follicles</td>
<td>136</td>
</tr>
<tr>
<td>3.11</td>
<td>Sequencing results for β-actin</td>
<td>137</td>
</tr>
<tr>
<td>3.12</td>
<td>Expression of HGF in non-balding scalp hair follicles</td>
<td>139</td>
</tr>
<tr>
<td>3.13</td>
<td>Sequencing results for HGF</td>
<td>140</td>
</tr>
<tr>
<td>3.14</td>
<td>Expression of MSP in non-balding scalp hair follicles</td>
<td>142</td>
</tr>
<tr>
<td>3.15</td>
<td>Sequencing results for MSP</td>
<td>143</td>
</tr>
<tr>
<td>3.16</td>
<td>Expression of RON in non-balding scalp hair follicles</td>
<td>145</td>
</tr>
<tr>
<td>3.17</td>
<td>Sequencing results for RON (MSP receptor)</td>
<td>146</td>
</tr>
<tr>
<td>3.18</td>
<td>Expression of c-Met in non-balding scalp hair follicles</td>
<td>148</td>
</tr>
<tr>
<td>3.19</td>
<td>Sequencing results for c-Met (HGF receptor)</td>
<td>149</td>
</tr>
<tr>
<td>3.20</td>
<td>Human hair bulb components</td>
<td>151</td>
</tr>
<tr>
<td>3.21</td>
<td>Isolated human hair bulb components</td>
<td>152</td>
</tr>
<tr>
<td>3.22</td>
<td>Expression of β-actin in non-balding scalp hair follicle components</td>
<td>154</td>
</tr>
<tr>
<td>3.23</td>
<td>Expression of RON in non-balding scalp hair follicle components</td>
<td>156</td>
</tr>
<tr>
<td>3.24</td>
<td>Expression of HGF in non-balding scalp hair follicle components</td>
<td>158</td>
</tr>
<tr>
<td>3.25</td>
<td>Expression of c-Met in non-balding scalp hair follicle components</td>
<td>160</td>
</tr>
<tr>
<td>3.26</td>
<td>Immunostaining of cytokeratin 6 in human hair follicle</td>
<td>162</td>
</tr>
<tr>
<td>3.27</td>
<td>Immunostaining of cytokeratin 6 in cross section of human hair follicle</td>
<td>163</td>
</tr>
<tr>
<td>3.28</td>
<td>Immunohistochemistry of c-Met in human hair follicle</td>
<td>165</td>
</tr>
</tbody>
</table>
Figure 3.29 Immunohistochemistry of c-Met in human hair follicle dermal papilla
Figure 3.30 Phase contrast images of human cultured dermal papilla cells
Figure 3.31 Expression of β-actin in human cultured dermal papilla cells
Figure 3.32 Sequencing results for β-actin RT-PCR product
Figure 3.33 Expression of HGF in human cultured dermal papilla cells
Figure 3.34 Sequencing results for HGF RT-PCR product
Figure 3.35 Expression of MSP in human cultured dermal papilla cells
Figure 3.36 Sequencing results for MSP RT-PCR product
Figure 3.37 Expression of RON in human cultured dermal papilla cells
Figure 3.38 Sequencing results for RON RT-PCR product
Figure 3.39 Expression of c-Met in human cultured dermal papilla cells
Figure 3.40 Sequencing results for c-Met RT-PCR product
Figure 3.41 Electrophoretic separation of total RNA from normal & balding follicles
Figure 3.42 Electropherogram of non-balding hair follicle RNA samples
Figure 3.43 Electropherogram of balding hair follicle RNA samples
Figure 3.44 Box plot: the distribution of microarray data of balding & normal follicles
Figure 3.45 The correlation of gene expression between normal and balding follicles
Figure 3.46 Principal components analysis of microarray data.
Figure 3.47 Gene expression profile of individual balding and non-balding samples
Figure 3.48 Gene expression profile of balding vs non-balding groups
Figure 3.49 The number of genes changed significantly in balding vs normal follicles
Figure 3.50 Volcano plot showing gene expression obtained from microarray data
Figure 3.51 The number of genes changed significantly in balding (t-test & fold change)
Figure 3.52 The total number of genes changed in balding at different levels of significance
Figure 3.53 Comparison of keratin gene expression in balding vs non-balding follicles
Figure 3.54 Comparison of KRTAP gene expression in balding vs non-balding follicles
Figure 3.55 Comparison of AR expression in balding vs non-balding follicles
Figure 3.56 Comparison of TGFβ1 expression in balding vs non-balding follicles
Figure 3.57 Comparison of SCF expression in balding vs non-balding follicles
Figure 3.58 Comparison of IGF-I & IGF-IR expression in balding vs normal follicles
Figure 3.59 Comparison of IGFBP3 expression in balding vs non-balding follicles
Figure 3.60 Comparison of IL-1β, IL-1α & IL-6 expression in balding vs normal follicles
Figure 3.61 Fold change analysis of gene expression of paracrine factors in hair follicles
Figure 3.62 Comparison of HGF and c-Met expression in balding vs non-balding follicles
Figure 3.63 Comparison of MSP and RON expression in balding vs non-balding follicles
Figure 3.64 Annealing temperature optimization for HGF and c-Met primers
Figure 3.65 Annealing temperature optimization for MSP and RON primers
Figure 3.66 Melt-curve analysis for HGF and c-Met in non-balding follicles
Figure 3.67 Relative expression levels for HGF & c-Met in non-balding follicles
Figure 3.68 Melt-curve analysis for MSP and RON in non-balding follicles
Figure 3.69 Relative expression levels of MSP & RON in non-balding follicles
Figure 3.70 Checking the quality of non-balding follicles amplified RNA
Figure 3.71 Checking the quality of balding follicles amplified RNA
Figure 3.72 Melt-curve analysis for HGF in balding and non-balding follicles
Figure 3.73 Relative expression levels of HGF in balding and non-balding follicles
Figure 3.74 Melt-curve analysis for c-Met in balding and non-balding follicles
Figure 3.75 Relative expression levels of c-Met in balding and non-balding follicles
Figure 3.76 Melt-curve analysis for MSP in balding and non-balding follicles
Figure 3.77 Relative expression levels of MSP in balding and non-balding follicles
Figure 3.78 Melt-curve analysis for RON in balding and non-balding follicles
Figure 3.79 Relative expression levels of RON in balding versus non-balding
List of tables

Table 2.1 Samples used for isolation of whole lower anagen hair follicles
Table 2.2 Samples used for isolation of individual hair bulb components
Table 2.3 Primer sequences and PCR conditions for each specific gene
Table 2.4 Samples used for isolation hair follicles from balding individuals
Table 2.5 Thermal cycling conditions used for preparing for DNA microarray
Table 2.6 PCR conditions for each specific gene used for real-time PCR.
Table 3.1 Interpretation of Sacpic staining colour
Table 3.2 The number of genes changed significantly in balding follicles (t-test filter)
Table 3.3 The number of genes changed significantly in balding follicles (FC filter)
Table 3.4 The number of genes changed in balding (combination between t-test and FC)
Table 3.5 Annealing temperature optimization for different primers used for real-time PCR
Table 3.6 The concentrations of amplified RNA from balding and non-balding samples
1- Introduction
1- Introduction

1.1 Hair functions & its importance:

Mammals have hair all over their bodies as a unique character which differentiates them from other animals in the Animal Kingdom (Valkovic, 1977; Porter, 2003). Previously, mammals were named Pilifera or hair bearers, from the latin word "pilus" for hair (Ebling, 1976). Most human skin produces hair, except for some particular parts of the body, the glabrous skin, including the lips, palms of the hands and soles of the feet (Ebling, 1986).

Hairs are greatly variable in colour, diameter, length, and cross-sectional shape (Schlake, 2007). There are three main types of hair: lanugo, vellus and terminal. Lanugo hair is soft and fine, and is formed in foetal life. This type of hair starts growing on human embryo skin at about the 20th week of pregnancy, and then sheds in uterus at about the 36th week of pregnancy. After birth, an infant has two types of hair, vellus and terminal. Vellus hair is short, small, fine, unmedullated and unpigmented hair. It is located in areas of the body described as hairless regions, for instance the facial hair of a child or adult women (Blume et al., 1991). Terminal hair is long, thick and pigmented, often with a medullated hair shaft. It grows on various parts of the human body such as the scalp, face, eyelashes, eyebrows, arms, legs and axillae (Gray & Dawber, 1998; Randall, 2007).

In mammals, hair performs several functions. It plays an important role in thermal insulation and regulation of body temperature by trapping a layer of air next to the skin or by allowing heat loss when flattened (Vaughan, 1986). Hair provides protection from
extreme temperatures to enable some mammals to live in some of the most difficult environments on earth, for example polar bears and seals at the North Pole. Hair also serves as a physical barrier to protect the skin from harmful rays such as ultraviolet rays (Oliver & Jahoda, 1989). Hair provides, through its coloration, camouflage for animals against their environment (Flux, 1970; Stenn and Paus, 2001). Animals change their coats seasonally to adapt to seasonal changes in their surrounding environment. For example, the arctic hare produces a brown coat during the summer and a white coat during the winter to remain camouflaged (Severaid, 1945). In addition, hair may play sensory functions in certain mammals and act as a neuroreceptor, for example whiskers. Hair may also serve as a visible signal which may play a role in social and sexual communication, for instance the production of the mane in the lion (West and Packer, 2002).

However, in human beings many of these functions are reduced. The main functions of human hair are protection and communication. Eyebrows and eyelashes protect eyes from incoming objects (Martini, 1998). Scalp hair protects the head and neck from the harmful effects of sunlight, ultraviolet light, cold and physical damage (Goodhart, 1960; Martini, 1998; Costin and Hearing, 2007). In addition, scalp hair provides insulation for the head due to the lack of adipose tissue on the scalp. Thermoregulatory function is less important in human beings, however the small amount of terminal hair on the human body helps to cool the body through evaporation from sweat glands (Ebling, 1985).

One of the most important functions of human hair is social and sexual communication. Hair plays a role in beauty; strong healthy hair indicates good health to others, whereas sparse and brittle growth of the hair indicates poor nutrition and bad health (Bradfield,
Hair removal generally has strong depersonalizing roles, for instance shaving prisoners and Christian or Buddhist monks, while long uncut hair has positive connotations like strength and its association with religion in Sikhs (Randall, 2008). There are clear differences in hair type and distribution between males and females, and between adults and children. The growth of pubic and axillary hair is a sign of maturation and puberty (Reynolds, 1951; Marshall and Tanner, 1969, 1970; Randall, 2007).

In addition, hair disorders such as hirsutism or excessive hair growth and alopecia or hair loss can have negative effects on the quality of life such as social restriction and psychological distress to those who suffer from them (Randall, 2008). Although hair loss is not life threatening in human beings, the important communication roles of hair explain its importance for most people (Jansen and Van Baalen, 2006). Since hair plays a vital role in a person's appearance, self image and communication, this provides an insight into why it is possible for hair growth disorders to be at the root of serious psychological distress (Girman et al., 1998; Gulec et al., 2004). Unfortunately, many hair disorders can not currently be effectively treated, mainly due to our lack of understanding of hair follicle function. Therefore a greater understanding of the mechanisms regulating hair growth, leading to the control of these conditions is of great interest worldwide.

1.2 Hair structure:

Hair is a fully keratinised flexible structure of epithelial cells which originates from hair follicles that extend down to various lengths into the dermis and often into the subcutaneous adipose tissue (Montagna & Van Scott, 1958; Montagna, 1976). The hair follicle has a complex structure which results from epithelial-mesenchymal interactions
initiated around the 3rd month of embryonic development (Sengel, 1983; Hardy, 1992; Gorpinich and Nozdrin, 2007). A typical hair consists of two main parts; the first part is the hair shaft which is located above the level of the epidermis. The other part is the hair root which is enclosed within the hair follicle under the level of the epidermis (Slobodan and Snezana, 1998), (figure 1.1).

**Figure 1.1** A schematic diagram showing the hair follicle and associated structures.
The hair shaft is formed of a type of protein called keratin. Outside the skin, hair consists of dead epithelial cells which form flexible, thin, fully keratinized tubes. The hair shaft is composed of three layers: the medulla in the centre of the hair shaft, which is surrounded by another layer called the cortex, which is surrounded by an outer layer called the cuticle (Chase, 1954; Schlake, 2007).

The cortex is made of millions of parallel microfibrils which are assembled in bundles to make macrofibrils, which run parallel to the long axis of the hair (Gray & Dawber, 1998). Macrofibrils are intertwined like a heavy rope, to give the hair strength and elasticity. Cortical cells appear without nuclei, although sometimes there are some visible remains of nuclei in the centre of each cell. Each cortical cell is about 100 µm in length and no more than 5 µm in width, but this varies between species (Ryder, 1973). The cortex is the major site of keratinization and therefore it is essential for hair shaft strength (Langbein et al., 2001).

The medulla is an air space which is located in the centre of the hair. The medulla is missing in much human hair, but is well developed in animals to provide the insulator properties of animal hair. Not all types of hair have medulla, such as thin hair, however it is most obviously present in large thick terminal hair and it can be continuous or discontinuous. Hair fibres with a continuous medulla, such as eyelashes, are stiff in character (Forslind, 2000). There are three main kinds of medulla: the latticed type, non latticed type, and ladder type (Ryder, 1973).
The cuticle is the thin outer layer which covers the hair. This layer is colorless and protects the cortex. It has flat, thin, unpigmented and transparent cells which overlap to give a structure like scales (figure 1.2). There are tiny regular spaces between cuticle cells, almost 10 µm. Cuticle cells overlap from the root to the tip of the hair shaft to make the outer layer of the hair. The cuticle thickness is nearly 6-10 cells (Valkovic, 1977). This layer can easily become damaged by mechanical or chemical treatments.

The hair root is a part of the hair which is within the skin under the level of the epidermis and enclosed within the hair follicle. Each hair arises from a long narrow pocket inside the skin which is called the hair follicle. Hair follicles are continuous with the epidermis; their cylindrical epithelial layers grow to various depths downwards into the dermis and in some species project into the subcutaneous adipose layer. Each follicle can be divided morphologically into three parts: the upper infundibulum, the middle isthmus and the inferior part which includes the hair bulb (Sperling, 1991; Gray and Dawber, 1998).

Figure 1.2 Scanning electron micrograph of the hair shaft shows the cuticle which appears as scales. Reproduced from Wolfram (2003), with the author's permission.
The follicle is surrounded by two main layers: the inner and outer root sheaths. The inner root sheath surrounds the hair fibre from the bulb up to the level of the sebaceous gland allowing the hair fibre elasticity (Forslind, 2000). It is in contact with the hair cuticle and contains three sub-layers: the inner layer is the cuticle which is in touch with the hair cuticle to form a structure like a zip. The middle layer is Huxley's layer which contains square cells that are arranged in one row. The outer layer of the inner root sheath is Henle's layer; its cells are polygonal, flattened and arranged in one row (Hardy, 1992; Kaufman, 2003; Alibardi, 2004). Around the outside of the outer root sheath is a layer of connective tissue which separates the follicle from the dermis. It divided into two parts: the outer reticular and inner papillary layers (Bartosova et al., 1984). There is a vitreous membrane between the outer root sheath and the connective tissue sheath (Bernard, 2006), (figure 1.3).

The bottom of the follicle is the hair bulb where the epithelial cells surround the mesenchyme-derived dermal papilla, a pear-shaped structure located within its centre. The dermal papilla plays an important role throughout the hair follicle development and life cycle, as it contains specialised fibroblasts, known as dermal papilla cells, which are surrounded by extracellular matrix containing mucopolysaccharides and basement membrane proteins (Couchman, 1986; Couchman et al., 1990). The non-cellular part of the dermal papilla includes collagen types I and III, and fibronectin (Messenger et al., 1991). The size of the dermal papilla and its cells is proportional to the size of the hair follicle, and therefore the hair fibre produced (Van Scott and Ekel, 1958; Ibrahim and Wright, 1982; Elliott et al., 1999). The dermal papilla is almost completely surrounded by the epithelial matrix, except a small gap at the base of the hair bulb, to allow blood
vessels and nerves to enter the papilla. The epithelial matrix is separated from the dermal papilla by a trilaminar basement membrane (Nutbrown and Randall, 1995). It is believed that the dermal papilla plays main roles in controlling the hair follicle development and the production of hair fibres, both during embryogenesis and during the hair growth cycle (Jahoda, 1992; Jahoda, 2003); see section 1.5.

Figure 1.3 The structure of the hair root

Isometric view of a human hair follicle cut away to show component parts. Reproduced with permission, Randall (1994).
The epithelial hair matrix contains very actively dividing cells which can differentiate, under a high level of organisation and control, into the different cell types of the inner root sheath and the hair fibre (Camacho et al., 2000; Bernard, 2006). The production of hair in the actively growing follicle is a result of rapid cell division of the epithelial keratinocytes in the hair matrix. The critical level is an imaginary line across the widest diameter of the follicular bulb into two distinctive regions (Auber, 1952). Below the critical level, the matrix cells have a high rate of mitosis but appear undifferentiated. Above the critical level, the rate of mitotic activity is decreased and cells become differentiated into either the inner root sheath or hair fibre cells.

Within the hair matrix, there are a number of melanocytes located above and to the sides of the dermal papilla. Follicular melanocytes are derived from epidermal melanocytes during hair follicle morphogenesis (Magerl et al., 2001; Tobin, 2008). These cells are responsible for production of the hair pigment. Pigmentation is a highly controlled process, as melanin incorporation occurs only in the hair shaft. The amount and type of pigment, melanin, and the balance of brown/black eumelanin and red/yellow phaeomelanin in the hair shaft determines the hair colour (Slominski et al., 2004; Slominski et al., 2005; Rousseau et al., 2007). Every division of the matrix cells results in a migration of the cells upward from the bulb to the narrower linear region of the follicle, cells become elongated and keratinisation takes place. This process continues as the cells migrate through the follicle, eventually becoming fully keratinised by the time they reach the surface of the skin.
There are some important structures of ectodermal or mesodermal origin which are associated with the hair follicle. The sebaceous gland and the arrector pili muscle, as part of the pilosebaceous unit, are important parts of organized follicular unit structure (Poblet et al., 2004). The sebaceous glands produce a waxy secretion into the follicle through the sebaceous duct, inducing a waterproof coating over the hair fibre. Apocrine sweat glands are also present above the sebaceous ducts in some follicles such as those found in axillae and genital areas (Hurley, 2001). The apocrine glands discharge into the upper portion of the hair follicle and can be a possible origin of pheromones (Spielman et al., 1995). The hair follicle contains stem cells, involved in the regeneration of the follicle during hair growth cycle. These cells are located in the outer root sheath and in the bulge area, the site of arrector pili muscle attachment (Taylor et al., 2000; Oshima et al., 2001; Waters et al., 2007; Morris, 2007). This muscle allows adjustment of the hair for thermoregulation, for instance erection of hair on skin in response to cold temperature.

1.3 Hair growth cycle:

The hair follicle grows in cycles. However, hair cycles in human beings do not occur at the same time for all hairs. Morphologically, the hair cycle process is well understood, but is still not clarified at the molecular level. Each hair cycle consists of successive stages of growth and involution, which include tissue regression and regeneration. The hair growth cycle includes three main stages: the growth phase, anagen, the transitional phase, catagen, and the resting phase, telogen (Chase, 1954; Kligman, 1959), (figure 1.4). A fourth phase, exogen, is involved in releasing the old hair via an active enzymatic releasing process (Forslind, 2000; Stenn, 2005, Higgins et al., 2009). The duration of each stage varies between species.
Anagen, or the growth phase, extends from the termination of the resting phase, which is called telogen, to the beginning of the regression phase, which is called catagen. The duration of anagen determines the length of the hair, and depends on the hair follicle location. For instance, eyebrow hair follicles are in anagen for about two to three weeks producing short hairs, whereas scalp hair follicles are in anagen from 2-8 years resulting in long hair (Saitoh et al., 1970; Paus and Cotsarelis, 1999). During anagen, the hair grows in length by addition of cells to the bottom end, the hair bulb lies deep inside the skin and elongates, the hair matrix cells divide to produce cells which grow upward and become longer and keratinize to make the growing hair. Melanocyte cells begin to give the hair colour. In the meantime, the inner root sheath, which covers the tip of the hair matrix, is replaced by the old club hair.
growing hair, ruptures to enable the growing hair to penetrate the skin and grow up outside the skin (Jarrett, 1977).

The anagen phase is followed by the catagen phase which is a highly controlled process involving regression, degradation and cessation of new hair cell production. This phase lasts about 2-3 weeks in human scalp (Kligman, 1959). During this phase, the dermal papilla becomes smaller, containing less extracellular matrix and its cells have only a little amount of cytoplasm (Sugiyama et al., 1976). The dermal papilla is released from the bulb, the inner root sheath is lost, the pigmentation process and cell division are stopped. The lower part of the hair becomes short with an enlarged end, and becomes fully keratinized to form a "club" hair which shrinks and moves towards the skin surface until it reaches the level of the sebaceous gland. The dermal papilla forms an assembly of flattened and compact dermal cells. At the end of catagen, the ball of the dermal papilla cells moves up in the skin to settle below the swollen end of the club hair, under the area of hair germ (Montagna and Ellis, 1985). If the dermal papilla fails to reach the bulge level, the cycle stops and the hair is lost (Paus and Cotsarelis, 1999). The involution of the club hair results in apoptosis of the epithelial components of the hair follicle (Weedon and Strutton, 1981; Lindner et al., 1997; Matsuo et al., 1998; Botchkareva et al., 2006).

The catagen phase is followed by a period of rest called telogen. During this phase, the follicle becomes inactive and the activities of the dermal papilla are stopped. A telogen hair is distinguished by its fully keratinized club hair which is surrounded by thick epithelial sac. Below this lies the condensed dermal papilla waiting for the signal to start a new hair cycle (Sperling, 1991; Schmidt-Ullrich and Paus, 2005; Jahoda, 2001). The
telogen phase lasts around approximately 3 months on the human scalp before re-entering anagen phase (Kligman, 1959). After the end of telogen, the hair cycle starts again by beginning cell division that leads to reactivation of the hair follicle to begin a new growth cycle. The dermal papilla cells are reactivated and move back down into the dermis with associated keratinocytes. A new hair bulb is formed, a new hair grows up, the existing hair is shed. Previously, it was believed that the new hair pushed the old hair out as a result of the physical movement of the new hair upward. Recently, the exogen stage has been suggested, which includes an active shedding process of the old hair involving enzymatic release (Stenn et al., 1998; Stenn and Paus, 2001; Stenn, 2005; Higgins et al., 2009). Some recent studies suggest that it is not unusual for the previous hair to be retained for more than one hair cycle in rodents (Milner et al., 2002), so this suggests that the anagen phase and exogen phase are separate events (Randall, 2007), (figure 1.5). Overall, the hair follicle exhibits a highly complex organised architecture and represents an attractive experimental system because of its accessibility, dispensability and self-renewal capacity (Schlake, 2007).

![Figure 1.5](image_url) Diagram showed the different stages of human hair growth cycle including anagen, catagen, telogen and exogen. Reproduced with the author’s permission, Randall (2008).
1.4 Hair follicle embryogenesis:

Human hair follicles first develop late in the second trimester of pregnancy in the regions of the developing eyebrow, chin, and upper lip (Pinkus, 1958) and their development is completed by the 6th month of foetal life (Gorpinich and Nozdrin, 2007). The process of hair formation is a highly co-ordinated process, involving cell-cell communication, cell division and cell differentiation. The development of embryonic hair follicles starts as a result of interaction between epithelial and mesenchymal cells (Mikkola and Millar, 2006; Fuchs, 2007). An early embryonic epidermis comprises the periderm, the intermediate layer and the basal layer. The pre-germ formation is considered the first event of the embryonic development of the hair follicle. This stage initiates as a result of localized thicking of the basal layer of the epidermis and an aggregation of mesenchymal cells at the junction between the epidermis and dermis (Schmidt-Ullrich and Paus, 2005); (figure 1.6). Epidermal and mesenchymal cells continue to develop and start to grow downward at an oblique angle to form a slight bulge on the underside of the epidermis called the hair germ (Pinkus, 1958; Holbrook and Minami, 1991) which elongates and grows deeper into the dermis to form a column of cells called the hair peg.

The free end of the hair peg flattens and enlarges to form a concave-shaped structure which is called a bulbous hair peg that eventually encloses the mesenchymal cells to form the dermal papilla (Breathnach & Smith, 1968; Robins and Breathnec, 1969). By this stage, the melanocytes are mainly localised in the epithelial part of the follicle to form a cap over the dermal papilla (Mishima and Widlan, 1966). The hair matrix arises from the epidermal cells surrounding the dermal papilla. As the bulbous hair peg elongates, the
matrix cells differentiate into the different cell types of the inner root sheath and the hair fibre. The development of epithelial cells, which connect the matrix to the interfollicular epidermis, forms the outer root sheath, whereas mesenchymal cells, which surround the epithelial cells, form the connective tissue sheath. On the posterior side of the follicle, two epithelial buds or swellings begin to grow. The most superficial one evolves into one or more sebaceous glands, whereas the lower bud forms the bulge area to which the arrector pili muscle attaches. In addition, at some certain areas of the body such as axilla and groin, follicles develop a third bud situated above the sebaceous gland to form apocrine glands.

The hair canal is formed by degeneration of cells in the centre of the bulbous hair peg. Meanwhile, epithelial cells almost completely surround the dermal papilla which remains attached by a narrow stalk to a basal plate of dermal cells. At this stage, the hair fibre and canal begin keratinisation. The different components of the hair follicle proceed to differentiate and the hair fibre continues to grow upwards until the hair emanates from the epidermis. The hair fiber eruption is preceded by elevation of the epidermis and avulsion of the canal roof. In the mean time, the hair follicle keeps its elongation and continues to grow downward into the epidermis until it attains its full size within the skin (Muller et al., 1991; Hardy, 1992; Paus et al., 1999; Wu-Kuo and Chuong, 2000; Schmidt-Ullrich and Paus, 2005; Fuchs, 2007). Hair follicles can therefore serve as an excellent model for investigating aspects of cell-cell interaction, patterning processes, cell differentiation, stem cell biology and cell lineage specification (Schlake, 2007).
Figure 1.6 Development of the hair follicle. Schematic diagram represents the different stages of hair follicle embryogenesis. Adapted from Randall (1994), with the author's permission.
1.5 The role of the dermal papilla in hair follicle growth and maintenance:

The hair follicle consists of structures derived from ectodermal and mesenchymal origins. Epithelial and mesenchymal interactions are important for both hair follicle development and cycling. There is a series of messages between the dermal and epithelial cells of the hair germ during the early stage of hair follicle development (Fuchs, 2007). These early messages have been illustrated by several tissue recombination experiments on embryonic skin from several species (Sengel, 1983; Hardy, 1992; Ferraris et al., 2000).

The dermal papilla is an important feature of hair follicle development and a permanent, discrete entity throughout the hair cycle of the hair follicle. The adult dermal papilla demonstrates similar features to its mesenchymal origin (Messenger, 1993; Rendl et al., 2005). Dermal papilla cells play a crucial role in inducing new follicular development and determining the type of hair produced (Jahoda et al., 2001). When the dermal papillae were amputated from anagen vibrissae follicles, this led to hair growth cessation (Oliver, 1966; Link et al., 1990), indicating that the dermal papilla plays an important role in hair follicle regulation.

Similarly, it has been reported that re-implanting cultured dermal papilla cells into the base of hair follicles after removal of their lower parts, enabled such follicles to reconstruct; the re-implantation led to organisation of the outer root sheath cells around the dermal papilla to form an epithelial matrix and reformation the hair bulb (Oliver, 1967; Jahoda et al., 1984). When the germinative epithelium, from the hair bulb matrix, was co-cultured with dermal papilla cells, growth occurred and organoid structures were formed, but no growth was observed when these cells were cultured alone without dermal
papilla cells or even with 3T3 feeder cell layers (Reynolds and Jahoda, 1991). This suggests the capability of dermal papilla to induce hair growth. More evidence to testify the importance of the dermal papilla in hair growth is that implanting cultured rat vibrissae dermal papilla cells superficially in the skin stimulated new hair follicle formation (Jahoda and Oliver, 1984). In other experiments, when cultured rat pelage dermal papilla cells were implanted into rat foot-pad skin, which does not contain any hair follicles normally, they also stimulated new follicle development indicating that the adult dermal papilla still has the capability to stimulate new hair follicle growth (Reynolds and Jahoda, 1991; 1992).

Importantly, it has been observed that the dermal papilla cells derived from rat vibrissae follicles induced the formation of new vibrissae follicles in rat ear wound (Jahoda, 1992). This indicates that the determination of the hair follicle and then the type of hair produced depends on the original site of which the dermal papilla was taken, not the transplantation site (Reynolds and Jahoda, 1992; Jahoda et al., 1993). This observation concurs with the concept of the surgical transplantation of hair follicles in which transferring hair follicles from non-balding scalp areas to cover balding sites of the scalp (Orentreich and Durr, 1982; Orentreich, 1985; Epstein 2007). Dermal papilla signals can also cross tissue and species. Embryonic dermal cells were able to reprogram adult corneal epithelial cells to form epidermis or epidermal appendages (Ferraris et al., 2000). A recent study showed that the dermal papilla cells derived from rat and mouse vibrissae also had the ability to induce new hair follicle structures in rabbit corneal epithelium (Richardson et al., 2007) and human dermal papilla cell signals can alter rodent hair follicle cells both in vitro and in vivo (Hamada and Randall, 2006).
Some studies indicated that the germinative epithelium and dermal sheath can play an inducible role in hair follicle formation. When germinative epithelial cells and vibrissae dermal sheath cells were implanted into the skin of rat ear, they failed to stimulate hair growth individually, but when they were implanted together into the ear wound they stimulated the formation of new follicles (Reynolds and Jahoda, 1996). This suggests that the cells of the dermal sheath require a signal from the germinative epithelial cells to modify their differentiation status to behave as dermal papilla cells and stimulate hair follicles construction. More recently, using fluorescently-labelled cells in vivo, it was observed that cells from the dermal sheath cup surrounding the hair bulb had a similar role as dermal papilla cells in inducing hair follicle (McElwee et al., 2003). It has been reported that this transformation of dermal sheath cells into dermal papilla cells is not completely unidirectional when labelled dermal papilla cells formed new dermal sheath in induced follicles (Inamatsu et al., 2006). Overall, these experiments clearly demonstrate an important regulatory role for the mesenchyme-derived dermal papilla and the dermal sheath surrounding the hair bulb in regulating the type of hair produced by a follicle.
1.6 The involvement of androgens in human hair growth:

The hair follicle growth cycle allows the hair produced from the follicle to change and adapt to seasonal variation or changes in sexual development. The mechanisms involved in the complicated processes of the hair growth cycle are not well understood.

In mammals, seasonal variation can be detected in the changing of coat thickness and colour induced by temperature, day length, and nutrient availability (Chase, 1954; Galbraith, 1998). These changes in the environment are translated to the follicle via the endocrine system, mainly through the pineal and hypothalamus-pituitary route (Ebling et al., 1991; Randall, 2007). The hormones involved differ between the species, but generally include gonadal hormones, thyroid hormones, corticosteroids, prolactin, and melanocyte stimulating hormone (MSH).

In human beings, a few weeks after birth a wave of hair shedding occurs (Pecoraro, 1968), after this the hair cycle is asynchronous except for the local groups of three follicles called DeMijère trios (Saitoh et al., 1970). Circannual variation in the rate of the human hair growth has been observed on the scalp, thigh, and beard (Orentreich, 1969; Randall and Ebling, 1991; Courtois et al., 1996). Hair growth in human beings is affected by various factors such as glucocorticoids (Stenn et al., 1993), thyroid hormones (Jackson et al., 1972), pregnancy (Lynfield, 1960), circulating hormones (Randall, 2007) and nutrient levels (Bradfield, 1971; Rushton, 2003). However, androgens are the major regulating hormones of hair growth (Ebling, 1986; Randall et al., 2008). Human hair follicles, which are distributed in various parts of the body, have an innate tendency for androgen-dependent growth (Nyholt et al., 2003). Almost all human hair follicles are
influenced by androgens except some specific areas of the body which produce terminal hair in childhood such as eyelashes, eyebrows, and many scalp follicles where androgens have no obvious effect (Ebling, 1986; Randall, 2007).

Androgens can exert opposite functions in human hair growth depending on the body site. They can transform small vellus hair follicles into large terminal hair follicles, as seen during the growth of beard, pubic hair, and axillary hair after puberty (Hamilton, 1958; Marshall and Tanner, 1969; 1970), whereas they can also transform large terminal hair follicles into small vellus hair follicles as seen on the scalp in genetically predisposed individuals with androgenetic alopecia (male pattern baldness) in which the follicles undergo progressive miniaturisation (Hamilton, 1942; 1960; Nyholt et al., 2003), see figure 1.7. These paradoxical effects of androgens on human hair growth have long been a mystery. These changes occur gradually with several hair growth cycles occurring before the type of hair produced is changed (Randall, 1994a; Randall, 2005). In addition, these changes appear to be androgen dependent and determined by the presence of sufficient circulating androgens (Hamilton, 1942), as well as the degree of genetic predisposition (Nyholt et al., 2003).

One of the first signs of puberty is the gradual replacement of small vellus hair with larger, more pigmented hair in the pubis and axillae in both sexes (Marshall and Tanner, 1969; 1970; Randall, 2007). These changes parallel the rise in plasma androgens which occurs earlier in girls than in boys (Winter and Faiman, 1972; 1973). During male puberty, the growth of beard initially starts in the moustache area and the middle of the chin and later spreads over the lower part of the face and parts of the neck (Marshall and
Tanner, 1970). In Japanese and Caucasian men, beard and axillary hair both grow rapidly, however while beard growth continues until the seventies, axillary hair growth peaks in the mid-twenties and then decreases rapidly in both races (Hamilton, 1958). This indicates that the hair follicles in different anatomical locations of the body have different responses and sensitivities to androgen.

However, the range of responses of hair follicles to androgens is genetically intrinsic (Nyholt et al., 2003) and depends upon body site (Randall et al., 1991; Randall, 2007), as well as levels of circulating androgens, for example beard hair growth in males needs much higher levels of androgens than post-pubertal axillary and pubic hair in females. The effects of androgens on hair growth are clearly illustrated by patients with testicular feminisation who have no functional androgen receptors. These individuals do not develop beards, axilla or pubic hair. There have been no reports of these patients suffering from androgenetic alopecia (Griffin & Wilson, 1989; Imperato-McGinley et al., 1991; McPhaul, 2004).
Figure 1.7 Effects of androgens on human hair follicles. Schematic diagram represents the paradoxical effects of androgens on human hair follicles of different body sites. Reproduced from (Randall, 2000), with the author's permission.
1.7 Androgen-related hair disorders:

Because of its importance in social and sexual communications, hair plays an important role in the standard of a person's appearance and self-image. Therefore, hair disorders may cause psychological distress and reduced quality of life as discussed in section 1.1. However, hair disorders are not physically painful or life threatening. The two main androgen-related problems associated with hair growth are the loss of hair on the scalp in males and females, which is more prevalent in males (androgenetic alopecia), and excessive hair growth on the face and body of women in the male pattern (hirsutism).

Androgenetic alopecia:

Androgenetic alopecia is a common type of hair loss. It is also called common baldness or male pattern baldness which mainly affect men (Hamilton, 1951), but can also affect women (Ludwig, 1977, Messenger and Sinclair, 2006). Androgenetic alopecia affects up to half of the male Caucasian population by middle age and almost all Caucasian men in old age (Ellis, 2001). This type of hair disorder is characterised by the gradual transformation of thick, pigmented hairs on the scalp to short, thin pale hairs, in both men and women (reviewed in Randall, 2005). In androgenetic alopecia and also during puberty, the gradual changes in the type of hair produced by the hair follicles are related to changes in the stages of the hair growth cycle (Randall, 2007). Therefore, during the disorder progression, the anagen phase of hair growth cycle shortens whereas the telogen phase remains constant (Hamilton, 1951; Norwood, 1975; Randall, 2005).
The distinctive pattern of hair loss was first described by Hamilton (1951) and later by Norwood (1975). The hair loss severity was classified into seven classes, starting from pre-pubertal scalp (Type I) through gradual recession of bitemporal hairline and thinning of the vertex (Type VII). This classification system was later modified by Norwood to improve the grading of the middle balding types. This type of male pattern baldness can occur in women, however a different pattern of hair loss is more common. It was described and graded by Ludwig (1977) as the gradual hair loss in the crown, with preservation of the frontal hair line (Sinclair et al., 1999; Price, 2003, Bionodo et al., 2004).

Figure 1.8 The pattern of hair loss in androgenetic alopecia in men. The Hamilton scale (as modified by Norwood) is used to classify the type and extent of common baldness in men. Reproduced from Randall (2000), with permission.
There are two main factors involved in the pathogenesis of androgenetic alopecia including androgen and genetic predisposition. There is a great deal of evidence to support the involvement of androgen in the development of androgenetic alopecia. For example, androgenetic alopecia does not occur in male castrated prior to puberty and progression is stopped if postpubertal males are castrated (Hamilton, 1960). In castrated men, hair loss can be stimulated using testosterone replacement therapy, however withdrawal of the hormone led to a cessation in hair loss progression (Hamilton, 1942). The role of androgen in hair loss is confirmed by the absence of androgenetic alopecia in people with complete androgen insensitivity syndrome in which individuals lack functional androgen receptors (McPhaul, 2004). In balding men, testosterone levels are normal (Phillipou and Kirk, 1981; Pitts, 1987) and the response to androgen seems to be intrinsic to the hair follicle (reviewed by Randall, 2005).

Genetic predisposition is required for the onset of alopecia alongside androgen (Birch and Messenger, 2001; Ellis and Harrap, 2001; Nyholt et al., 2003). Androgenetic alopecia has been described as an autosomal dominant trait with variable penetrance (Bergfeld, 1995). A number of genes have been investigated for an association with androgenetic alopecia, it has been reported that male-pattern baldness associated with genetic variants in the androgen receptor gene (Ellis and Harrap, 2001; Prodi et al., 2008). In addition, recent studies showed susceptibility variants for androgenetic alopecia on chromosome 20p11 (Hillmer et al., 2008; Richards et al., 2008). This indicates that the inheritance pattern of androgenetic alopecia seems to be polygenic.
There are a range of treatments for androgenetic alopecia such as surgery, wigs and hairpieces, hormonal, and non-hormonal therapy. The surgical treatments are based on the intrinsic response of hair follicles to androgens. It involves the relocation of hair follicles from the non-balding occipital and parietal areas of the scalp to cover the bald sites (Orentreich and Durr, 1982; Epstein, 2007). This type of treatment is long lasting, painful and expensive, as well as it may require further surgery as the hair loss develops around the transplanted regions.

The main hormonal treatments include anti-androgens and 5α-reductase inhibitors. Anti-androgen treatment blocks the androgen binding to the androgen receptors; however, this is not a desirable treatment for men as it has impractical effects on male masculinity. 5α-reductase inhibitors, such as finasteride, act by blocking the conversion of testosterone to its more active form 5α-dihydrotestosterone (DHT) which binds the same receptor as testosterone. It has been observed that finasteride slowed down gradual hair loss and promoted hair growth in men under 42 with stage II to V hair loss (Kaufman et al., 1998). Another study showed the efficacy of finasteride in older men (Whiting et al., 2003). Recently, it has been revealed that dutasteride, a type I and type II 5α-reductase inhibitor, increased scalp hair growth in men with androgenetic alopecia more rapidly than finasteride, which only inhibits type II 5α-reductase (Olsen et al., 2006). To date, 5α-reductase inhibitors are the best current treatment for androgenetic alopecia in men, however similar to all hormonal treatments, they require long-term continuation.

The most commonly used non-hormonal treatment for androgenetic alopecia in men and women is minoxidil (Olsen et al., 2002; Dawber, 2000; Messenger and Rundegren,
2004). It belongs to a group of drugs known as potassium channel openers. It was initially developed for hypertension treatment, but it was observed to have hypertrichosis (excessive hair growth) as a common side effect (Shapiro and Price, 1988; Dawber and Rundegren, 2003) and then it was remarked as a hair loss treatment. It is unclear how minoxidil itself functions to stimulate hair growth, however a recent study suggested that the mechanism of minoxidil action in hair follicle is via the opening the ATP-sensetive potassium channels in the membranes of hair follicle cells (Shorter et al., 2008).

**Hirsutism:**

Hirsutism is the excessive hair growth on areas of the body of women in the pattern of a normal post-pubertal male (Dawber, 1994; Azziz, 2003). In normal women, these sites of the body are covered by fine hair for instance above the lip, on the chin, chest, forearms, back and abdomen. This abnormal hair growth may be triggered by excess production of androgen. In these cases androgens cause the transformation of fine, small hairs into thick, pigmented hairs. This is a paradoxically different effect of androgens compared to their involvement in androgenetic alopecia. It has been reported in a study investigating hirsutism in British women between 15 and 64 years of age, that about 10% have hair on the chest, 22% on the chin, and 49% on lips (Ehrmann, 2005). There are several conditions which can cause hirsutism such as polycystic ovary syndrome (PCOS) which is characterised by excessive production of androgen (Bardin and Lipsett, 1967; Franks, 1989; Ehrmann, 2005). However, a small portion of cases have idiopathic hirsutism, with no detectable hormonality (Simpson and Barth, 1997; Elghblawi, 2008).
Treatment of hirsutism can involve cosmetic and/or pharmacological approaches. Pharmacological methods include treatment with anti-androgen drugs such as cyproterone acetate (CPA), spironolactone (Hammerstein, 1987; Shapiro and Lui, 2005) and flutamide (FLU) (Osculati and Castiglioni, 2006). This type of treatment may take 6-9 months before detecting any effect on hair growth and there may only be partial improvement. In addition, there is also a risk of feminisation of a male foetus with anti-androgen treatment (Hughes and Cunliffe, 1988). Finasteride is also used as a competitive inhibitor of 5α-reductase, and has been shown to be effective for treating hirsutism with fewer side effects (Moghetti, 2000). The cosmetic approach employs various methods which physically remove or lighten excess hair to make it less conspicuous; such treatment includes shaving, waxing, bleaching, depilatory creams, electrolysis and laser hair removal (Claman et al., 2002).

1.8 Mechanism of androgen action in the hair follicle:

The main circulating androgen in the human body is testosterone which is derived from cholesterol like other steroid hormones. Testosterone is primarily made and secreted by leydig cells in the testes of human men and the ovaries of women; the adrenal glands in both sexes can also secrete low level of androgens. However, the amounts of testosterone produced by human males are much more than those produced by females (Baird, 1970). In addition, the concentration of circulating testosterone produced by males is estimated as 10-35 nmol whereas it is <3.5 nmol in females (Leshin and Wilson, 1981). Androgens are carried in the blood either free or bound to specific proteins such as albumin, and sex-hormone binding globulin (SHBG), (Selby, 1990; Rommerts, 2004).
Systemic androgens can enter the hair follicle via its blood vessels into the dermal papilla and the dermal sheath. Because androgens are lipid soluble (lipophilic molecules), they can diffuse directly from the blood into the cytoplasm of target cells through the cell membrane and then enter the nucleus (Randall et al., 2000). In the cytoplasm, testosterone can be metabolised to 5α-dihydrotestosterone (DHT) depending on the site of the follicle in the presence of the enzyme 5α-reductase.

Androgens exert their activities on the target cells by binding to specific intracellular receptors. Each receptor contains a ligand-binding site and DNA-binding site. Both testosterone and 5α-dihydrotestosterone can bind to the ligand-binding domain, which causes a conformational change in the receptor, as well as activation of the DNA binding site (Janne et al., 1993, Narayanan et al., 2008). However, 5α-dihydrotestosterone is a more potent androgen and binds to the androgen receptor with a higher affinity than testosterone to modulate gene expression (Randall, 1994b, Randall 2007). The receptor-androgen complex then binds to a specific DNA sequence of the target gene which is called a hormone response element (Handelsman, 2005). This binding process results in activation of androgen-dependent genes, and DNA transcription which leads to altering the production of specific proteins. The summary of this process is shown in figure 1.9.
Androgen insensitivity syndrome:

The mechanism of androgen action in hair follicles has been illustrated by various forms of androgen insensitivity syndrome. Individuals with this syndrome are characterised by an XY genotype but they exhibit a female external phenotype. These individuals lack functional androgen receptors. Although they have normal or raised circulating androgen levels, they lack beard, pubic, and axillary terminal hair and they do not undergo androgen-dependent scalp hair thinning (McPhaul, 2004; Randall, 2007). This confirms that the terminal hair growth in these sites of the body is androgen dependent, whereas follicles in other sites such as scalp, eyebrows, and eyelashes are androgen independent.

Figure 1.9 Mechanism of androgen action.
Schematic diagram represents the mechanism of action of testosterone and 5α-dihydrotestosterone (DHT). Reproduced with permission, Randall et al., (2000).
The enzyme 5α-reductase:

Two isoforms of the enzyme 5α-reductase type I and type II, have been identified (Jenkins et al., 1992; Blanchard et al., 2007). The distribution of these isoenzymes varies between tissues, however both types are found in scalp follicles (Eicheler et al., 1995) and a recent study showed that the genes for 5α-reductase type I and type II were expressed in cultured dermal papilla cells derived from both beard and scalp hair follicles (Liu and Yamauchi, 2008). Type I is mainly found in the liver, however its role is not obvious. Absence of 5α-reductase type II results in the syndrome of 5α-reductase deficiency, a rare form of male pseudohermaphroditism. Individuals with this syndrome are born genetic males (XY) with ambiguous external genitalia and they are unable to convert testosterone (Andersson et al., 1991). Individuals with 5α-reductase type II deficiency do not produce male patterns of body hair, in spite of their circulating androgens after puberty. They only produce female patterns of pubic and axillary hair and show an absence or reduction of beard growth, although they do develop a masculinized male body shape (Imperato-McGinley et al., 1991; Wilson et al., 1993; Randall, 2008). This suggests that pubic and axillary follicles respond to testosterone, but the male secondary sexual hair growth demands metabolism of testosterone to 5α-dihydrotestosterone (Randall, 2007). It is unclear why some follicles require testosterone and others 5α-dihydrotestosterone to induce the same cell biological changes. This suggests that the cells use different intracellular coactivating proteins to act with the receptor (Randall, 2008).
Figure 1.10 Human hair patterns under various endocrine conditions. reproduced from Randall (2008) with the author's permission.
1.9 Current hypothesis of androgen action in hair follicles:

The molecular mechanisms involved in the control of hair growth are not well understood. Androgens are the major regulating hormones of human hair growth (Randall, 2008). It is believed that androgens act on the hair follicle via the mesenchyme-derived dermal papilla which interprets the response indirectly for the other follicular components. The current hypothesis of androgen action in hair follicles was proposed by Randall (1994). This hypothesis proposed that circulating androgens enter the dermal papilla via its well developed blood supply. Within the dermal papilla cells of androgen dependent hair follicles, androgens bind to specific intracellular androgen receptors and then cause alteration in their production of paracrine factors. These factors then act on other components of the hair follicles, altering the type of hair produced (figure 1.11).

Paracrine factors are those for which the target cells are different from, but near to the signal-releasing cell. These factors could be soluble mitogenic factors such as growth factors and/or extracellular matrix components. Elucidation of these factors may lead to a better understanding of hair biology and the mechanisms involved in androgen dependent hair disorders. These factors may act on different follicular targets including the melanocytes which modify the amount of pigments produced, the keratinocytes which propagate to produce the hair fibre and different sheaths, and the follicular endothelial cells and the cells of the follicular connective tissue sheath (Randall et al., 2007). In addition, another possible target for these factors could be the dermal papilla itself via an autocrine regulatory system (Hamada and Randall, 2006), as any change in the dermal papilla size leads to a change in hair follicle size and the type of hair produced (Van Scott
and Ekel, 1958; Elliot et al., 1999). Therefore, the dermal papilla cells would be the direct target for androgen action, whereas the other follicular components would be controlled by androgens indirectly.

This hypothesis has received much support by several experimental studies. Using autoradiographic localisation, the highest concentrations of radio-labelled testosterone were detected in the dermal papilla cells and sebaceous glands in rat skin. This indicates that these cells are the main androgen targets of skin (Stumpf and Sar, 1976). It has been reported that androgen receptors are localised in human dermal papilla cells, but not hair follicle keratinocyte cells using immunohistochemistry (Choudhry et al., 1992; Itami et al., 1992).

Figure 1.11  Diagrammatic representation of the hypothesis of androgen action in the hair follicles. Circulating androgens enter the dermal papilla via its blood capillaries, causing the production of paracrine factors which act on other follicular components. Reproduced with permission (Randall, et al., 2008). T: Testosterone, ?: unknown paracrine factors.
al., 1995; Thornton et al., 2003). Based on *in vitro* studies, dermal papilla cells derived from androgen-sensitive regions, such as human balding scalp and beard, contain higher levels of specific, saturable androgen receptors than those derived from androgen-insensitive regions such as non-balding scalp (Randall et al., 1992; Hibberts et al., 1998). This is also reflected in the red deer follicles where androgen receptors have been localised by immunohistochemistry in the dermal papilla cells derived from mane follicles taken during the breeding season, whereas they were not detected in those taken during non-breeding season (Thornton et al., 2001). The gene for the androgen receptor was expressed in cultured human dermal papilla cells taken from axillary and beard hair follicles, whereas it was only present at a low level in cells derived from occipital scalp hair (Ando et al., 1999). This parallels individuals without functional androgen receptors, i.e., with androgen insensitivity syndrome, who exhibit no beard, axillary, and pubic terminal hair, and do not develop androgenetic alopecia (McPhaul, 2004). This indicates that the dermal papilla plays a main role in androgen-mediated effects in the hair follicles.

Further evidence implicating the dermal papilla as an androgen target is that 5α-reductase type II mRNA was expressed in the dermal papilla and the dermal sheath cells from both balding and non-balding scalp follicles (Asada et al., 2001). Based on *in vitro* study, the metabolism of testosterone has also been examined, and varies between dermal papilla cells taken from different body sites. Cultured dermal papilla cells from non-balding scalp, beard, pubic and axillary follicles were incubated with radio-labelled testosterone. All cells and media contained testosterone and androstenedione, but only beard dermal
papilla cells contained the metabolite 5α-dihydrotestosterone (Itami et al., 1990; Thornton et al., 1993; Hamada et al., 1996).

Itami and colleagues (1991; 1994; 1995a) used a co-culture system of dermal papilla cells and outer root sheath cells to investigate the DNA synthesis level. They observed an increase in DNA synthesis measured by ³H-thymidine uptake compared to cells cultured separately. Addition of testosterone stimulated ³H-thymidine uptake in the co-culture system of both beard and axillary dermal papilla cells with outer root sheath cells. However, there was no effect for testosterone on cells cultured alone, or in the co-culture of occipital scalp dermal papilla cells and outer root sheath cells. In addition, the stimulation of ³H-thymidine uptake under the influence of testosterone was antagonised by the addition of anti-androgen, cyproterone acetate. This strongly suggests that the stimulatory effects in the co-culture system of both beard and axillary were mediated via an androgen receptor pathway.

The production of paracrine factors by cultured dermal papilla cells was examined in the presence and absence of androgen (Hibberts and Randall, 1996). Conditioned media produced by cultured dermal papilla cells derived from beard, balding and non-balding scalp were collected. All media promoted DNA synthesis in epithelial cell line of human skin. After incubation with testosterone, media from balding scalp dermal papilla cells had a reduced ability to induce the epithelial cell growth compared to untreated media from non-balding, balding, and beard dermal papilla cells. This may have been due to the production of inhibitory paracrine factors under the influence of testosterone.
Another study used a similar experimental design to examine the production of paracrine and autocrine factors by androgen-insensitive non-balding scalp and androgen-sensitive beard dermal papilla cells (Thornton et al., 1998). The conditioned media by the dermal papilla cells were collected and its effects on \(^3\)H-thymidine incorporation into other dermal papilla cells were investigated. From the study it was found that both non-balding and beard dermal papilla conditioned media have inducible effect for both cell types, but the response to the factor was greater in beard dermal papilla cells. After incubation with testosterone, the production of mitogenic factors was only increased with the conditioned media from beard dermal papilla cells. It was observed that only beard dermal papilla cells were able to respond to these factors. This suggested that both cell types secreted autocrine factors and the testosterone stimulation of autocrine factors production by beard dermal papilla cells \textit{in vitro} reflected the response of these cells to androgens \textit{in vivo}.

Recently, the production of inhibitory autocrine factors by balding dermal papilla cells was investigated (Hamada and Randall, 2006). This study revealed that conditioned medium, produced by dermal papilla cells derived from either non-balding or balding scalp had growth promoting effect on non-balding scalp dermal papilla cells \textit{in vitro}. However, balding medium was found to stimulate to a lesser extent. In addition, only conditioned medium from non-balding scalp stimulated rat vibrissae dermal papilla cells, and no significant stimulation was seen with conditioned medium from balding scalp. This was due to the secretion of soluble inhibitory factors by balding cells and this study also revealed that the conditioned medium from balding dermal papilla cells inhibited mouse hair growth \textit{in vivo}, indicating that this medium contained inhibitory autocrine factors.
1.10 Paracrine factors in the hair follicle:

The development of epithelial appendages demands cell signaling and interaction between epithelial cells, and between epithelia and mesoderm-derived dermis (Sengel, 1983; Chuong et al., 2001). It is believed that cell signals may play a key role in regulation of cell growth, cell differentiation and epithelial appendage formation (McElwee and Hoffmann, 2000). Polypeptide growth factors are a variety of proteins which play important roles in the regulation of cell growth, movement and differentiation, and perform their activities, for example control cell proliferation and differentiation, by binding to specific membrane receptors on the cell membrane to transduce the growth factor signals (Ullrich and Schlessinger, 1990; Olsnes et al., 2003; Carpenter and Liao, 2009)

These proteins can be classified into several main families including; Plasminogen-Related Growth Factors (PRGFs) (Donate et al., 1994; Comoglio and Boccaccio, 2001), Platelet-Derived Growth Factors (PDGFs) (Heldin et al., 1993; Alvarezet al., 2006), Nerve Growth Factors (NGFs) (Ebendal, 1992; Wiesmann and de Vos, 2001), Epidermal Growth Factors (EGFs) (Derynck, 1988; Leahy, 2004), Insulin Growth Factors (IGFs) (Humbel, 1990; Dupont et al., 2003), Fibroblast Growth Factors (FGFs) (Burgess and Maciag, 1989; Goldfarb, 2005), Transforming Growth Factors (TGFs) (Massague, 1990; Kim et al., 2005) and Vascular Endothelial Growth Factor (Kowanetz and Ferrara, 2006).

The identification of paracrine factors produced by dermal papilla cells in androgen dependent follicles has been the focus of much research. A number of growth factors and cytokines have been implicated in the regulation of the hair follicle (Blume-Peytavi and
Mandt, 2000; Paus, 2000; Philpott, 2000). Only some of these have been implicated in androgen action so far, generally by studying cultured dermal papilla cells; the main ones include:

Insulin-like growth factor-I (IGF-I) is a highly potent mitogen which maintains cultured anagen scalp hair follicles at physiological concentrations in vitro (Philpott et al., 1994). Abnormal patterns of hair growth and differentiation have been observed when IGF-I actions were blocked in the IGF-I receptor deficient mouse (Liu et al., 1993). Itami and others (1995b) observed an increase in gene expression of IGF-I when beard dermal papilla cells were cultured with testosterone in vitro. They also reported that androgens increased the proliferation of outer root sheath cells when co-cultured with dermal papilla cells in vitro. This stimulation by androgens was intercepted when IGF-I action was blocked using antibodies.

Transforming growth factor-β1 (TGF-β1) is another possible paracrine factor which inhibits hair growth in vitro and may play a role as a negative regulator of hair follicle growth (Philpott, 2000). In organ culture, it has been found that TGF-β1 inhibits human hair growth in vitro (Philpott et al., 1990). In addition, it was reported that TGF-β1 was expressed in mouse dermal papilla cells using immunohistochemistry (Heine et al., 1987). Inui et al., (2002; 2003) used a co-culture system to investigate TGF-β1 in dermal papilla cells of balding scalp with transfected androgen receptors and keratinocytes. When a synthetic androgen was added to the culture, a significant inhibition was noticed in keratinocyte growth. They also found that TGF-β1 was induced by androgen in cultured balding dermal papilla cells. Furthermore, the inhibition of keratinocyte growth
was reversed using anti-TGF-β1-antibody in a dose-dependent manner. This suggests that androgens stimulate the expression of TGF-β1 in balding dermal papilla cells leading to inhibition of epithelial cell growth. TGF-β1 has also been implicated in the control of catagen. Premature catagen was induced when TGF-β1 was injected into the back skin of mice (Foitzik et al., 2000) and a suppressor of TGF-β1 delayed the progression of catagen in mice (Tsuji et al., 2003). Androgen also stimulated balding cultured dermal papilla cells to produce TGF-β2 (Hibino and Nishiyama, 2004) which is associated with the transition from anagen to catagen in human hair follicles in vitro (Soma et al., 2002). Therefore, androgen stimulation of dermal papilla production of TGF-β could shorten anagen.

Another possible androgen-sensitive paracrine factor is Stem cell factor (SCF), also called c-kit ligand, mast cell growth factor or steel factor. SCF is known to play a main role in development of epidermal (Williams et al., 1992; Grichnik et al., 1998) and hair pigmentation (Geissler et al., 1988; Fleischman et al., 1991). Dermal papilla cells derived from beard, non-balding and balding scalp follicles can produce SCF (Hibberts et al., 1996b; Randall et al., 2008). However, beard cells secrete more SCF than those from non-balding scalp suggesting that androgens may increase the production of SCF by beard dermal papilla cells leading to formation of darker facial hair (Hibberts et al., 1996b). Similarly, cells derived from the paler balding follicles secreted less SCF than normal scalp cells (Randall et al., 2008). The SCF receptor, c-kit, was expressed in adult scalp hair follicle melanocytes (Randall, 2001b, Randall et al., 2008). This suggests that changes in the amount of SCF produced by the dermal papilla cells may alter hair pigmentation by acting on the hair follicle melanocytes.
Vascular endothelial growth factor (VEGF) is another possible paracrine factor which acts as a modulator of angiogenesis and vascular permeability. Anagen terminal hair follicles have a good vascular system, suggesting microvascular angiogenesis may occur at an early stage of every new anagen phase (Montagna and Van Scott, 1958). The gradual changes in the size of androgen-responsive follicles, for instance during puberty, are reflected in the different size of their blood vessels (Montagna and Ellis, 1958). It has been reported that cultured dermal papilla cells express the gene for VEGF (Lachgar et al., 1996; Hibberts et al., 1996a; Merrick, 1999). Cultured dermal papilla cells derived from catagen and telogen follicles of human scalp were found to express low levels of VEGF using in situ hybridization (Lachgar et al., 1996). Recently, it has been found that the levels of VEGF were decreased in patients undergoing androgen deprivation therapy for prostate cancer compared to its levels before treatment indicating an androgen-regulation of VEGF expression (Aslan et al., 2005).

Better understanding of these factors should elucidate androgen action in hair follicles and therefore lead to the development of treatments for androgen dependent hair disorders such as androgenetic alopecia and hirsutism. Members of the hepatocyte growth factor family, hepatocyte growth factor (HGF) and macrophage stimulating protein (MSP), are also potential candidates for paracrine factors produced by dermal papilla cells of human hair follicles.
1.11 HGF & MSP as possible paracrine factors produced by hair follicles:

There are currently two plasminogen-related growth factors (PRGFs), hepatocyte growth factor (HGF), which is also called scatter factor (SF), and macrophage stimulating protein (MSP), which is also known as hepatocyte growth factor-like (HGFL). Structurally, the domain organization of the PRGFs is remarkably similar to that of the blood proteinase plasminogen (Donate et al., 1994; Carafoli et al., 2005) (figure 1.12). HGF and MSP have been implicated in the regulation of hair growth (Lindner et al., 2000; McElwee et al., 2004) and are possible paracrine factors produced by androgen dependent hair follicles under androgen regulation.

1.11.1 Structure of HGF and MSP:

Interestingly, HGF and MSP are structurally related and their overall structure is similar to that of the blood protease, plasminogen. HGF, MSP and plasminogen belong to a family of polypeptides characterised by the presence of at least one kringle domain, a serine protease domain and an activation part. A kringle domain is a triple disulfide-bonded loop structure composed of 80 amino acids, that is thought to play an important role in protein-protein interactions. However, among the members of this family, HGF and MSP lack proteolytic activity and are ligands for transmembrane tyrosine kinases (Waltz et al., 1997).

The secondary structure of HGF and MSP shows a mature heterodimeric form composed of two subunits, alpha and beta, linked together by a disulfide linkage between C487 (α) and C604 (β) (Furlong, 1992). Starting from the amino terminal, the α-chain is a heavy
chain which consists of 440 amino acids and contains a hairpin loop of about 27 amino acids similar to the preactivation peptide of plasminogen (Trusolino et al., 1998) followed by four kringle domains. Each kringle domain possesses its respective three disulfide linkages. The β-chain is a light chain which consists of 234 amino acids and contains an inactive serine protease like structure (Lokker et al., 1992; Ultsch 1998) that includes a triad of glutamine, aspartate and tyrosine in HGF, whereas in MSP these are glutamine and two tyrosines. In contrast, plasminogen contains five kringle domains and includes histidine, aspartate and serine lying within the catalytic site of serine protease (Trusolino et al., 1998). In addition, there is a cleavage site between α and β chains.

Figure 1.12  The structure of HGF, MSP, and plasminogen.
A diagram of the structure of HGF and MSP, compared with plasminogen. reproduced from (Trusolino et al., 1998), with the author's permission. Signal peptide (SP), hairpin loop (HL), Kringle (K), dibasic argnine-valine (R-V), histidine (H), serine (S), glutamine (Q), tyrosine (Y), aspartate (D), and disulphide bond (S-S).
1.11.2 Receptors for hepatocyte growth factor & macrophage stimulating protein:

c-Met or HGFR (hepatocyte growth factor receptor) and RON (Recepteur d'origine Nantaise) are the receptors of HGF and MSP respectively. They belong to a distinct subfamily of receptor tyrosine kinases (RTKs) (Bottaro et al., 1991; Gaudino et al., 1994). These tyrosine kinases play an important role in performing cell functions by transmitting extracellular signals such as growth factors and peptide hormones (Sakamoto et al., 1997; Carpenter and Liao, 2009). They all consist of three main domains including an extracellular ligand binding domain, a single membrane-spanning domain and a cytoplasmic domain (Hanks et al., 1988; Yarden and Ullrich, 1988).

Interestingly, c-Met and RON are structurally related (Forgie et al., 2003) and both receptors have a complex multi-domain architecture (Gherardi et al., 2003). In addition, RON has a significant homology to hepatocyte growth factor receptor c-Met (Bottaro et al., 1991; Ronsin et al., 1993). Both are synthesised as a single-chain precursor and then are cleaved by proteolytic processing to generate the mature disulfide linked α/β heterodimer, consisting of two subunits. The α-chain is an extracellular, glycoprotein whilst the β-chain is a transmembrane protein responsible for the tyrosine kinase activity (Gaudino et al., 1995; Iwama et al., 1995), figure 1.13.

Full activation of c-Met and RON requires the complete ligand α-β heterodimer (Okigaki et al., 1992; Wang et al., 1997). However, the high affinity receptor binding site in HGF is located in the α-chain, whereas MSP binds with a high affinity to its receptor RON via the β-chain (Danilkovitch et al., 1999). Binding of c-Met and RON by their ligands results in tyrosine kinase activity of the cytoplasmic domain and initiates intracellular
signal transduction (Muraoka et al., 1999). Activation of c-Met and RON by binding to their ligands, elicits multiple biological responses, which include cytoskeletal reorganisation, proliferation, cell-polarisation and tubular structure formation in epithelial and endothelial cells (Medico et al., 1996).

The MSP receptor (RON) is mainly found in several tissues such as skin, lungs, central and peripheral nervous systems, digestive tract epithelia and bones (Gaudino et al., 1995; Okino et al., 2003). More recently it has been found in various reproductive tissues including uterus, placenta, testes and epididymis (Hess et al., 2003). Overexpression of the HGF receptor, c-Met, has also been associated with a number of epithelial malignancies (Wilson and McPhaul, 1994; Marshall and Kornberg, 1998) and with a significant rate of prostate adenocarcinoma in men (Inoue et al., 1998).

**Figure 1.13 The structure of c-Met.**
Diagrammatic representation of the structure of hepatocyte growth factor receptor (c-Met). Reprinted by kind permission of the authors from Gentile et al., (2008).
1.11.3 Hepatocyte growth factor (HGF) and hair growth:

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a multifunctional peptide which acts as a mitogen (Sonnenberg et al., 1993), motogen (Stoker et al., 1987; Weidner et al., 1990) and morphogen (Montesano et al., 1991) and has effects on various epithelial and endothelial cells. HGF interacts with responsive cells by binding to the cell surface receptor, c-Met, activating a tyrosine kinase signaling cascade leading to regulation of cell growth, cell proliferation, cell motility, and morphogenesis.

HGF is secreted by mesenchymal cells as a single inactive polypeptide precursor (Naldini et al., 1992) which consists of 728 amino acids (Seki et al., 1990; Weidner et al., 1991). This HGF precursor is cleaved by proteolytic digestion at a specific site called a dibasic site within two amino acids (Arg494-Val495) in the presence of urokinase, which is crucial in morphogenesis, tissue repair, and tumor invasion, or by different proteases, such as HGF activator, to produce the mature active form of HGF which is a disulfide-linked heterodimer composed of a 69 kDa α-chain and a 34 kDa β-chain (figure 1.14) (Lokker et al., 1992; Birchmeier and Gherardi, 1998). The molecular mass (Mr) of the mature form of human HGF and HGF precursor are 76,879 and 83,126 respectively (Nakamura et al., 1989).

Hepatocyte growth factor may act as a paracrine factor secreted by mesenchymal-derived cells in a variety of organs and acts primarily upon epithelial cells and endothelial cells (Rubin et al., 1991; Matsumoto and Nakamura, 1992; Sulpice et al., 2009). HGF also plays an important role in embryonic organ development and in adult organ regeneration (Stern et al., 1990), for example in lung formation and repair (Ohmichi et al., 1998;
Mizuno et al., 2005; Lassus et al., 2006), the early development of the kidney and kidney repair (Woolf et al., 1995; Van Adelsberg et al., 2001; Baer and Geiger, 2006), and angiogenesis and vascular repair (Montesano et al., 1991; Rosen et al., 1991; McKinnon et al., 2006). HGF may interact with other paracrine factors. Based on in vitro studies, basic fibroblast growth factor (bFGF), which plays a role in embryonic development and wound repair, has been found to stimulate HGF secretion in many of mesenchymal-derived cell lines in human beings (Roletto et al., 1996).

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**Figure 1.14 HGF structure.**
HGF has been implicated in hair growth control. HGF has been observed to stimulate growth of human scalp hair follicles (Jindo et al., 1995) as well as mouse vibrissae (Jindo et al., 1994) in organ culture. Human recombinant HGF can also stimulate the growth of mouse pelage hair follicles in vivo and retards murine hair follicles regression into catagen (Jindo et al., 1998). When recombinant mouse HGF was injected under the back skin of mice, the result showed that HGF significantly delayed catagen development during both early and late stages of hair follicle regression in vivo (Lindner et al., 2000). The gene for HGF was strongly expressed in rat anagen tissue and slightly in telogen tissue, and HGF mRNA was detected in rat cultured dermal papilla cells, using RT-PCR (Yamazaki et al., 1999). HGF has also been observed to localise to the dermal papilla cells of mouse pelage anagen follicles using immunohistoreactivity (Lindner et al., 2000). These results suggest that HGF can regulate rodent hair follicles and is produced by their dermal papilla.

Another study showed that HGF can increase DNA synthesis in cultured keratinocytes derived from human scalp hair bulb in a dose-dependent manner in vitro (Shimaoka et al., 1995). In addition, HGF stimulated the proliferation of rat vibrissae dermal papilla cells with maximal effect at 50 ng/ml in a concentration-dependent manner, whereas there was minimal effect on cultured fibroblasts (Yu et al., 2004). Cultured dermal papilla cells of human scalp hair follicles were found to express HGF, which has stimulatory effects on human scalp hair follicle growth in vitro (Shimaoka et al., 1995). No effects were noticed in HGF gene expression when balding, non-balding scalp and beard cultured dermal papilla cells were cultured with testosterone (Merrick, 2000). However, HGF gene expression was very low in balding scalp cells whereas beard cells showed a greater
expression than non-balding scalp cells. This differential expression suggests that HGF may play a role in maintaining large follicles and the levels of HGF in androgen dependent follicles may be changed by in vivo androgen exposure (Randall et al., 2001). The gene for the HGF receptor, c-Met, was expressed in human scalp and rat vibrissae cultured dermal papilla cells as observed by the RT-PCR technique, and it was also found in rat vibrissae follicular matrix keratinocytes by in situ hybridization (Yu et al., 2004). c-Met was also prominently localised in the inner root sheath and outer root sheath in mouse pelage anagen follicles using immunohistochemistry (Lindner et al., 2000).

1.11.4 Macrophage stimulating protein (MSP) and hair growth:

Another member of hepatocyte growth factor family recently implicated in hair follicle growth is macrophage stimulating protein (MSP), also called scatter factor 2 (SF2) and hepatocyte growth factor-like protein (HGFL). MSP is a soluble cytokine that belongs to plasminogen-related growth factor family (PRGFs) (Stella et al., 2001) (see section 1.11); it was initially considered as a serum factor which stimulated chemotaxis of peritoneal resident macrophages (Brunelleschi et al., 2001).

MSP is released as a single-chain inactive protein called MSP precursor or pro-MSP which is cleaved into biologically active MSP after proteolytic digestion to give a disulfide-linked heterodimer in the presence of proteases (Bezerra et al., 1998; Wang et al., 1996a; Danilkovitch-Miagkova and Leonard, 2001). The mature form of MSP is a heterodimer protein (78 kDa) consisting of two chains: large α and small β held together by a single disulfide bond (Carafoli et al., 2005).
MSP exerts its actions on target cells by binding to a cell surface receptor called RON (Recepture d'origin Nantaise) in human beings (Camp et al., 2007), also known as macrophage stimulating 1 receptor (MST1R). In mice, the receptor is named STK (Stem cell derived tyrosine kinase) (Ronsin et al., 1993; Wang et al., 1994). RON belongs to the Met proto-oncogene family, a distinct subfamily of the receptor tyrosine kinase family (Wang et al., 2003).

Several functions for MSP have been determined, including the ability to act as an inflammatory cytokine to activate macrophages (Skeel et al., 1991), promote bone resorption (Kurihara et al., 1996; 1998), induce cell motility (Santoro et al., 1996), promote cellular proliferation (Gandino et al., 1994), stimulate liver cell morphogenesis and motogenesis (Medico et al., 1996), promote the growth and motility of keratinocytes (Wang et al., 1996b), stimulate maturation of megakaryocytes (Banu et al., 1996), and prevent cellular apoptosis (Iwama et al., 1996) of epithelial cells separated from the extracellular matrix (Danilkovitch-Miagkova and Leonard, 2001) Based on these studies, MSP has been proposed as participating in a wide range of biological process, such as inflammation, wound healing, bone formation and hematopoiesis.

Both MSP and HGF belong to the same family, the hepatocyte growth factor family. As discussed in section 1.11.3, HGF may act as a powerful modulator of hair growth and be involved in morphogenesis, and cycling. More recent studies investigated the effect of MSP on hair growth due to the close relationship between MSP and HGF. In an in vivo study, MSP was incubated with agarose beads, then injected subcutaneously into mice, which induced telogen follicles to enter the anagen growth phase (McElwee et al., 2004).
In addition, exposure to different concentrations of MSP \textit{in vitro}, gave an increase in human hair follicle length in organ culture. Using immunohistochemistry, the MSP receptor, RON, was localised in human hair follicles. This localisation was with higher intensity in the outer and inner root sheath, and hair matrix, whereas it was found to a lesser extent in the dermal papilla (McElwee et al., 2004). Therefore MSP may also play a role as a paracrine modulator of hair growth.

1.12 Aims and experimental design:

Androgens are an important regulator of human hair growth. Human hair follicles in various sites of the body have a variable individual, inherited susceptibility for androgen dependent growth. Androgens regulate the type of hair produced in human follicles, which can cause psychological distress in cases of excess large hairs, e.g. in hirsutism, or reduced visible hairs, e.g. in androgenetic alopecia (see section 1.7). These disorders are poorly controlled. Better understanding of the mechanism of androgen action and the structure, functions and growth of hair follicles should lead to better treatment. Changes in hair follicle and hair size are believed to involve signalling between the hair follicle components via paracrine factors.

The overall aim of this study was to identify key paracrine factors which may be involved in androgen regulated changes in the hair follicles to improve our understanding of the mechanism of androgen action in human hair follicles. In addition, this study aimed to investigate differences in gene expression between balding and non-balding scalp hair follicles. Previous research on paracrine factors in human hair follicles has focussed on cultured dermal papilla cells whose gene expression is altered from that \textit{in vivo} as soon as
the cells are established (O’Shaughnessy et al., 2004). It has also been suggested that balding dermal papilla cells are degenerate and therefore not really a valid model system (Bahta et al., 2007). Therefore, the aim of this study was to determine gene expression of paracrine factors which may be involved in androgen regulated signalling in individual human hair follicles themselves after dissection from human normal and balding scalp.

Since hepatocyte growth factor (HGF) family members, HGF and macrophage stimulating protein (MSP), are implicated in hair growth (see sections 1.11.3 and 1.11.4), they seem possible candidates for roles as androgen-regulated paracrine factors. HGF has been shown as a modulator of hair growth which plays an important role in morphogenesis and cycling in rodent follicles (Jindo et al., 1995; Lindner et al., 2000). It has also been reported that MSP has a stimulatory effect on hair growth in vitro in organ culture and induced telogen hair follicles in mice to enter the anagen growth phase in vivo (McElwee et al., 2004). This study was designed to investigate whether, and where, these genes were expressed in human hair follicles and whether their expression was altered in balding.

Initial experiments focused on investigating the gene expression of these two members of the hepatocyte growth factor family and their respective receptors using reverse transcription polymerase chain reaction (RT-PCR). For these purposes, firstly deer and human hair follicles were individually microdissected. Total RNA, mRNA and cDNA were prepared from them and used to check if RNA for specific genes including hepatocyte growth factor (HGF), macrophage stimulating protein (MSP) and their respective receptors, c-Met and RON, were expressed by human hair follicles. β-actin
was used as a positive control to assess the quality of cDNA samples (Davies, 2001; Croft, 2002). The detection of this housekeeping gene indicated that the isolated RNA is of sufficient quality for RT-PCR to be performed successfully. The products of the RT-PCR were confirmed as appropriate by agarose gel electrophoresis and sequencing.

Deer hair follicles were microdissected at the beginning of the work to get practical experience to learn these techniques. This is because they are larger than human follicles and easy to practise on, dissect and section. Deer hair growth is also synchronous, which means that in any given area the hair growth would be at the same hair cycle stage. This was followed by investigation of human scalp anagen follicles.

In an attempt to localize the expression of each of the genes in human scalp follicles, the three main components of the hair bulb: the epithelial hair matrix, the dermal sheath and the regulatory dermal papilla, were also investigated separately by isolating them individually by microdissection. Further investigation was done using monoclonal antibodies to determine the location of the HGF receptor, c-Met, protein in human hair follicles by immunohistochemistry of frozen skin sections. Prior to that, the expression of cytokeratin 6 was investigated to gain practical experience with the immunohistochemistry technique.

To investigate what changes androgens may cause to gene expression in human hair follicles, the work focused on comparing the gene expression in matched non-balding and balding scalp hair follicles from the same men with androgenetic alopecia to determine whether there are significant differences between them using an advanced, powerful
comparative method, DNA microarray analysis. Complex data analysis was used to compare expression in these matched sets of follicles.

To confirm these differences for specific genes including HGF, MSP, c-Met, and RON in balding scalp hair follicles relative to the same sequence in matched non-balding samples from the same individuals, relative quantitative real-time PCR was used to calculate relative quantities of nucleic acid sequences of targets. In quantitative real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target gene is first detected rather than by the amount of target accumulate at the end of PCR (Freeman et al., 1999; Raeymaekers, 2000). Therefore, data are collected throughout the PCR process rather than at the end of the PCR process. This gives reliable quantitative assessment. Due to the limited sample availability and the lower yield of total RNA, the extracted total RNA from all six samples was amplified to produce high-quality RNA for use in quantitative real-time PCR. Hopefully, this research should give us a better understanding of the mechanism of androgen action in human hair follicles which could lead to better treatment for hair disorders.
2- Materials & Methods
2. Materials and Methods

2.1 Identification of hepatocyte growth factor family members and their receptors in isolated human scalp hair follicles and their components:

2.1.1 The Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

2.1.1.1 Biological materials:

Human skin samples from non-balding individuals were obtained from healthy donors as waste products of cosmetic dermatological surgeries, with full written donor consent. All operations took place in private clinics under the University of Bradford Ethical Committee approval for such procedures for all research in Division of Biomedical Sciences/Centre for Skin Sciences. Tissues were collected into 50ml Falcon tubes containing Rnalater™ (Sigma-Aldrich Ltd.) to inhibit RNases. The tubes were labeled with details of date, sample origin, gender and age and then the tubes were placed on ice for the period of transportation. Upon arrival at the university, the samples were stored in the fridge at 4°C overnight to allow the Rnalater™ to penetrate the tissues. The next day, the tissues were transferred to the freezer and stored at -20°C until required.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Gender</th>
<th>Age</th>
<th>Status</th>
<th>Body region</th>
<th>No. of follicles dissected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>male</td>
<td>30</td>
<td>non-balding</td>
<td>Occipital scalp</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>male</td>
<td>29</td>
<td>non-balding</td>
<td>Occipital scalp</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>male</td>
<td>37</td>
<td>non-balding</td>
<td>Occipital scalp</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>female</td>
<td>37</td>
<td>non-balding</td>
<td>Occipital scalp</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>female</td>
<td>32</td>
<td>non-balding</td>
<td>Occipital scalp</td>
<td>60</td>
</tr>
</tbody>
</table>
2.1.1.2 Microdissections and tissues preparation:

2.1.1.2.1 Microdissections of whole anagen follicles:

The Leica MZ8 dissecting microscope (Leitz, Germany) was used for the dissection of the hair follicles from the skin samples. All the plastic, glass and dissection tools were sterile. All work areas were cleaned using 70% alcohol to prevent any contamination. The tissues were stabilised using RNA stabilization reagent, RNaLater™. If frozen, the skin samples were thawed on ice approximately at 4°C then transferred to a petri dish containing RNaLater™ that was previously cooled in ice to ensure equal temperature to the tissues. The skin sample was cut at the junction between the epidermis and dermis using a sterile scalpel blade and the dermal layer transferred into a petri dish (60mm x 15mm) containing RNaLater™. Under the dissecting microscope, the hair follicles were plucked from the skin gently using fine forceps and then transferred into another petri dish containing fresh RNaLater™ kept on ice. The isolated hair follicles were cleaned of any dermis or fat debris under a higher magnification using syringe needles (27G1/2 tuberculin syringe; Sigma). The clean follicles were transferred to fresh RNaLater™ placed on ice. A total of 60 follicles were collected from each sample. Photographs of the dissection steps are displayed in figure 2.1. Photographs were taken using the MDG17 light microscope (Wild Heerbrugg, Switzerland).

Table 2.2 Samples used for isolation of individual hair bulb components; the dermal sheath, matrix and dermal papilla (three healthy donors):

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Gender</th>
<th>Age</th>
<th>Status</th>
<th>Body region</th>
<th>No. of follicles dissected</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>male</td>
<td>35</td>
<td>non-balding</td>
<td>Occipital scalp</td>
<td>150</td>
</tr>
<tr>
<td>7</td>
<td>male</td>
<td>29</td>
<td>non-balding</td>
<td>Occipital scalp</td>
<td>150</td>
</tr>
<tr>
<td>8</td>
<td>female</td>
<td>45</td>
<td>non-balding</td>
<td>Occipital scalp</td>
<td>150</td>
</tr>
</tbody>
</table>
Figure 2.1 Microdissection steps of human hair follicle.

The human skin sample (I), scale bar (500 µm), was cut at the junction between the epidermis and dermis using a sterile scalpel blade (II), scale bar (496 µm). The hair follicles were plucked from the skin gently using fine forceps but still have some of the dermis and subcutaneous fat attached (III), scale bar (264 µm). The isolated hair follicles were cleaned of any dermis or fat debris under a higher magnification using syringe needles (IV), scale bar (275 µm). Cleaned isolated follicles were transferred to fresh RNAlater™ and placed on ice prior to total RNA extraction. Photographs were taken using the MDG17 light microscope (Wild Heerbrugg). Magnification: (x20, x18, x259, x266, respectively).
2.1.1.2.2 Microdissections of hair bulb components:

For further investigation of the expression of specific genes including HGF, MSP, c-Met and RON, to try to localise them within human hair follicles, whole cleaned lower human anagen hair follicles were dissected to isolate the dermal sheath, matrix and dermal papilla. A total of 150 hair follicles were isolated from each sample. Whole lower follicles were dissected in RNASAFE™ placed on ice as described above. Under the dissecting microscope Leica MZ8 (Leica Germany), each clean follicle was cut above the bulb using needles (27G1/2 tuberculin syringe; Sigma) to separate the bulb from the rest of the follicle. The bulb then was transferred to another petri dish (60mm x 15mm) containing fresh RNASAFE™ and the upper part of the follicles were discarded. Holding the root sheath with one needle inside, a vertical cut was made towards the side of the dermal sheath avoiding the dermal papilla. The dermal sheath was then inverted to expose the bulb matrix and the dermal papilla which was still attached to the sheath. The dermal papilla was then cut across its base to detach it from the sheath. The clean isolated dermal sheath, matrix and dermal papilla were transferred to their respective Eppendorf tubes containing 100 µl RNASAFE™. Photographs of the dissection steps are displayed in figure 2.2. Photographs were taken using the MDG17 light microscope (Wild Heerbrugg).
Figure 2.2 Microdissection steps of human hair bulb components.

Whole follicles were dissected from scalp skin as described in figure 2.1. Each cleaned follicle (I), scale bar (275 µm), was cut above the bulb using needles to separate the bulb from the rest of the follicle (II), scale bar (281 µm). The bulb was transferred to another petri dish containing fresh RNAlater™ and the upper part of the follicle was discarded (III), scale bar (189 µm). Holding the root sheath with one needle inside, a vertical cut was made towards the side of the dermal sheath avoiding the dermal papilla (IV), scale bar (195 µm). The dermal sheath was then inverted to expose the matrix and the dermal papilla which was still attached to the sheath (V). The dermal papilla was then cut across its base to detach it from the sheath. The clean isolated dermal sheath (VI) matrix (VII) and dermal papilla (VIII) were transferred to their respective collection tubes containing RNAlater™. Photographs were taken using the MDG17 light microscope (Wild Heerbrugg). Magnification: (x266, x268, x288, x284, x284, x284, x286, x300, respectively).
2.1.1.3 Total RNA isolation for RT-PCR:

The isolated hair follicles were placed in a glass homogeniser (3 ml) containing lysis solution which consisted of 5 µl of 2-mercaptoethanol (14 mM) with 500 µl of lysis solution (Total RNA isolation kit, Sigma-Aldrich). The lysis solution contained guanidine thiocyanate to inactivate endogenous RNases which may have been introduced during the procedure. The follicles were homogenised until there were no visible solid remains.

Total RNA extraction was carried out using the GenElute Mammalian Total RNA kit (Sigma, UK). The homogenate was then transferred to a GenElute filtration column and centrifuged for 2 minutes at 13000 g (Eppendorf 5415 R) to remove any cellular debris and shear the DNA. After centrifugation, the column was discarded and an equal amount of 70% alcohol was added to the filtrate. The sample was then mixed thoroughly by vortexing.

The filtrate/alcohol mixture (700 µl) was placed in the binding column and then centrifuged for 15 seconds at 13000 g. Following centrifugation, the flow-through was discarded and the RNA was bound to the column. The binding column was then transferred to a new collection tube.

Wash solution 1 (500 µl) was applied to the binding column and then centrifuged for 15 seconds. The binding column was placed in a new collection tube. After that, 500 µl of wash solution 2 was added to the column and centrifuged for 15 seconds at 13000 g. The flow-through was discarded. Another 500 µl of wash solution 2 was added again to the
binding column and centrifuged for 2 minutes to remove the ethanol. The purpose of this washing was to remove any contaminants.

The purified RNA was eluted from the binding column by adding 50 µl of Elution solution (10 mM Tris-HCl, pH 7.4) to the centre of the binding column. The column was centrifuged for one minute at 13000 g. The column was discarded and the flow-through which contained purified RNA was used immediately or stored at -80°C until required.

2.1.1.3.1 Checking the quality of total RNA:

2.1.1.3.1.1 Agarose gel preparation:

The quality of total RNA was checked by agarose gel electrophoresis using 1.5% (w/v) agarose gel prepared using 1.5 g of agarose powder (Invitrogen Ltd, Paisley, UK) dissolved in 100 ml of 1X TAE (Tris Acetate EDTA) buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.3). The mixture was dissolved with the aid of the microwave (Proline Microchef ST44). The mixture was cooled to around 50°C then 25 µl of ethidium bromide (1 mg/ml; Sigma Aldrich Ltd) was added to the mixture. The gel was directly poured into a plastic gel tray and gel comb to form wells. The gel was left around 40 minutes to set and form wells before the gel comb was gently removed.
2.1.1.3.1.2 Agarose gel electrophoresis:

The running buffer was prepared by adding 75 µl of 0.5 µl/ml ethidium bromide to 300ml 1X TAE buffer (40 mM Tri-Acetate, 1 mM EDTA, pH 38.3). The gel tray containing the prepared gel was placed in the electrophoresis tank and the running buffer was poured into the tank. Total RNA (10 µl) was mixed with 2 µl of blue/orange 6X loading dye (Promega, Ltd, Southampton, UK). This mixture was loaded on to the gel. The purpose of using the dye was to assist loading and monitor the resolution of the sample. The tank was set to run at 100 V for 35-45 minutes and then total RNA was visualised using the Uvitec gel documentation system (Uvitec Limited, Cambridge, UK) at 312 nm wavelength and the image captured.

2.1.1.4 Poly (A) RNA isolation:

The process of poly (A) RNA isolation was carried out by using GenElute mRNA Miniprep Kit (Sigma-Aldrich Ltd). The isolated total RNA was brought up to 250 µl with RNase-free water and then vortexed thoroughly. The 2X binding solution (250 µl) and oligo (dT) beads (15 µl) were added to the mixture and mixed thoroughly before incubating for 30 minutes at 70°C for RNA denaturation. The mixture was left at room temperature for 10 minutes before centrifugation to pellet the beads: poly (A) RNA complex for 2 minutes at 13000 rpm. Following centrifugation, the supernatant was removed.

Wash solution (500 µl) was added to resuspend the pellet and then transferred to a spin filter-collection tube. The suspension was centrifuged for 2 minutes at 13000 rpm. The
flow-through was discarded and 500 µl of wash solution was added again for a second wash and then centrifuged for 2 minutes.

The spin filter column was placed into a new collection tube and 50 µl Elution solution (10 mM Tris-HCl, pH 7.4, preheated to 70°C) was added to the filter column and incubated for 5 minutes at 70°C. The sample was centrifuged for 1 minute at 13000 rpm. The elution steps mentioned above were repeated by adding a further 50 µl Elution solution. The mRNA present in elute was either used immediately or stored in the freezer at -80°C.

2.1.1.5 DNase treatment of poly (A) RNA samples:

In order to ensure that the RNA samples to be used for cDNA synthesis were free of any contaminating DNA, the sample was treated with the DNA amplification Grade I Kit (Invitrogen Ltd). A reaction mixture was prepared inside a 0.5 ml RNase free eppendorf tube. The mixture consisted of 8 µl of mRNA, 1 µl of 10X DNase I reaction buffer (200 mM Tris-HCl, 25 mM MgCl₂, 50 mM KCl) and 1 µl of DNase I amplification Grade (1 U/µl, containing enzyme oligodeoxyribonuclease I which digests single and double stranded DNA and oligodeoxyribonucleotides containing 5' phosphatase). The reaction mix was vortexed thoroughly and then incubated for 15 minutes at room temperature. EDTA (1 µl of 25 mM) was added to the mixture to inhibit the DNA digestion and then the mixture was incubated for 10 minutes at 65°C. The DNase-treated poly (A) RNA sample was then added directly to a reverse transcription reaction, or stored in the freezer at -80°C until required.
2.1.1.6 **cDNA synthesis by reverse transcription:**

cDNA synthesis was carried out by using an Avian Myeloblastosis Virus (AMV) reverse transcription system (Promega, Southampton, UK) to produce single strand cDNA from DNase-treated poly (A) RNA. All reagents were defrosted, mixed and kept on ice during preparation. The mixture was done in 0.5 ml eppendorf tube containing 2 µl of reverse transcription 10X buffer (25 mM Tris-HCl, pH 8.3, 25 mM KCl, 25 mM spermidine, 50 mM DTT), 4µl magnesium chloride (25 mM), 1 µl of oligo (dT) primer (0.5 µg/µl), 2 µl deoxynucleotide triphosphate (dNTP mix 10 mM: dATP, dTTP, dGTP, dCTP), 0.5 µl of recombinant RNase® ribonuclease inhibitor (1 U/µl), 0.8 µl of AMV reverse transcriptase (15 U/µl), and 10 µl poly (A) RNA sample.

The mixture was incubated in a PCR machine-sprint thermal cycler (Thermo Hybrid, Ashford, UK) and set to run the reverse transcription programme which is 1 hour at 42°C to allow cDNA synthesis from poly (A) RNA by reverse transcription, 5 minutes at 99°C to inhibit the reverse transcriptase, followed by 5 minutes at 4°C for cooling. The sample was then vortexed and centrifuged for a few seconds to collect the cDNA at the base of the tube. The cDNA was then kept on ice for immediate use or aliquoted (10 µl) into tubes to be stored at -20°C.

2.1.1.7 **Polymerase Chain Reaction (PCR):**

PCR components were prepared in 0.5ml eppendorf tubes. The reaction mixture consists of: 1µl of dNTP mix (dATP, dCTP, dGTP and dTTP, 10mM; Promega), 1.5-2.5 mM MgCl₂ depending upon primer set (50 mM; Invitrogen Ltd), 5µl of 10X PCR buffer (20
mM Tris-HCl, pH 8.4, 50 mM KCl; Invitrogen), 1-2.5 µl of forward and reverse target primers (10 µM), 3-10 µl of cDNA and 0.5 µl of Taq DNA polymerase (5 units/µl; Invitrogen Ltd). The volume of the mixture was topped up to 50 µl with nuclease free water and then vortexed thoroughly. For each PCR reaction, a negative control was set up replacing the cDNA with nuclease free water. To prevent evaporation of the reaction mixture, one drop of mineral oil (Sigma-Alrich Ltd) was added on the top of the mixture. The tubes were transferred to the PCR thermal cycler.

RT-PCR was performed using specific forward and reverse primers for each cDNA target sequence. Each target primer set during PCR required optimisation of the annealing temperature and MgCl₂ (Magnesium chloride) concentration. Each different gene required a specific PCR programme for amplification. The RT-PCR products were analysed by agarose gel electrophoresis as described before (section 2.1.1.3.1.2). The primer sequences used for RT-PCR had been previously optimised and published. The primer sequences, their optimised RT-PCR conditions and expected amplicon size are detailed in table 2.3.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Optimal thermocycling Conditions</th>
<th>Conc. of MgCl2</th>
<th>Expected amplicon Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-actin</td>
<td>F: 5’ ATCTGGCACCAACCTTTACTAAAGCTG 3’&lt;br&gt;R: 5’CGTCATACTCTGTGCTGGATCCACATCTG 3’</td>
<td>95°C for 5 min&lt;br&gt;95°C for 1 min&lt;br&gt;56°C for 1 min&lt;br&gt;72°C for 1 min&lt;br&gt;72°C for 11 min&lt;br&gt;4°C hold&lt;br&gt;35 cycles</td>
<td>2.5 mM</td>
<td>838 bp</td>
<td>Davies et al., 2005</td>
</tr>
<tr>
<td>HGF</td>
<td>F: 5’TTCAACCAATCCAGAGGTACGC 3’&lt;br&gt;R: 5’GAGGGTGATTAGCAGGACCAG 3’</td>
<td>94°C for 5 min&lt;br&gt;94°C for 1 min&lt;br&gt;63°C for 1 min&lt;br&gt;72°C for 1 min&lt;br&gt;72°C for 2 min&lt;br&gt;4°C hold&lt;br&gt;45 cycles</td>
<td>2.5 mM</td>
<td>261 bp</td>
<td>Shimaoka et al., 1995</td>
</tr>
<tr>
<td>MSP</td>
<td>F: 5’AGGAGGATGTCGCAGATGC 3’&lt;br&gt;R: 5’GATTTGATGCCAGCTCT 3’</td>
<td>95°C for 5 min&lt;br&gt;95°C for 1 min&lt;br&gt;62°C for 1 min&lt;br&gt;72°C for 1 min&lt;br&gt;72°C for 11 min&lt;br&gt;4°C hold&lt;br&gt;35 cycles</td>
<td>1.5 mM</td>
<td>415 bp</td>
<td>Shorter, 2007</td>
</tr>
<tr>
<td>RON</td>
<td>F: 5’TCAACCCACATCCACTCTCTG 3’&lt;br&gt;R: 5’AGTGAACGCCAGGTCCTCT 3’</td>
<td>95°C for 5 min&lt;br&gt;95°C for 1 min&lt;br&gt;56°C for 1 min&lt;br&gt;72°C for 1 min&lt;br&gt;72°C for 11 min&lt;br&gt;4°C hold&lt;br&gt;35 cycles</td>
<td>2.5 mM</td>
<td>217 bp</td>
<td>Matsuzaki et al., 2005</td>
</tr>
<tr>
<td>c-Met</td>
<td>F: 5’ACTGCCCTGAAAACCAAGCC 3’&lt;br&gt;R: 5’GGCTTACACTCGCAGCTTAC 3’</td>
<td>94°C for 1 min&lt;br&gt;94°C for 1 min&lt;br&gt;60°C for 1 min&lt;br&gt;72°C for 1 min&lt;br&gt;72°C for 1 min&lt;br&gt;4°C hold&lt;br&gt;30 cycles</td>
<td>2.5 mM</td>
<td>536 bp</td>
<td>Imaizumi et al., 2003</td>
</tr>
</tbody>
</table>

**Table 2.3** Primer sequences and PCR conditions for each specific gene used for RT-PCR
2.1.1.8 DNA sequencing of PCR products:

After analysis of PCR products by electrophoresis, the identity of DNA fragments were verified and confirmed by sequencing. The PCR products were separated by using a low melting point agarose gel to allow the use of a low temperature for dissolving the gel and to facilitate the isolation of DNA fragments from the gel. The DNA fragments were extracted from the gel using a MinElute Gel Extraction Kit (Qiagen, Crawly, UK). The DNA fragments were excised from the agarose gel by using a clean and sharp scalpel blade. The gel slices were transferred to a pre-weighed 1.5 ml colorless eppendorf tube. Three volumes of buffer QG (contained guanidine thiocyanate) were added to one volume of gel. The tube was incubated for 10 minutes at 50°C or until the gel slices were completely dissolved. During the incubation, the gel was vortexed every 2-3 minutes to help dissolving.

One volume of isopropanol was then added to the sample and mixed thoroughly. The sample was applied to a MinElute column in a 2 ml collection tube and then centrifuged for 1 minute to allow DNA binding to the column. The flow-through was discarded and the column was placed again into the same collection tube. An additional amount of 500 µl of buffer QG was added to the column and centrifuged for 1 minute. The flow-through was discarded and the column returned back to the collection tube.

Buffer PE (700 µl, composition not available due to trade secret) was added to the column for washing with incubation for 2-5 minutes at room temperature before being centrifuged for 1 minute. The flow-through was discarded and an additional centrifugation was done for 1 minute to remove residual ethanol from buffer PE. The
column was then transferred to a new 1.5ml eppendorf tube. Buffer EB (10 µl of 10 mM Tris-HCl, pH 8.5) was added to the centre of the column and then incubated at room temperature for 1 minute. The sample was then centrifuged to elute the DNA from the column.

The purified sample of PCR product was sent to Complement Genomics (Sunderland, UK) for sequencing. The sequencing data were compared to the previously identified gene sequences using the NCBI BLAST programme available at the website: (http://www.ncbi.nlm.nih.gov/blastlb12seq/wblast2.cgi). The chromatogram of the sequencing data was produced using the Chromas Lite software (version 2.0) available from http://www.technelysium.com.au/. Any non-matching nucleotides between the sequenced and known data were analysed using the chromatogram. Each base is allocated a different colour, in the chromatogram, and the sequence is determined by the highest peak.
2.1.2 Histological examination of hair follicle structure and localization of the HGF receptor by immunohistochemistry:

2.1.2.1 Tissue samples:

2.1.2.1.1 Human skin samples:

Samples of full depth human scalp skin were obtained from non-balding healthy donors as a waste from routine surgery procedures, with full consent and Ethical Committee approval. The tissue samples were transferred into collection medium RPMI (Gibco, Paisly, UK). Samples were kept on ice and transported to the University. The samples were cleaned, trimmed of excess fat and dissected into small pieces approximately 1 cm² each and then washed in phosphate buffer saline (PBS). Each piece was transferred into 1.5 ml eppendorf tube containing O.C.T (Raymond Lamb Ltd, Sussex, UK) and then placed in the freezer at -20°C until used.

2.1.2.1.2 Deer skin samples:

Deer skin samples were gathered from the neck-mane region of healthy animals routinely killed for food in Yorkshire. The samples were collected after animal death and dissected into strips. The tissue were placed into 50 ml falcon tubes containing collection medium which contained 500 ml RPMI 1640, 100 U/ml penicillin, 2 mM L-glutamine, 5X amphotericin, 100 µg/ml streptomycin (Gibco). The samples were kept on ice during the period of transportation. At the University, the samples were washed in sterile phosphate buffered saline (PBS), cleaned, trimmed of excess fat and cell debris, shaved, dissected into approximately 1 cm² pieces and frozen in O.C.T at -20°C until required.
2.1.2.2 Preparation of slides:

Prior to tissue sectioning, glass slides (76 x 26 x1 mm; BDH, Lutterworth, UK) were cleaned and coated with poly-l-lysine (Sigma Aldrich, Ltd) to assist and ensure adherence of the cut sections. The slides were transferred and placed individually into a plastic slide holder, and cleaned by immersing in Pyroneg (Diversey, Lever Ltd, Northampton, UK) and distilled water for approximately 30 minutes. To remove the detergent, the slides were rinsed in distilled water and then soaked for 5 minutes in absolute alcohol, and dried in the drying cupboard. The slides were allowed to dry at room temperature. When dry, the slides were placed in 10% (v/v) poly-l-lysine in distilled water for approximately 10 minutes. When dry, the slides were placed in the original box and stored at room temperature until use. These slides were used for general histology and immunohistochemistry.

2.1.2.3 Preparation of frozen tissue sections:

Using a cryostat (Leica CM 1800 cryostat, Germany), sections of the skin samples were prepared at -27°C for general histology and immunohistochemistry. The skin samples were placed in the cryostat. O.C.T was applied to the metal holder to form an even disc and placed in the cryostat to freeze solid, ensuring all bubbles were removed. The frozen O.C.T disc was then placed into the sample holder of the cryostat and sliced until even. The skin sample was dissected and placed onto the O.C.T disc orientated so that longitudinal follicles could be sectioned. The sample was orientated to its side to ensure the blade concurrently cuts through the dermis and fat. O.C.T was applied to the sample until no longer visible, and allowed to freeze solid. Sections (5µm diameter) were cut and
mounted on to labelled poly-l-lysine coated slides and placed into a labeled slide holder wrapped in foil, at -20°C until histological analysis.

2.1.2.4 Investigation of hair follicle structure:

2.1.2.4.1 Haematoxylin & eosin staining:

Sections of human and deer skins were treated with haematoxylin and eosin staining to clearly visualise the normal histology of different parts of hair follicle. Prior to tissue staining, frozen slides were allowed to dry at room temperature for 45-60 minutes. The slides were fixed in ice cold acetone (Fisher Scientific Loughborough, UK) for approximately 10 minutes to fix the proteins. The sections were then rinsed in PBS three time for 3 minutes each. The slides were then flooded with haematoxylin for 2-5 minutes depending on how the stain seemed to penetrate the tissue and then rinsed in distilled water until excess haematoxylin was removed. The slides were then placed in Scott's tap water and allowed to blue for 2 minutes, followed by 5 minutes staining in 1% (w/v) eosin. The slides were rinsed in distilled water to remove excess eosin. The sections were then dehydrated by immersion in ascending concentrations of ethanol 50%, 70%, 95% for 1 minute in each. Coverslips (VWR international, Leicester, UK) were then mounted using histomount (VWR international).

2.1.2.4.2 Sacpic staining:

Frozen sections of human and deer skin were stained with the Sacpic staining technique (Nixon, 1993; Nutbrown and Randall, 1996) in order to better differentiate the different components of hair follicle as an aid to interpretation of immunohistochemical staining. Different solutions were prepared prior to staining which include Celestine blue, safranin,
picric acid, ethanol, picro-indigo carmine, Scott's tap water and Gill's haematoxylin (Appendix I).

The slides were removed from -20°C and allowed to dry at room temperature for 45-60 minutes. To fix the proteins, the sections were placed into ice-cold acetone (Fisher Scientific) for 15 minutes. The sections were placed in celestine blue to mordant for 5 minutes before rising in tap water. They were then flooded with Gill's haematoxylin for 5 minutes, followed by a rinse in distilled water for 5 minutes. The sections were then blued by washing in Scott's tap water for 2 minutes, followed by staining in safranin for 5 minutes. The sections were then dehydrated by rinsing in 70% ethanol and 95% ethanol for 1 minute each and differentiated in the picric acid/ethanol for 3 minutes. The slides were then rehydrated by immersing in 95% ethanol, 70% ethanol and then tap water for 1 minute each. The sections were stained in picro-indigo carmine for 1 minute and then rinsed with tap water. The water was removed by dehydrating the sections in ascending alcohol 70% and 95% ethanol for 5 minutes each. The sections were cleared in histoclear/ethanol (50:50 v/v) and then absolute histoclear (National Diagnostic, Hull, UK) for 4 minutes each. Coverslips were then mounted using histomount.

2.1.2.5 Immunohistochemistry:

2.1.2.5.1 Detection of cytokeratin 6 expression by immunohistochemistry:

Frozen tissue sections of human skin were removed from -20°C and fixed in cold acetone on ice. The sections were allowed to dry at room temperature. The sections were washed in sterile PBS three times. Each section was isolated by drawing a circle around it using a
Vector ImmEdge pen (Vector Laboratories, Peterborough, UK) to minimise the volume of antibody used and to treat each section individually.

The sections were incubated with 0.3% (v/v) hydrogen peroxide (Sigma-Aldrich Ltd) made up in methanol (Fisher Scientific) for 30 minutes to eliminate endogenous peroxidase activity, followed by a wash in PBS for 5 minutes. The sections were incubated for 20 minutes in 5% (v/v) normal mouse serum (NMS; Sigma-Aldrich Ltd) in PBS to block non-specific protein binding sites in the tissue. The excess serum was blotted away from the slides.

The primary antibody, which was a goat polyclonal anti-cytokeratin 6 antibody (Novocastra Laboratories, Newcastle upon Tyne, UK), was reconstituted to the stock recommended by the manufacturer using sterile water and then stored at -20°C until use. The primary antibody was then diluted 1:20 with 1.5% (v/v) normal mouse serum in PBS. After blocking, the sections were incubated with 150-200 µl of the diluted primary antibody per section for approximately 24 hours at 4°C. The slides were washed in PBS twice for 10 minutes each to remove excess unbounded primary antibody.

The sections were incubated with secondary biotinylated antibody (monoclonal antigoat IgG) 1:20 normal mouse serum in PBS for 30 minutes, followed by two washes in PBS for 10 minutes. The sections were then incubated with extravidin-peroxidase 1:20 normal mouse serum in PBS for 30 minutes. Extravidin binds to the secondary antibody and provides more sites for chromogen binding to enhance specific staining. The sections were washed twice by rinsing in PBS for 5 minutes each.
The peroxidase 3-amino-9-ethylcarbazole (AEC; Vector Laboratories) was added to the sections for 20-30 minutes to visualise and locate the antibody binding sites. The reaction of the AEC substrate with the peroxidase enzyme produces a red-brown chromogen. At this stage, progress of the colour development was monitored to prevent overstaining. After sufficient colour had developed, the reaction was stopped by rinsing in distilled water. The slides were allowed to dry and then mounted with an aqueous mountant, Aquamount (VWR international).

A number of negative controls were used to assess the results; in the first one, the sections were incubated with PBS instead of the secondary antibody to check that the primary antibody was not activating the AEC without the presence of the secondary antibody. The second negative control involved replacing both the primary and secondary antibodies with PBS to check that the blocking had been efficient and there was no endogenous peroxidase activity remaining. The third negative control was by replacing the primary antibody with PBS to check that the secondary antibody was not binding non-specifically to the tissue without the presence of the primary antibody.

2.1.2.5.2 Detection of the HGF receptor, c-Met, by immunohistochemistry:

The immunostaining procedure of c-Met was similar to that for cytokeratin 6 as mentioned before (section 2.1.2.5.1). However, sections were blocked with 5% (v/v) bovine serum albumin (BSA) in PBS for 20 minutes. A range of antibody concentrations were examined between 1:10 and 1:100 until the optimum one was achieved. A mouse monoclonal anti human c-Met antibody (Novocastra Laboratories Ltd) was applied to the sections at a dilution of 1:20 with 1.5% (v/v) BSA/PBS and left overnight at 4°C. The
slides were washed in PBS two times for 10 minutes each to remove excess unbounded primary antibody. The sections were then incubated with the secondary biotinylated antibody (Dako, Cambridge, UK) for 20 minutes, followed by two washes in PBS for 10 minutes. The sections were then incubated with streptavidin for 20 minutes. The negative controls were as described in the cytokeratin 6 section (2.1.2.5.1).

2.1.2.6 Visualising the staining:

All stained sections, Sacpic, haematoxylin & eosin and immunohistochemistry, were examined using the Orthodox II light microscope (Leitz, Germany). The sections were captured using a Nikon Coolpix 4500 digital camera. The images then were transferred to the computer using Nikon View 5 software.
2.1.3 Investigation of the expression of genes for specific molecules in human cultured dermal papilla cells using RT-PCR:

2.1.3.1 Dermal papilla cell lines:

The dermal papilla cells were provided from Professor Randall’s stock, Centre for Skin Sciences, University of Bradford. The cells were stored frozen in liquid nitrogen. The dermal papilla cells were originally derived from non-balding areas (scalp) of two healthy women aged 34 and 37 years, undergoing elective cosmetic surgery operations with full donor consent.

2.1.3.2 Cell culture procedure:

2.1.3.2.1 Maintenance of cell cultures:

The cryovials containing dermal papilla cells were removed from the liquid nitrogen and placed in a water bath at 37.5°C to defrost. Following the completion of the thawing, the contents of each cryovial were placed into a polystyrene rectangular canted-neck cell culture flask with a surface area of 25 cm² (Thomas Scientific, USA). Each flask contained 10 ml RPMI 1640 growth medium (Gibco, Paisly, UK). The growth media was routinely supplemented with penicillin (10 units/ml), streptomycin (100 µg/ml), fungizone (2.5 ng/ml), 2 mM L-glutamine and fetal calf serum (20%, v/v). The flasks were placed in the incubator for 24 hours to allow the cells to attach to the surface of the flasks. Cell culture was carried out in a humidified atmosphere at 37°C in 95% air and 5% CO₂ using a Heraeus B5060 EK incubator. The medium was changed every 3 days. The
maintenance of the dermal papilla cells was performed in a Class II MDH laminar flow cabinet.

2.1.3.2.2 Visualising cell cultures:

Growing cultures were viewed daily using an inverted, phase contrast microscope (Leitz, Wetzlar, Germany) to observe general morphology and growth conditions. Photographs of cultures were taken under a phase contrast microscope (Leitz).

2.1.3.2.3 Passaging of cell cultures:

Passaging was carried out when cell cultures reached approximately 90% confluence. Trypsinisation was performed to detach cells from the flask by proteolysis. The medium was emptied from the flasks and the cells were rinsed three times in a suitable amount of sterile PBS (10-15 ml), to remove any excess serum protein which could possibly inactivate the trypsin. An appropriate volume of trypsin/EDTA, approximately 500 µl, was added to each cell culture flask (25 cm²) and the flasks were replaced in the incubator for approximately 2-3 minutes until the cells had rounded up and detached. The cell detaching was viewed under a phase contrast microscope (Leitz) until all cells had detached from the surface of the flask. The cells of each flask were transferred and divided between three 25 cm² flasks containing 10 ml of fresh growth media. The flasks were placed back in the incubator and the media changed every 3 days.
2.1.3.2.4 Collection and storage of cultured cells:

When cultured dermal papilla cells reached confluence, they were collected from the flasks using a cell scraper (Bioscience Technology, USA) and transferred to universal tubes. The tubes were spun down in a Sanyo Harrier 15/80 centrifuge (Jepson Bolton & Co Ltd, UK) at 1200 rpm for about 10 minutes until the cell pellets were formed. The supernatant was carefully removed to avoid disruption of the cell pellets. The pellets were resuspended in 2 ml of stabilization reagent, RNA Later, and used immediately or stored at -80 until required. The resulting pellets were used for RNA isolation and later cDNA synthesis as described in sections 2.1.1.3 and 2.1.1.6. Following cDNA synthesis, RT-PCR was carried out to investigate gene expression for HGF, MSP and their receptors c-Met and RON, as described in section 2.1.1.7. After analysis of PCR products by electrophoresis, the identity of DNA fragments were verified and confirmed by sequencing, as described in section 2.1.1.8.
The cells were transferred into a culture flask (25 cm²) containing growth media & placed in an incubator

Medium was changed every 3 days

Cells were passaged when reached confluence

After trypsinisation to detach cells from the flask, the contents were divided into 3 flasks

When confluent, the cells were collected for total RNA isolation, cDNA synthesis & RT-PCR

Figure 2.3 Cell culture procedure
Schematic diagram displaying the dermal papilla cell culture steps
2.2 Comparison of the gene expression of normal and balding scalp hair follicles from men with androgenetic alopecia:

2.2.1 Biological materials:

Samples of human scalp skin from occipital and frontal regions of balding individuals were obtained from healthy donors as a result of routine cosmetic dermatological surgeries, with full donor consent and ethical committee approval. Samples were collected as full depth skin of about 0.2 cm². Tissues were transferred to 50ml falcon tubes containing 30 ml RNA stabilisation reagent, RNAlater™, to inhibit RNAses, which had been pre-cooled on ice (4°C). Tubes were labelled with details of date, sample origin, gender and age. The tubes were kept in ice for the duration of transportation (about two hours). Upon arrival at the university, the samples were stored in the fridge at 4°C overnight to allow the RNAlater™ to penetrate the tissues. The next day, these samples were transferred into clean RNAlater™ and stored at -20°C until required.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Gender</th>
<th>Age</th>
<th>Sample no.</th>
<th>Sample status</th>
<th>Body region</th>
<th>Hamilton stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>male</td>
<td>51</td>
<td>1</td>
<td>non-balding balding</td>
<td>Occipital scalp</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>Frontal scalp</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>male</td>
<td>56</td>
<td>3</td>
<td>non-balding balding</td>
<td>Occipital scalp</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td>Frontal scalp</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>male</td>
<td>55</td>
<td>5</td>
<td>non-balding balding</td>
<td>Occipital scalp</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td></td>
<td>Frontal scalp</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4 Samples used for isolation of whole anagen hair follicles from occipital and frontal areas of balding individuals (three healthy donors)
2.2.2 Total RNA isolation for DNA microarray & real-time PCR:

Following dissection of the required number of hair follicles, the process of total RNA isolation was carried out in accordance to the manufacturer’s instructions using the RNeasy Mini kit (Qiagen Ltd, Sussex, UK). The microdissected hair follicles were placed in a glass homogeniser (3 ml) containing lysis solution which consisted of 3 µl of β-mercaptoethanol (14 mM) with 350 µl of buffer RLT which contained guanidine thiocyanate. The hair follicles were disrupted and homogenised until they became undetectable and there were no visible solid remains. The homogenate was placed in a filtration column and centrifuged at 12000 g for 3 minutes (Eppendorf 5415 R) to remove any cellular debris and shear the DNA. Following centrifugation, the filtration column was discarded and an equal volume of 70% ethanol treated water was added to the cleared filtrate. The sample was mixed thoroughly by pipetting.

The lysate/ethanol mixture (700 µl) was transferred to a RNeasy spin column placed in a 2 ml collection tube and then centrifuged for 15 seconds at 8000 g. Following centrifugation, the flow-through was discarded and the RNA was bound to the column. The remaining sample was added to the binding column and the procedure repeated. The spin column was then transferred to a new collection tube.

DNA digestion was carried out to eliminate any genomic DNA contamination using RNase-free DNase kit (Qiagen Ltd) containing the enzyme oligodeoxyribonuclease I which digests single and double strand DNA. DNase I stock solution was prepared by dissolving the lyophilized DNase I (1500 kunitz units) in 550 µl of RNase-free water. To avoid loss of DNase I, the RNase-free water was injected into the vial using a RNase-free
needle and syringe. Since DNase I is especially sensitive to physical denaturation, the mixture was mixed gently by inverting the vial without vortexing. The reconstituted DNase I was divided into single-use aliquots and stored at -20ºC until required.

Buffer RW1 (350 µl, composition not available due to trade secret) was added to the RNeasy spin column and centrifuged for 15 seconds at 8000 g to wash the spin column membrane. DNase I stock solution (10 µl) was added to buffer RDD (70 µl, composition not available) and mixed by gently inverting the tube; residual liquid was collected from the sides of the tube by centrifuging briefly. The DNase I incubation mix (80 µl) was transferred directly to the RNeasy spin column membrane and placed on the benchtop at 20-23ºC for 15 minutes. Buffer RW1 (350 µl) was added to the spin column and centrifuged at 8000 g for 15 seconds. Following centrifugation, the flow-through was discarded and the spin column was transferred into a new collection tube.

To wash the spin column membrane, buffer RPE (500 µl, composition not available) was added to the column and centrifuged for 15 seconds at 8000 g; the flow through was discarded. Another 500 µl of buffer RPE was added to the column and centrifuged for 2 minutes at 8000 g to remove the ethanol. After centrifugation, the RNeasy spin column was removed carefully and placed in a new 2 ml collection tube, and centrifuged for 1 minute at 11,000 g. Long centrifugation dried the spin column membrane and ensured that no residual ethanol remained. The column was then transferred to a new 1.5 ml collection tube. The purified RNA was eluted from the column by adding the RNase-free water (30 µl) directly to the centre of the column and centrifuged for 1 minute at 8000 g. The column was discarded and the flow-through which contained purified RNA was used immediately or stored at -80ºC until used.
2.2.2.1 Assessing yield and purity of total RNA:

The quality and integrity of total RNA was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) which uses a fluorescent assay involving electrophoretic separation to evaluate RNA samples qualitatively. The Bioanalyzer provides more sensitive qualitative analysis from less RNA than the traditional methods such as using a spectrophotometer and gel electrophoresis. It is faster than gels, saving time and materials while using small amount of RNA, as little as 1 µl of total RNA.

The Bioanalyzer measures the amount of fluorescence as the RNA sample is pulsed through a microchannel over time. It calculates the ratio of ribosomal bands in total RNA samples and shows the percentage of ribosomal impurities in mRNA samples (Agilent). The Bioanalyzer software creates a graph called an electropherogram, which diagrams fluorescence over time and creates a gel image to accompany the graph for each sample.
2.2.3 Comparing gene expression in isolated hair follicles in balding and non-balding individuals using DNA microarray:

2.2.3.1 DNA microarray procedure:

The biological materials used for DNA microarray analysis were described in section 2.2.1. The hair follicles were microdissected from both balding and non-balding samples and then total RNA was extracted. Following total RNA isolation using the RNeasy Mini kit (Qiagen Ltd) as described in section 2.2.2, the quality of the resulting total RNA was checked using the Bioanalyzer 2100 (Agilent) (see section 2.2.2.1). All the subsequent technical procedures, including checking the quality and concentration measurement of RNA, cDNA synthesis, labelling, hybridization, washing and scanning of the microarray were done at the Sheffield Microarray Core Facility, The Medical School, University of Sheffield, UK. Initially, the Ambion WT expression kit (Ambion, Austin, USA) was used to prepare RNA samples for Affymetrix microarray analysis. This kit was designed to generate amplified sense-strand DNA without bias. The procedure was performed, according to the manufacturer’s instruction, in the following successive steps:

2.2.3.1.1 First-strand cDNA synthesis:

The first-strand master mix was prepared in a sterile tube (0.5 ml) by combining the first-strand buffer mix (4 µl, composition not available due to trade secret) with the first-strand enzyme mix (1 µl) at room temperature. The contents were mixed thoroughly by gently vortexing and centrifuged briefly at 8000 g for about 5 seconds to collect the mix at the bottom of the tube. The master mix (5 µl) was transferred to a PCR reaction tube. The total RNA sample (5 µl) was added to the mix and the tube was centrifuged briefly at 8000 g for a few seconds. The tube was incubated in a thermal cycler for 1 hour at 25°C,
then 1 hour at 42°C and then for at least 2 minutes at 4°C. Following the incubation, the tube was centrifuged briefly as above for a few seconds and then placed in ice for 2 minutes to cool the tube because transferring the second-strand master mix to a hot tube or holding the first-strand cDNA for longer than 10 minutes at 4°C may significantly reduce the cRNA yield.

**2.2.3.1.2 Second-strand cDNA synthesis:**

In a nuclease-free tube, the second-strand cDNA master mix (50 µl) was prepared by combining nuclease-free water (32.5 µl), second-strand buffer mix (12.5 µl, composition not available due to trade secret) and second-strand enzyme mix (5 µl). The tube was mixed thoroughly and centrifuged briefly. The master mix (50 µl) was transferred to a reaction tube containing 10 µl of the resulting first-strand cDNA from the previous step. The tube was incubated in a thermal cycler for 60 minutes at 16°C, for 10 minutes at 65°C and for at least 2 minutes at 4°C. The tube was centrifuged at 8000 g for 5 seconds to collect the cDNA at the bottom of the tube and then placed in ice.

**2.2.3.1.3 cRNA synthesis by in vitro transcription:**

cRNA was synthesized and amplified by *in vitro* transcription (IVT) of the second-strand cDNA template using T7 RNA polymerase. At room temperature, the IVT master mix was prepared by adding 24 µl of IVT buffer mix (composition not available) to 6 µl of IVT enzyme mix in a nuclease-free tube. The tube was mixed and spun down briefly for few seconds. The total volume of master mix (30 µl) was added to 60 µl of second-strand cDNA and mixed thoroughly by vortexing before centrifuging briefly at 8000 g. The tube was incubated in a thermal cycler for 16 hr at 40°C and then overnight at 4°C.
The resulting cRNA was purified to improve its stability and to remove any unincorporated nucleotides, salts and inorganic phosphate. In a sterile tube, the cRNA binding mix was prepared by combining nucleic acid binding beads (10 µl) to nucleic acid binding buffer concentrate (50 µl) and mixed thoroughly. The resulting binding mix (60 µl) was added to the cRNA sample and mixed by pipetting up and down 3 times. Isopropanol (100%; 60 µl) was added to the mix and then transferred to a well of a U-bottom plate. The plate was placed in a MixMate shaker (Krackeler Scientific Inc, US) and shaken gently for at least 2 minutes. This incubation allowed the cRNA to bind to the nucleic acid binding beads. The plate was placed in a magnetic stand to capture the magnetic beads. During this process, the nucleic acid binding beads formed pellets against the magnets in the stand. The supernatant was discarded leaving the beads in the plate. Nucleic acid wash solution (500 µl) was then added to the sample and shaken for 1 minute. Following that, the plate was placed again in the magnetic stand to capture the beads and the supernatant was removed. To evaporate residual ethanol, the plate was shaken vigorously for 1 minute. The purified cRNA was eluted by adding 40 µl preheated elution solution (55 to 58ºC) and incubated for 2 minutes. After incubation, the nucleic acid binding beads were dispersed by vigorously shaking the plate for 3 minutes and then recaptured using the magnetic stand. The supernatant which contained the eluted cRNA was transferred to a new collection tube and used immediately or stored at -80ºC until required.

2.2.3.1.4 2nd cycle cDNA synthesis:

Sense-strand cDNA was synthesized by the reverse transcription of cRNA using random primers. The cRNA (10 µg; 22 µl) was combined with 2 µl random primers and mixed
thoroughly by gently vortexing and centrifuging at 8000 g for 5 seconds to collect the reaction mix at the bottom of the tube. The tube was then incubated in a thermal cycler for 5 minutes at 70°C, 5 minutes at 25°C, and then 2 minutes at 4°C. In a nuclease-free tube, the 2nd-cycle master mix was prepared from 2nd-cycle buffer mix (8 µl, composition not available due to trade secret) and 2nd-cycle enzyme mix (8 µl), and then added to 24 µl of cRNA/random primer sample. The reaction mixture was incubated in a thermal cycler for 10 minutes at 25°C, 90 minutes at 42°C, 10 minutes at 70°C, and for at least 2 minutes at 4°C. After incubation, the tube was placed in ice and used for subsequent steps. On ice, RNase H (2 µl) was added to the 2nd-cycle cDNA to degrade the cRNA, leaving only the single-strand cDNA. After mixing by pipetting the tube was incubated in a thermal cycler for 45 minutes at 37°C, 5 minutes at 95°C, and for at least 2 minutes at 4°C.

The resulting 2nd-cycle cDNA was purified to remove unincorporated dNTPs, salts and enzymes. The cDNA binding mix was prepared at room temperature in a sterile tube by mixing nucleic acid binding beads (10 µl) to nucleic acid binding buffer concentrate (50 µl). Nuclease free water (18 µl) and the binding mix (60 µl) were added to the reaction mixture and mixed thoroughly. The contents were transferred to a well of a U-bottom plate and 120 µl of 100% ethanol was added to the mix and mixed by gently shaking using a MixMate shaker (Krackeler Scientific) for 2 minutes so that the cDNA in the sample bound to the beads during this incubation process. The plate was transferred to the magnetic stand to capture the magnetic beads and the supernatant removed. The nucleic acid wash solution (100 µl) was added to the contents and mixed by shaking for 1 minute, the beads were captured using the magnetic stand. Vigorous shaking for 1 minute
evaporated the residual ethanol. The purified cDNA was eluted from the beads by incubating at room temperature for 2 minutes with 30 µl of preheated (55-58°C) elution solution. The plate was shaken vigorously for 3 minutes to disperse the beads which were captured using the magnetic stand. The supernatant which contained the eluted cDNA was transferred to a new collection tube and used then for fragmentation labelling steps.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-strand cDNA synthesis</td>
<td>25°C, 60 min</td>
<td>42°C, 60 min</td>
<td>4°C, 2 min</td>
<td></td>
</tr>
<tr>
<td>Second-strand cDNA synthesis</td>
<td>16°C, 60 min</td>
<td>65°C, 60 min</td>
<td>4°C, 2 min</td>
<td></td>
</tr>
<tr>
<td>cRNA synthesis (in vitro transcription)</td>
<td>40°C, 16 hrs</td>
<td>4°C, hold</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd-cycle cDNA denaturation</td>
<td>70°C, 5 min</td>
<td>25°C, 5 min</td>
<td>4°C, 2 min</td>
<td></td>
</tr>
<tr>
<td>2nd-cycle cDNA synthesis</td>
<td>25°C, 10 min</td>
<td>42°C, 90 min</td>
<td>70°C, 10 min</td>
<td>4°C, 2 min</td>
</tr>
<tr>
<td>RNase H hydrolysis</td>
<td>37°C, 45 min</td>
<td>95°C, 5 min</td>
<td>4°C, 2 min</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.5* Thermal cycling conditions used for preparing samples for DNA microarray analysis.
2.2.3.2 Fragmentation & labelling of single-strand DNA:

This process was carried out using the GeneChip terminal labelling and hybridization kit (Affymetrix, Santa Clara, CA). The fragmentation master mix was prepared by combining RNase-free water (10 µl), 10X cDNA fragmentation buffer (4.8 µl), uracil-DNA glycosylase (UDG) (1 µl) and apurinic/apyrimidinic endonuclease 1 (APE1) (1 µl) to recognize and fragment the cDNA at dUTP residues. The prepared master mix was then added to the sample tube which contained single-strand DNA (5.5 µg) and RNase-free water (31.2 µl). The reaction mixture was vortexed gently, and spun down at 8000 g for a few seconds and then incubated in the thermal cycler for 60 minutes at 37ºC, for 2 minutes at 93ºC, and then for 2 minutes at 4ºC.

Following this incubation, labeling of fragmented single-strand DNA was performed. The labeling reaction mixture contained 5X TdT buffer (12 µl, 50 mM Sodium Cacodylate, pH 6.8, 5 mM Cobalt Chloride and 0.5 mM DTT), deoxynucleotidyl transferase (TdT) (2 µl) and DNA labeling reagent contained Allonamid triphosphate (1 µl) to give the total volume 15 µl which added to the fragmented single-strand DNA (45 µl). The tube was incubated in the thermal cycler for 60 minutes at 37ºC, 10 minutes at 70ºC, and then for at least 2 minutes at 4ºC.

2.2.3.3 Hybridization:

The hybridization process was carried out using the GeneChip hybridization, wash and stain kit (Affymetrix). In a 1.5 ml RNase-free eppendorf tube, the hybridization cocktail was prepared by combining 60 µl of the fragmented and labeled DNA, 3.7 µl of control oligonucleotide B2 (3 nM), 11 µl of 20X eukaryotic hybridization control, 110 µl of 2X
hybridization mix, 15.4 µl DMSO, and nuclease-free water (220 µl). The tube was vortexed gently and spun down for few seconds. The hybridization cocktail was heated at 99°C for 5 minutes and cooled to 42°C for 5 minutes, and then centrifuged at 12,000 g for 1 minute. The GeneChip Human Exon 1.0 ST Array (Affymetrix) was equilibrated to room temperature immediately before use. The array was labelled with the name of the sample to be hybridized. An appropriate amount of hybridization mix was then injected into the array through one of its septa. The array was placed in a 45°C hybridization oven at 60 rpm and incubated for about 17 hours.

Figure 2.4 GeneChip® probe array and its parts
Images were adapted and modified from the Affymetrix manual.
2.2.3.4 Washing, staining & scanning of hybridized array:

After 17 hours of hybridization, the array was removed from the hybridization oven. Wash and stain steps were carried out using the GeneChip hybridization, wash and stain kit (Affymetrix). The probe array was vented by inserting a clean pipette tip into one of the septa and the hybridization cocktail was extracted using a pipettor through the other septum. The array was refilled completely with a suitable volume of wash buffer A (6X SSPE, 0.01% Tween-20).

The staining reagents consisted of streptavidin phycoerythrin (SAPE) solution and antibody solution. The SAPE solution mix was prepared by combining: 1X stain buffer (600 µl, 10 mM MES, 0.1 M NaCl, 0.01% Tween-20), 2 mg/ml BSA (48 µl), 10 µg/ml streptavidin phycoerythrin (SAPE) (12 µl) and deionized water (540 µl). Since SAPE is sensitive to light, it was stored in the dark at 4 °C and wrapped with foil. The antibody solution mix consisted of 2X stain buffer (300 µl), 2 mg/ml BSA (24 µl), 0.1 mg/ml goat IgG (6 µl), 3 µg/ml biotinylated antibody (3.6 µl), deionized water (266.4 µl). The wash and stain process was then carried out automatically in the Fluidics Station 450/250 (Affymetrix) using Affymetrix® GeneChip Command Console (AGCC) software.

After the wash protocols were completed, the intensity of the fluorescence of the array was scanned and measured using the Affymetrix® GeneChip® Scanner 3000 (Affymetrix). The laser was warmed up by turning it on at least 10 minutes prior to scanning. The scanner was controlled by the AGCC software. After completing the scanning procedure, the scanned probe array image was ready for analysis.
Figure 2.5 Preparation of microarray and microarray procedure
Image was reproduced and adapted from Somasundaram et al., (2002) with the author’s permission.
2.2.3.5 Microarray data analysis:

To study the changes in gene expression in balding versus non-balding scalp hair follicles, the Affymetrix GeneChip human exon 1.0 ST array containing 28,869 genes was used. A total of six chips were used in this study; one chip per sample. Following the scanning procedure, the scanned probe array image files were processed and analyzed using GeneSpring GX 11 software (Agilent technologies). The data was normalized to standard microarray data as per-gene normalization using the robust multi-array analysis (RMA) method (Irizarry et al., 2003). The normalization process aims to remove certain systematic biases from microarray data. The data was transformed to the log scale to stabilize the variation across the expression ranges associated with the probe concentration (Quackenbush, 2002; Cui & Churchill, 2003). The data distribution in balding and non-balding samples was visualized using box and whiskers plot. The correlation between the two conditions was plotted on a scatter diagram. The data quality was further investigated using principal component analysis (PCA) method (Komura et al., 2004; Shlens, 2009).

Replicate data was organized in hierarchical clustering by both individual samples as well as grouped data. A t-test filter was used to determine which genes are significantly differentially expressed and to exclude all probe sets with differential expression t-test P-values of less than 0.05. A fold change filter was also used for further investigation.
2.2.4 Comparison of the expression of specific genes in non-balding and balding hair follicles using quantitative real-time PCR:

2.2.4.1 RNA amplification:

Due to the limited starting materials obtained from the androgenetic alopecia patients, the isolated total RNA (section 2.2.2) was amplified using the Smart™ RNA amplification kit (Clontech laboratories, USA) to synthesize high-quality RNA for use in quantitative real-time PCR. The amplification procedure was done in accordance to the manufacturer’s instructions in the following successive steps:

2.2.4.1.1 First-strand cDNA synthesis:

In a sterile 0.5 ml reaction tube, 1-3 µl of RNA sample (0.1-5 µg) was added to 1 µl of cDNA synthesis (CDS) primer II A (12 µM) and a suitable amount of nuclease-free water to give the total volume of 4.25 µl. The contents were mixed and centrifuged briefly at 8000 g for few seconds in a eppendorf 5415 microcentrifuge. The tube was incubated in a thermal cycler for 3 minutes at 70ºC and then the temperature was reduced to 42ºC for 2 minutes. The master mix was prepared by combining the following components in a 0.5 ml RNase-free eppendorf tube in the order shown:

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X First-Strand Buffer (25 mM Tris-HCl, pH 8.3, 15 mM MgCl2, 20 mM spermidine, 35 mM KCl)</td>
<td>2.00</td>
</tr>
<tr>
<td>DTT (100 mM)</td>
<td>0.50</td>
</tr>
<tr>
<td>RNase inhibitor (1 U/µl)</td>
<td>0.25</td>
</tr>
<tr>
<td>SMART™ T7 Oligonucleotide (10 µM)</td>
<td>1.00</td>
</tr>
<tr>
<td>50X dNTP mix (dATP, dGTP, dCTP, dTTP; 10 mM)</td>
<td>1.00</td>
</tr>
<tr>
<td>Smartscibe Reverse Transcriptase</td>
<td>1.00</td>
</tr>
</tbody>
</table>
The contents were mixed by vortexing and centrifuged briefly in the microcentrifuge. Following centrifugation, the master mix (5.75 µl) was added to the reaction tube prepared previously. The contents were mixed thoroughly by pipetting and centrifuged briefly. The reaction tube was returned immediately to the thermal cycler and incubated at 42°C for 1.5 hr. The reaction was terminated by heating at 68°C for 10 minutes.

2.2.4.1.2 Second-strand cDNA synthesis:

Following first-strand cDNA synthesis, the master mix for second-strand cDNA was prepared by combining the following components in a 0.5 ml RNase-free eppendorf tube in the order shown:

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>73</td>
</tr>
<tr>
<td>10X Advantage® 2 PCR buffer (10 mM Tris-HCl, 15 mM MgCl₂, 50 mM KCl)</td>
<td>10</td>
</tr>
<tr>
<td>50X dNTP mix (dATP, dGTP, dCTP, dTTP; 10 mM)</td>
<td>2.0</td>
</tr>
<tr>
<td>T7 extension primer (10 µM)</td>
<td>2.0</td>
</tr>
<tr>
<td>RNase H (10 U/µl)</td>
<td>1.0</td>
</tr>
<tr>
<td>50X Advantage® 2 polymerase mix</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The contents were mixed by vortexing and the tube centrifuged briefly at 8000 g for few seconds. This master mix (90 µl) was added to the reaction tube containing the first-strand cDNA obtained from the previous step (section 2.2.4.1.1) and mixed by vortexing. The reaction tube was placed in a thermal cycler using the following program: 37°C for 15 minutes, 95°C for 2 minutes, 60°C for 1 minute, 68°C for 10 minutes.
### 2.2.4.1.3 Purification of double-strand cDNA:

Prior to performing *in vitro* transcription, the double-strand cDNA was purified using the Atlas Nucleospin® Extraction II kit (Clontech). Two volumes of buffer NT (200 µl, contained chaotropic salt) was added to one volume of sample (100 µl PCR reaction mix). The sample was loaded on to a Nucleospin extract II column which was placed into 2 ml collecting tube. The mixture was centrifuged for 1 minute at 11,000 *g*; the flow-through was discarded and the column was placed into a new collecting tube.

Buffer NT3 (600 µl) was added to the reaction mixture and centrifuged at 11,000 *g* for 1 minute. The flow-through was discarded and the column was placed back into the collecting tube. To remove buffer NT3, the column was centrifuged for 2 minutes at 11,000 *g*. The spin column was placed in a clean 1.5 ml tube.

Buffer NE (50 µl, 5 mM Tris-HCl; pH8.5) was added to the column and was left to stand for 1 minute, before the tube was centrifuged at 12,000 *g* for 1 minute. A second aliquot (50 µl) of buffer NE was added to the column and the process repeated. The Nucleospin column was discarded and the eluted cDNA was centrifuged at 12,000 *g* for an additional 3 minutes. The supernatant was transferred to a new collection tube.

Linear acrylamide (3 µl), sodium acetate (10 µl) and 100% ethanol (250 µl) were mixed and added to the reaction tube which was placed at -20°C overnight to precipitate the cDNA. The next day the tube was centrifuged for 20 minutes at 12,000 *g*. The supernatant was removed by pipetting and the pellet washed once in 100 µl of 70% ethanol for 10 minutes before dissolving in 9 µl nuclease-free water. The purified double-strand DNA was used immediately or stored at -20°C until required.
2.2.4.1.4 Synthesis of cRNA (in vitro transcription):

Following purification of double-strand cDNA, in vitro cRNA synthesis was carried out. The transcription master mix was prepared by combining the following components at room temperature, in the order shown below:

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T7 transcription buffer (20 mM Tris-HCl; pH 7.5, 50 mM NaCl₂, 10 mM spermidine, 30 mM MgCl₂)</td>
<td>2.0</td>
</tr>
<tr>
<td>3X rNTP mix (ATP, CTP, GTP, UTP; 10 mM)</td>
<td>7.0</td>
</tr>
<tr>
<td>RNase inhibitor (1 U/µl)</td>
<td>1.0</td>
</tr>
<tr>
<td>T7 RNA polymerase (1,000 U/µl)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The tube was mixed by vortexing and centrifuged briefly at 8000 g for few seconds in the microcentrifuge. The transcription master mix (11 µl) was added to the purified double-strand cDNA (9 µl) and mixed by vortexing before incubating at 37°C for 12 hr. The resulting cRNA was purified as described below.

2.2.4.1.5 Purification of cRNA:

The cRNA was purified to remove any unincorporated ribonucleotides and small cDNA and RNA fragments. The purification process was carried out using the NucleoSpin® RNA II purification kit (Clontech). Buffer RA1 (300 µl, composition not available) was added to cRNA (20 µl) in a 0.5 ml RNase-free eppendorf tube and mixed well by pipetting. Ethanol (100%; 240 µl) was added to the mixture and mixed thoroughly. The
sample was loaded into a Nucleospin column already inside a 2 ml collection tube. The tube was centrifuged for 60 seconds at 8000 g, and the flow-through discarded.

The cRNA was washed by adding buffer RA3 (750 µl, composition not available) to the Nucleospin column and centrifuged at 14,000 g for 1 minute; the flow-through was discarded and the column placed into a new 2 ml collection tube. This process was repeated twice using 250 µl of buffer RA3 each. The tube was centrifuged at 14,000 g for a further minute to remove any residual wash buffer and dry the filter column, which was then transferred to a new 1.5 ml collection tube. The RNA was eluted by adding 30 µl of nuclease-free water to the centre of the column. After elution, the spin column was discarded and the eluted RNA was transferred to a new collection tube. The purified cRNA was either used immediately or stored at -80ºC until required.

2.2.4.1.6 Checking the quality of purified cRNA:

The quality and purity of the resulting RNA was evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Loughborough, UK) before synthesis of cDNA was carried out as described in section 2.1.1.6. The automatic path length capability of the NanoDrop 2000 spectrophotometer allowed for measurement of samples using the instrument’s standard method, with no special sample preparation required. All samples were measured on the NanoDrop 2000 without dilution. The elution solution (1.2 µl) was used as a control to set the background as zero and then 1.2 µl of each RNA sample was used for measurement of the RNA concentration. The NanoDrop 2000 created a graph for each sample measured. Figure 2.6 shows the basic use of NanoDrop 2000 instrument.
Figure 2.6 NanoDrop 2000 instrument (A) and checking the quality of RNA (B)

(1) The sampling arm was raised and both the upper and lower pedestals were wiped using a dry, lint-free laboratory wipe.
(2) The elution solution (1.2 µl) was used as a control to set the background as zero and then the RNA sample (1.2 µl) was loaded onto the lower measurement pedestal.
(3) The sampling arm was lowered and a spectral measurement was initiated using the software on the PC which was completed automatically.
(4) When the measurement was completed, the sampling arm was raised and the sample was wiped from both the upper and lower pedestals to prevent sample carryover and to avoid residue buildup in subsequent measurements.

Images were adapted and modified from the Thermo Scientific manual.
2.2.4.2 Primer design used for real-time PCR:

The Ensemble genome browser was used to find the human genomic sequences for GAPDH, HGF, MSP, c-Met and RON, and the exon information part of each gene sequence was used for this purpose. Primers, forward and reverse, for each gene were selected from the exon part of the genomic sequence. The primer combination was selected using two different exons, crossing an intron, to ensure that the correct sized product would only be obtained from cDNA and not from any contaminating genomic DNA sequence that might be present in the PCR mixture. The designed primers for each gene were checked by comparing with the specific human gene sequence of the target gene using the NCBI BLAST programme (http://www.ncbi.nlm.nih.gov/blast/b12seq/wblast2/.cgi).

After designing the primers was completed for each gene, they were checked using the Ensemble genome browser against the human genome of the target gene to see if they were only specific to the desired target sequence. Ensemble blast was also used to check the exact chromosomal location of each primer, the length of matching area on the chromosome and the length of the gap between the end of the forward primer matching area and the start of reverse primer matching area, all done against human genome. The primers for GAPDH, HGF, MSP, c-Met and RON were synthesised by Sigma Genosys Biotechnologies Ltd (Pampisford, UK). The designed primers for each gene were first checked on human cDNA obtained from the universal human reference RNA (Stratagene, UK) which comprises a collection of RNA pooled from ten different cell lines for an optimal broad coverage of human genes (appendix II). The primer sequences, their optimised PCR conditions and expected amplicon size are detailed in table 2.6.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Optimal thermocycling conditions</th>
<th>Expected amplicon size</th>
</tr>
</thead>
</table>
| GAPDH | F: 5’ ATC ACT GCC ACC CAG AAG 3’  
R: 5’ CAG TGA GCT TCC CGT TCA 3’ | 95°C for 3 minutes  
95°C for 10 seconds  
60.5°C for 30 seconds  
72°C for 15 seconds  
40 cycles | 148 bp |
| HGF  | F: 5’ TTCACAAGCAATCCAGAGGTACGC 3’  
R: 5’ ATGATCCCCAGCCTGACAAATCTT 3’ | 95°C for 3 minutes  
95°C for 10 seconds  
58.3°C for 30 seconds  
72°C for 15 seconds  
40 cycles | 144 bp |
| MSP  | F: 5’ AGGAGGATGTGGCAGATGC 3’  
R: 5’ TTGAGTCCATGGCAGCAGT 3’ | 95°C for 3 minutes  
95°C for 10 seconds  
58.3°C for 30 seconds  
72°C for 15 seconds  
40 cycles | 116 bp |
| RON  | F: 5’ ACAGTGGACCCTATAAGGCGA 3’  
R: 5’ AGGGACTTTGGCCACACGTGA 3’ | 95°C for 3 minutes  
95°C for 10 seconds  
60.5°C for 30 seconds  
72°C for 15 seconds  
40 cycles | 117 bp |
| e-Met | F: 5’ ATTTGGATAGGCTTGTAAGTG 3’  
R: 5’ TGTCAGAGGATACTGCACTTTT 3’ | 95°C for 3 minutes  
95°C for 10 seconds  
56.5°C for 30 seconds  
72°C for 15 seconds  
40 cycles | 152 bp |

**Table 2.6** Primer sequences and PCR conditions for each specific gene used for real-time PCR.
2.2.4.3 The quantitative real-time PCR procedure:

Real-time PCR was performed using the MyiQ™ single-colour real-time PCR detection system (Bio-Rad, UK) and SYBER® Green PCR Master Mix (Applied Biosystem, USA). For each real-time PCR reaction, the following reaction mix was prepared and used: SYBER® Green PCR Master Mix (12.5 µl), the forward primer of the target gene (1 µl), the reverse primer of the target gene (1 µl), the cDNA template (1 µl) and the mixture was brought to 25 µl by adding nuclease-free water (9.5 µl). The reaction mixture was then transferred into an optical 96-well reaction plate (Applied Biosystem) which was already labeled. Each well of the reaction plate was also supplied with 25 µl of the appropriate PCR master mix was added. Each well was tightly covered using specific optical caps. The highly expressed housekeeping protein, GAPDH, was used as an endogenous control.

The plate was then transferred to a centrifuge (Eppendorf, 5810R) for centrifugation at 3000 rpm for about 10-15 minutes and checked to verify that each reaction mix was positioned in the bottom of the well to ensure that there was no air bubbles inside the well which would interfere with the reading, see figure 2.7. The plate was then placed into the real-time PCR machine.
Real-time PCR was performed using specific forward and reverse primers for each cDNA target sequence. The annealing temperature for each target primer set was initially optimized using the cDNA template, synthesized from the universal human reference RNA (Stratagene, UK) which composed of total RNA isolated from 10 cell lines representing different human tissues which were chosen to ensure a standard broad coverage of human genes (appendix II). The real-time PCR was performed under the following cycling conditions: 94°C for 3 minutes, followed by denaturing at 94°C for 15 seconds, annealing (gradient) of 55°C to 63°C for 30 seconds, followed by 72°C for 15 seconds; this was repeated for 40 cycles. The primer sequences, their optimized cyclic conditions and their expected amplicon size are detailed in table 2.6.

Figure 2.7 The different positions of reaction mix in 96-well plate.

(A) Correct position: the reaction mix is positioned in the bottom of the well

(B) Incorrect position: the reaction mix is positioned on the side wall due to the lack of centrifugation

(C) Incorrect position: presence of air bubbles at the bottom of the well due to the plate was not centrifuged with sufficient time or speed.
2.2.4.4 Data analysis:

Real-time PCR data and the differences between samples and controls were calculated using the Genex database software based on the comparative (ΔΔCt) equitation method (Livak and Schmittgen, 2001) to calculate relative quantities of a nucleic acid sequence. The Ct is the threshold cycle during which a reaction emits the threshold level of fluorescence. The detectable amount of fluorescence when a signal is significantly greater than background, is known as the threshold. Data was normalized to the corresponding values of an endogenous control, glyceraldehyde 3-phosphate dehydrogenase (abbreviated as GAPDH or less commonly as G3PDH). A statistically based method, Student’s t-test, was used to determine significant differences in gene expression between the two conditions with P<0.05.
3- Results
3- Results

3.1 Histological investigation of human and red deer hair follicles:

The initial stage of this investigation required familiarisation of the hair follicle structure. To do this, cryosections of human and red deer skin were examined by histological staining using Sacpic (Nixon, 1993; Nutbrown and Randall, 1996) and haematoxylin & eosin staining techniques to clearly visualise the normal histology of the hair follicle and to aid the interpretation the immunohistochemical staining.

In Sacpic staining, several different dyes were used to distinguish the different parts of the skin and hair follicle. In general, the Sacpic staining allowed the different structures of the hair follicles to be differentiated in both species (figures 3.1, 3.2, 3.3). The overall structure of the hair follicle was seen showing the connective tissue sheath as pale blue encasing the whole follicle, separating it from the dermis (blue/green). The fully keratinised hair fibre stained yellow surrounding by red inner root sheath and the outer root sheath (blue). The dermal papilla is situated in the hair bulb, surrounded by the hair matrix and attached to the connective tissue sheath at its base.

<table>
<thead>
<tr>
<th>Components</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermis</td>
<td>Blue/green</td>
</tr>
<tr>
<td>Collagen</td>
<td>Bright blue</td>
</tr>
<tr>
<td>Connective tissue sheath</td>
<td>Pale blue</td>
</tr>
<tr>
<td>Outer root sheath</td>
<td>Blue</td>
</tr>
<tr>
<td>Inner root sheath</td>
<td>Red</td>
</tr>
<tr>
<td>Fully keratinised hair fibre</td>
<td>Yellow</td>
</tr>
<tr>
<td>Pre-keratinised hair fibre</td>
<td>Pink</td>
</tr>
</tbody>
</table>

Table 3.1 Interpretation of Sacpic staining
3.1.1 Red deer skin

The histological staining of adult red deer stag neck (mane) skin with anagen hair follicles using Sacpic revealed a thin epidermal layer above a thick collagenous dermal layer stained blue/green; the hair follicles extended down into the dermis. The components of the hair follicles are highlighted by the Sacpic staining, the dermal papilla located within the hair bulb and surrounded by the hair matrix, the hair fibre surrounded by the inner root sheath and the outer root sheath. The connective tissue sheath encapsulated the entire follicle separating it from the dermis. Red deer hair follicles are larger than human scalp hair follicles and an obvious medulla was clearly seen within the hair (figure 3.1). Table 3.1 gives a summary of the Sacpic staining colours.
Figure 3.1 Histology of red deer hair follicle.

Histological staining of cryosections of red deer hair follicle using Sacpic staining. The hair follicle extends into the thick dermal layer. The sections show the structure of deer hair follicle components including the outer root sheath (ORS), the inner root sheath (IRS), the connective tissue sheath (CTS), The hair cortex (CO), the medulla (MD) in the centre of the follicle. The hair bulb extends deep within the skin and includes the dermal papilla (DP) which is surrounded by the hair matrix (M) and attached to the connective tissue sheath (CTS) at its base. Scale bar (A 75 µm, B 69 µm), magnification (A x70, B x95).
3.1.2 Human skin:

The histological staining of human scalp skin showed a thin epidermis, underlying collagenous dermis and a subcutaneous fat layer beneath into which the anagen hair follicles extend (figure 3.2). The hair follicles extend down into the subcutaneous adipose tissue. The different components of the hair follicle were highlighted by the Sacpic stain (figure 3.3) and haematoxylin & eosin (figure 3.5) which showed the hair shaft internal root sheath, outer root sheath, connective tissue sheath, hair matrix and derma papilla located within the hair bulb.

Many hair follicle layers can be seen in horizontal cross-section of hair follicle using Sacpic (figure 3.4) and haematoxylin & eosin (figure 3.5). The hair follicles were sectioned in the upper part of the follicles in which fully keratinised hair fibres are present. The Scapic stain revealed greater details of the hair follicle structure.
Figure 3.2 Longitudinal section of human scalp skin stained with Sacpic staining

(A) A section of a 42 year old female human full depth scalp biopsy shows a thin epidermis (E), a thick dermal layer (D) stained blue/green, composed of collagen, and a longitudinal hair follicle which extends deep into the subcutaneous fat (SF). The fully keratinized hair fibre (H) stained yellow. Attached to the hair follicles are sebaceous glands (SG) which consists of several lobes clearly visible within the dermal layer. Scale bar (205 µm), magnification (x16).
Figure 3.3  Longitudinal section of human scalp anagen hair follicle stained with Sacpic staining

Different components of the human scalp hair follicle are clearly visible which include the connective tissue sheath (CTS), the outer root sheath (ORS), and the inner root sheath (IRS) stained red. The hair bulb is located in the subcutaneous fat (SF) deep within the skin and includes the dermal papilla (DP) which is surrounded by the hair matrix (M) containing melanocytes and attached to the connective tissue sheath (CTS) at its base. Scale bar (80 µm), magnification (x163).
Figure 3.4  Cross section of human scalp skin stained with Saepic staining

(A) Cross section of human scalp skin (male, age 45) shows the collagen fibers (C) as a part of the thick dermal layer, sebaceous glands (SG) which consists of several lobes, and hair follicles (HF). Scale bar (199 µm), magnification (x42).

(B) Horizontal cross-section of a human scalp hair follicle shows different layers of the hair follicle including the hair fibre (F), the inner root sheath (IRS), the outer root sheath (ORS) and the connective tissue sheath (CTS). The inner root sheath has partially separated from the outer root sheath, probably during the staining procedure. Scale bar (25 µm), magnification (x196).
Figure 3.5 Histology of human scalp hair follicle using H & E.

(A) Cross section of human skin (male, age 45) shows the collagen fibers (C) as a part of the dermal layer, subcutaneous fat (SF), hair shaft (HS), and hair follicle (HF), scale bar (199 µm), (x45).

(B) Cross section of human scalp hair follicle shows the different layers of the hair follicles including the connective tissue sheath (CTS), the outer root sheath (ORS) and the inner root sheath (IRS), and the hair shaft (HS), scale bar (23 µm), magnification (x195).

(C) Longitudinal section of lower human hair follicle. The section shows hair follicle components including the outer root sheath (ORS), the inner root sheath (IRS), the connective tissue sheath (CTS). The hair bulb extends deep within the skin and includes the dermal papilla (DP) which is surrounded by the hair matrix (M) and attached to the connective tissue sheath (CTS) at its base. Scal bar (75 µm), magnification (x152).
3.2 **RT-PCR analysis of the expression of the genes for HGF, MSP and their receptors, c-Met and RON, in human scalp hair follicles:**

3.2.1 **Isolation of whole lower anagen hair follicles by microdissection:**

For the purpose of investigating the expression of HGF, MSP and their receptors, c-Met and RON, individual human hair follicles were isolated by microdissection as described in section 2.1.3.1. Dissection of the hair follicles proved to be extremely difficult and time consuming, requiring patience and dedication. Photographs of the dissection steps are displayed in figures 2.1, 3.6, 3.7, 3.8. The skin sample was cut at the junction between the epidermis and dermis using a sterile scalpel blade (figures 2.1, 3.5). Under the dissecting microscope, the hair follicles were plucked from the skin gently using fine forceps and then transferred into a petri dish containing RINAlater. The isolated hair follicles were cleaned of any dermis or fat debris under a higher magnification using syringe needles (figure 3.8). The clean follicles were transferred to fresh RINAlater placed on ice. A total of 60 follicles were collected from each sample. The resulting whole follicles from each sample were gathered for total RNA isolation, followed by poly (A) RNA extraction and then cDNA synthesis to be used in the PCR reactions for detecting gene expression.
Figure 3.6 Surface view of the lower part of human scalp skin.
Human scalp skin (female, age 37) after cutting the junction between the epidermis and dermis (D). The hair follicles appear as dark circular shapes, frequently in groups of the hair follicles. Scale bar (500 µm), mag. (x20).

Figure 3.7 Human scalp anagen hair follicles within the skin
The hair follicles extend deep within the skin and (female, age 37). The hair bulbs are located in the subcutaneous fat. Whole hair follicles were microdissected for total RNA isolation and later the investigation into their expression of specific molecules by RT-PCR. Scale bar (360 µm), magnification (x259).
Figure 3.8 Isolated human scalp hair follicle and its bulb.
Isolated lower human scalp anagen hair follicle (male, age 30) (A) and isolated human hair bulb (B), show the different parts of the hair follicle including the dermal sheath, hair matrix & dermal papilla. The hair follicles were microdissected for the purpose of total RNA isolation and later investigating the expression of specific molecules using RT-PCR. Some blood vessels can be clearly seen in the connective tissue sheath. (Scale bar: 290 µm & 185 µm, magnification: x266 & x284 respectively).
3.2.2 Checking the quality of total RNA:

The quality of total RNA for each sample was checked by agarose gel electrophoresis using 1.5% (w/v) agarose gel. Total RNA (10 µl) was mixed with blue/orange loading dye (2 µl) to assist loading and monitor the resolution of the sample. The electrophoresis tank was run at 100V for about 45 minutes and then the total RNA was visualised using the Uvitec gel documentation system at 312 nm wavelength and the image captured. Intact total RNA should have sharp 28S and 18S rRNA bands (Skrypina et al., 2003), with the 28S rRNA band should be approximately twice as intense as the 18S rRNA band (figure 3.9). This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Furthermore, partially degraded RNA will not exhibit a 2:1 ratio whereas completely degraded RNA will appear as very low molecular weight smear (Ambion, USA). The results for all five non-balding samples showed clear bands with much stronger bands for 28S than 18S.

![Figure 3.9 Gel electrophoresis of total RNA](image)

**Figure 3.9 Gel electrophoresis of total RNA**

Total RNA (10 µl) from five non-balding hair follicles samples (1-5) were loaded on a 1.5% agarose gel. The rRNA bands (28S and 18S) exhibited 2:1 ratio and are clearly visible in the total RNA gel.
3.2.3 **Checking the quality of cDNA using β-actin:**

Prior to investigating the expression of the genes for HGF, MSP and their receptors, c-Met and RON, RT-PCR was initially performed using primers specific for the positive control, housekeeping gene, β-actin. The β-actin PCR products from all five individuals samples were separated using agarose gel electrophoresis and visualised with ethidium bromide staining (figure 3.10). The detection of β-actin as a housekeeping gene would denote that the isolated RNA from all experimental samples was of sufficient quality for reverse transcriptase PCR to be performed successfully. The expression of the β-actin sequence amplified by RT-PCR in all five individual samples were similar in size to that expected from the human sequence which is 838 bp (see figure 3.10).

When nuclease free water was used as a template in the negative control instead of cDNA, no PCR products were detected. This demonstrated that there was no DNA contamination in the samples used for RT-PCR and that the β-actin PCR products were the result of the amplification of cDNA synthesized from the mRNA samples.

The identity of the β-actin PCR products was verified by sequence analysis, a band of expected size was excised from the gel, purified and sent for sequencing. The β-actin PCR products of the human hair follicles exhibited 96% correlation with the known human sequence through the use of the ensemble genome browser and the NCBI BLAST program (figure 3.11).
Figure 3.10  Expression of β-actin in non-balding scalp hair follicles.

Reverse transcriptase PCR demonstrated expression of β-actin in mRNA from five human hair follicle samples. Hair follicle cDNA samples were taken from non-balding scalp of five individuals (3 males aged 30, 29, 37 and 2 females aged 37, 32). RT-PCR was performed using specific primers for β-actin. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2-6 human hair follicle β-actin PCR products (30 µl/lane). Lane 7 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 838 bp in length as indicated.
Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (|). The β-actin PCR products from human hair follicles showed 96% correlation with the known human sequence.
3.2.4 Human hair follicles express the genes for HGF, MSP and their receptors, c-Met and RON:

3.2.4.1 Human hair follicles express the gene for HGF

The expression of the gene for HGF in human hair follicles was investigated using RT-PCR. The resulting PCR products for HGF were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. All five human hair follicle cDNA samples produced PCR products of the expected size 262 bp for the gene of HGF (figure 3.12).

The negative control, in which cDNA was omitted from the PCR reaction mix and replaced with nuclease free water, was clear of any bands. This demonstrated that all PCR products were originated from the amplification of the cDNA synthesis from the mRNA samples and demonstrated an absence of any contamination.

Sequence analysis was used to ascertain the identity of the PCR products for HGF. Bands of expected size of HGF gene were excised from the gel, purified and sent for sequencing. The homology of the sequenced HGF PCR products was compared to the known expected human sequence, obtained from the ensemble genome browser, using the NCBI BLAST program. The PCR products which produced from the experimental samples exhibited 95% homology to the known human sequence for HGF (figure 3.13).
Figure 3.12 Expression of HGF in non-balding scalp hair follicles.

Reverse transcriptase PCR demonstrated expression of HGF in mRNA from five human hair follicle samples. Hair follicle cDNA samples were taken from non-balding scalp of five individuals (3 males aged 30, 29, 37 and 2 females aged 37, 32). RT-PCR was performed using specific primers for HGF. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2-6 human hair follicle HGF PCR products (30 µl/lane). Lane 7 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 262 bp in length as indicated.
Figure 3.13 Sequencing results for HGF RT-PCR product amplified by specific primers

Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (i). The HGF PCR products from human hair follicles showed 95% correlation with the known human sequence.
3.2.4.2 Human hair follicles express the gene for MSP:

The expression of the gene for MSP in human hair follicles was investigated using RT-PCR. The resulting PCR products for MSP were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. All five human hair follicle cDNA samples produced PCR products of the expected size 415 bp for the gene of MSP (figure 3.14). When nuclease free water was used as a template in the negative control instead of cDNA, no PCR products were detected. This demonstrated that there was no DNA contamination in the samples used for RT-PCR and that the MSP PCR products were the result of the amplification of cDNA synthesized from the mRNA samples.

Sequence analysis was used to ascertain the identity of the PCR products for MSP. Bands of expected size of MSP gene were excised from the gel, purified and sent for sequencing. The homology of the sequenced MSP PCR products was compared to the known expected human sequence, obtained from the ensemble genome browser, using the NCBI BLAST program. The PCR products which were produced from the experimental samples exhibited 97% homology to the known human sequence for MSP (figure 3.15).
Reverse transcriptase PCR demonstrated expression of MSP in mRNA from five human hair follicle samples. Hair follicle cDNA samples were taken from non-balding scalp of five individuals (3 males aged 30, 29, 37 and 2 females aged 37, 32). RT-PCR was performed using specific primers for MSP. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2-6 human hair follicle MSP PCR products (30 µl/lane). Lane 7 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 415 bp in length as indicated.
Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (i). The MSP PCR products from human hair follicles showed 97% correlation with the known human sequence.
3.2.4.3 *Human hair follicles express the gene for RON:*

The expression of the gene for the MSP receptor, RON, in human hair follicles was investigated using RT-PCR. The resulting PCR products for RON were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. All five human hair follicle cDNA samples produced PCR products of the expected size 217 bp for the gene of MSP (figure 3.16).

The negative control, in which cDNA was omitted from the PCR reaction mix and replaced with nuclease free water, was clear of any bands. This demonstrated that all PCR products were originated from the amplification of the cDNA synthesis from the mRNA samples and demonstrated an absence of any contamination.

Sequence analysis was used to ascertain the identity of the PCR products for RON. Bands of expected size of RON gene were excised from the gel, purified and sent for sequencing. The homology of the sequenced RON PCR products was compared to the known expected human sequence, obtained from the ensemble genome browser, using the NCBI BLAST program. The PCR products which were produced from the experimental samples exhibited 94% homology to the known human sequence for RON (figure 3.17).
Figure 3.16 Expression of RON in non-balding scalp hair follicles.

Reverse transcriptase PCR demonstrated expression of RON in mRNA from five human hair follicle samples. Hair follicle cDNA samples were taken from non-balding scalp of five individuals (3 males aged 30, 29, 37 and 2 females aged 37, 32). RT-PCR was performed using specific primers for RON. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2-6 human hair follicle RON PCR products (30 µl/lane). Lane 7 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 217 bp in length as indicated.
Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (׀). The RON PCR products from human hair follicles showed 94% correlation with the known human sequence.
3.2.4.4 Human hair follicles express the gene for c-Met:

The expression of the gene for the HGF receptor, c-Met, in human hair follicles was investigated using RT-PCR. The resulting PCR products for c-Met were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. All five human hair follicle cDNA samples produced PCR products of the expected size 536 bp for the gene of c-Met (figure 3.18).

When nuclease free water was used as a template in the negative control instead of cDNA, no PCR products were detected. This demonstrated that there was no DNA contamination in the samples used for RT-PCR and that the c-Met PCR products were the result of the amplification of cDNA synthesized from the mRNA samples.

Sequence analysis was used to ascertain the identity of the PCR products for c-Met. Bands of expected size of c-Met gene were excised from the gel, purified and sent for sequencing. The homology of the sequenced c-Met PCR products was compared to the known expected human sequence, obtained from the ensemble genome browser, using the NCBI BLAST program. The PCR products which were produced from the experimental samples exhibited 95% homology to the known human sequence for c-Met (figure 3.19).
Figure 3.18 Expression of c-Met in non-balding scalp hair follicles.

Reverse transcriptase PCR demonstrated expression of c-Met in mRNA from five human hair follicle samples. Hair follicle cDNA samples were taken from non-balding scalp of five individuals (3 males aged 30, 29, 37 and 2 females aged 37, 32). RT-PCR was performed using specific primers for c-Met. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (range from 100-1,500) (10 µl was loaded), Lane 2-6 human hair follicle c-Met PCR products (30 µl/lane). Lane 7 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 536 bp in length as indicated.
Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (i). The c-Met PCR products from human hair follicles showed 95% correlation with the known human sequence.
3.3 RT-PCR analysis of the expression of HGF, RON and c-Met in human hair bulb components:

3.3.1 Isolation of whole lower anagen hair follicles by microdissection:

To investigate the expression of HGF, RON and c-Met and to possibly localise the expression of these genes in human hair follicles, whole cleaned lower anagen hair follicles were dissected to isolate the three cell types of the hair bulb including the dermal sheath, the epithelial matrix and the dermal papilla (see section 2.1.3.2), (figures 2.1, 3.20, 3.21). Three different individual samples were used for this investigation. A total of 150 hair follicles were isolated from each individual (see table 2.2). Equal numbers of the three cell types of the hair bulb were collected separately from each individual for the total RNA isolation, poly (A) RNA isolation and cDNA synthesis.
Figure 3.20 Human hair bulb components

Isolated human hair bulb (A) from anagen hair follicle (male, age 35) and its components (B), scale bar (195 μm & 189 μm respectively). Human scalp anagen hair follicles were further dissected to isolate the dermal sheath, hair matrix and dermal papilla for total RNA isolation and later RT-PCR analysis to investigate genes expression. The hair bulb components were microdissected from 150 follicles from each of 3 individuals and processed separately.
whole cleaned lower human anagen hair follicles were further dissected to isolate the dermal sheath (A), the epithelial hair matrix (B), and the dermal papilla (C). A total of 150 hair follicles were isolated from each individual (n=3) for the purpose of total RNA isolation and later investigating the expression of specific molecules using RT-PCR. Each sample of the three individuals was processed separately. (x284, x286, x300 respectively).

Figure 3.21 Isolated human hair bulb components
3.3.2 Checking the quality of cDNA, produced from human hair bulb components, using β-actin:

Prior to investigation the expression of the genes for HGF, RON and HGF receptor, c-Met, RT-PCR was initially performed using primers specific for the positive control, housekeeping gene β-actin. The resulting β-actin PCR products from each component were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. The β-actin PCR products from all three cell types of the hair bulb corresponded to the anticipated size of the β-actin (838 bp), (figure 3.22). When the same amounts of cDNA were used, the resulting β-actin PCR products from the bulb matrix cDNA in all three samples showed the strongest intensity bands, followed by the dermal sheath and then the dermal papilla cDNA which exhibited the weakest bands. The negative controls, in which the template cDNA was excluded from the reaction mix, were clear of any bands. This indicated that there was no DNA contamination in the samples used for RT-PCR (figures 3.22).
Figure 3.22  Expression of β-actin in non-balding scalp hair follicle components

Human hair bulb components: the dermal sheath, the matrix and the dermal papilla, were microdissected from 150 follicles from each of 3 individuals [male, age 35 (A); female, age 45 (B); male, age 29 (C)] and processed separately. mRNA was extracted and RT-PCR was performed using specific primers for β-actin. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane L denotes 100bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane DS dermal sheath β-actin PCR products (30 µl). Lane MX matrix β-actin PCR products (30 µl). Lane DP dermal papilla β-actin PCR products (30 µl). Lane N contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 838 bp in length as indicated.
3.3.3  RT-PCR analysis of the expression of RON in human hair bulb components:

To investigate the expression of RON in the hair bulb components, RT-PCR was further utilized using primers specific for RON. The RON PCR products, which produced from all three hair bulb components, corresponded to the expected size of (217 bp). They were all expressed much less strongly than β-actin. When the same amounts of cDNA were used, the RON expression was present with greater intensity in the matrix, followed by the dermal sheath cDNA and with the weakest band produced from the dermal papilla cDNA. This is a reflection of the β-actin bands produced from these three hair bulb components. The negative controls were free of any contaminating bands. Therefore, it seems that the dermal sheath, matrix and dermal papilla cells express the gene for RON (figures 3.23).
Figure 3.23 Expression of RON in non-balding scalp hair follicle components

Human hair bulb components: the dermal sheath, the matrix and the dermal papilla, were microdissected from 150 follicles from each of 3 individuals [male, age 35 (A); female, age 45 (B); male, age 29 (C)] and processed separately. mRNA was extracted and RT-PCR was performed using specific primers for RON. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane L denotes 100bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane DS dermal sheath RON PCR products (30 µl). Lane MX matrix RON PCR products (30 µl). Lane DP dermal papilla RON PCR products (30 µl). Lane N negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 217 bp in length as indicated.
3.3.4  *RT-PCR analysis of the expression of HGF in human hair bulb components:*

The expression of HGF in human hair follicles was further clarified by the investigation of its expression in the three cell types of the hair bulb using the RT-PCR. Bands corresponding to the expected size of HGF (261 bp) were produced from the dermal sheath and the dermal papilla samples. When the same amounts of cDNA were used, no bands of the anticipated size of HGF were produced from the matrix cDNA samples. The HGF bands produced from the dermal sheath cDNA were of greater intensity than those produced from the dermal papilla cDNA. This reflects the differences in the cDNA quantity observed in the β-actin (figure 3.22). This indicated that the dermal sheath and dermal papilla cells express the HGF but not the matrix cells (figures 3.24).
Figure 3.24 Expression of HGF in non-balding scalp hair follicle components

Human hair bulb components: the dermal sheath, the matrix and the dermal papilla, were microdissected from 150 follicles from each of 3 individuals [male, age 35 (A); female, age 45 (B); male, age 29 (C)] and processed separately. mRNA was extracted and RT-PCR was performed using specific primers for HGF. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane L denotes 100bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane DS dermal sheath HGF PCR products (30 µl). Lane DP dermal papilla HGF PCR products (30 µl). Lane MX matrix HGF PCR products (30 µl). Lane N contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 262 bp in length as indicated.
3.3.5 RT-PCR analysis of the expression of HGF receptor, c-Met, in human hair bulb components:

The gene expression of c-Met in the hair bulb components were further investigated using RT-PCR. The PCR products of the anticipated size for c-Met (536 bp) were produced from the dermal sheath and the dermal papilla samples but not from any of the matrix samples. When the same amounts of cDNA were used, the resulting c-Met PCR products produced from the dermal sheath was of greater intensity than the band produced from the dermal papilla (figures 3.25). This is a reflection of the β-actin PCR products produced from the hair bulb components (figure 3.22).
Figure 3.25 Expression of c-Met in non-balding scalp hair follicle components

Human hair bulb components: the dermal sheath, the matrix and the dermal papilla, were microdissected from 150 follicles from each of 3 individuals [male, age 35 (A); female, age 45 (B); male, age 29 (C)] and processed separately. mRNA was extracted and RT-PCR was performed using specific primers for c-Met. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane L denotes 100bp DNA molecular size marker (range from 100-1500) (10 µl was loaded), Lane DS dermal sheath c-Met PCR products (30 µl), Lane DP dermal papilla c-Met PCR products (30 µl), Lane MX matrix c-Met PCR products (30 µl). Lane N contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 536 bp in length as indicated.
3.4 Immunohistochemistry:

3.4.1 Localisation of cytokeratin 6 in human hair follicles:

The location of cytokeratin 6, a highly expressed cytoskeletal protein, was investigated in human hair follicles to learn the technique of immunohistochemistry using a highly expressed antigen. Immunohistochemistry was performed using a goat polyclonal anti cytokeratin 6 antibody which was diluted for use in human hair follicles at 1:20 with 1.5% (v/v) normal mouse serum in PBS. Prior to treatment with the primary antibody, sections were blocked using 5% (v/v) normal mouse serum in PBS to block non-specific protein binding sites in the tissue.

Three negative controls were included in each experiment to detect any non-specific binding to ensure that the colour detection system was not activated alone, to ensure that the secondary antibody is not binding non-specifically to the tissue, and that the primary antibody was not activating the red chromogen AEC solution without the presence of the secondary antibody.

All the negative controls were clear of any non-specific staining. Therefore only the photograph of the negative control, where the primary antibody was replaced with PBS, was included. Strong positive staining of cytokeratin 6 was present in the outer root sheath (figure 3.26, 3.27).
Figure 3.26 Expression of cytokeratin 6 in human scalp hair follicle.

Immunohistochemistry for cytokeratin 6, in 35 year old male scalp hair follicle, was performed using the goat polyclonal anti-cytokeratin 6 antibody (Novocastra Laboratories, UK) diluted 1:20 with 1.5% (v/v) NMS/PBS. Prior to treatment with the antibody, the tissue was blocked with 5% (v/v) normal mouse serum in PBS.

(A) & (B) Negative control for cytokeratin 6. The section is free from any uptake of stain. The primary antibody was replaced with PBS however the secondary antibody and the AEC solution were applied. Scale bar (32 µm), magnification (x110).

(C) & (D) Strong positive red staining of cytokeratin 6 can be seen in cells of the outer root sheath (ORS). Scale bar (29 µm), magnification (x120).
Figure 3.27 Expression of cytokeratin 6 in cross section of human scalp hair follicle.

Immunohistochemistry for cytokeratin 6, in scalp hair follicle, was from a 35 year old male performed using the goat polyclonal anti-cytokeratin 6 antibody (Novocastra Laboratories, UK) diluted 1:20 with 1.5% (v/v) NMS/PBS. Prior to treatment with the antibody, the tissue was blocked with 5% (v/v) normal mouse serum in PBS. Sections were counterstained with Harris's haematoxylin.

(A) Negative control for cytokeratin 6. The section is free from any uptake of stain. The primary antibody was replaced with PBS however the secondary antibody and the AEC solution were applied. Scale bar (27µm), magnification (x152).

(B) Strong positive staining of cytokeratin 6 can be seen in cells of the outer root sheath (ORS). Scale bar (27µm), magnification (x152).
3.4.2 Immunohistochemical investigation of HGF receptor, c-Met, in human hair follicles:

The expression of hepatocyte growth factor receptor, c-Met, was investigated in human hair follicles using immunohistochemistry. A mouse monoclonal anti-human c-Met antibody was applied to the sections with 1.5% (v/v) bovine serum albumin in PBS. A range of antibody concentrations were examined between 1:10 and 1:100 until the optimum one was achieved which is 1:20. Prior to treatment with the primary antibody, sections were blocked using 1.5% (v/v) bovine serum albumin in PBS.

Three negative controls were included in each experiment to detect any non-specific binding to ensure that the colour detection system was not activated alone, to ensure that the secondary antibody is not binding non-specifically to the tissue, and that the primary antibody was not activating the red chromogen AEC solution without the presence of the secondary antibody.

No positive staining was observed in all negative controls so only the photograph of the negative control, where the primary antibody was replaced with PBS, was included. The presence of c-Met was detected in the outer root sheath and the dermal papilla of human hair follicles (figure 3.28, 3.29).
Figure 3.28 Expression of the HGF receptor, c-Met, in human scalp hair follicle.

Immunohistochemistry for the HGF receptor, c-Met, in 35 year old male scalp hair follicle, was performed using the mouse monoclonal anti-human c-Met antibody (Novocastra Laboratories, UK) diluted 1:20 with 1.5% (v/v) BSA/PBS. Prior to treatment with the antibody, the tissue was blocked with 5% (v/v) bovine serum albumin in PBS.

(A) & (B) Negative controls staining for the HGF receptor, c-Met. The section is free from any uptake of stain. The primary antibody was replaced with PBS however the secondary antibody and the AEC solution were applied. Scale bar (30 µm, 25 µm), magnification (x115, x120).

(C) & (D) Positive red staining of HGF receptor, c-Met, can be seen in cells of the outer root sheath (ORS). Scale bar (27 µm), magnification (x132).

(E), (F) & (G) Positive red staining of c-Met in the ORS of cross sections of hair follicles. Scale bar (29 µm), magnification (x120, x149).
Figure 3.29 Expression of the HGF receptor, c-Met, in the dermal papilla of human scalp hair follicle.

Immunohistochemistry for the HGF receptor, c-Met, in the dermal papilla of human scalp hair follicle, was performed using the mouse monoclonal anti-human c-Met antibody (Novocastra Laboratories, UK) diluted 1:20 with 1.5% (v/v) BSA/PBS. Prior to treatment with the antibody, the tissue was blocked with 5% (v/v) bovine serum albumin in PBS.

(A) Negative controls staining for the HGF receptor, c-Met. The section is free from any uptake of stain. The primary antibody was replaced with PBS however the secondary antibody and the AEC solution were applied. Scale bar (17 µm), (x375).

(B) Positive red staining of HGF receptor, c-Met, can be seen in cells of the dermal papilla. Scale bar (17 µm), magnification (x375).
3.5 RT-PCR analysis of the expression of the genes for HGF, MSP and their receptors, c-Met and RON, in human cultured dermal papilla cells:

Cultured dermal papilla cells from two individuals were used to investigate the gene expression for HGF, MSP, c-Met and RON using RT-PCR. The dermal papilla cells in culture increased in number gradually to give multi-polar shapes and formed small clumps (figure 3.30). When confluent, they were collected for RNA extraction, cDNA synthesis and gene expression investigation.

![Phase contrast images of human cultured dermal papilla cells.](image)

**Figure 3.30** phase contrast images of human cultured dermal papilla cells.

Dermal papilla (DP) cells were cultured for the purpose of total RNA extraction and cDNA synthesis to investigate gene expression using RT-PCR, scale bar (25 µm).
3.5.1 Checking the quality of cultured DP cells cDNA using β-actin:

Prior to investigating the expression of the genes for HGF, MSP and their receptors, c-Met and RON, RT-PCR was initially performed using primers specific for the positive control, housekeeping gene, β-actin. The β-actin PCR products from the two individual samples were separated using agarose gel electrophoresis and visualised with ethidium bromide staining (figure 3.31). The detection of β-actin as a housekeeping gene would denote that the isolated RNA from the experimental samples was of sufficient quality for reverse transcriptase PCR to be performed successfully. The expression of the β-actin sequence amplified by RT-PCR in both individual samples were similar in size to that expected from the human sequence which is 838 bp (see figure 3.31).

When nuclease free water was used as a template in the negative control instead of cDNA, no PCR products were detected. This demonstrated that there was no DNA contamination in the samples used for RT-PCR and that the β-actin PCR products were the result of the amplification of cDNA synthesized from the mRNA samples.

The identity of the β-actin PCR products was verified by sequence analysis, a band of expected size was excised from the gel, purified and sent for sequencing. The β-actin PCR products of the human cultured dermal papilla cells exhibited 98% correlation with the known human sequence through the use of the ensemble genome browser and the NCBI BLAST program (figure 3.32).
Figure 3.31 Expression of β-actin in human cultured dermal papilla cells.

Reverse transcriptase PCR demonstrated expression of β-actin in mRNA from two human dermal papilla samples. The cDNA samples were taken from non-balding dermal papilla cells of two individuals. RT-PCR was performed using specific primers for β-actin. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2 & 3 contained human cultured dermal papilla β-actin PCR products (20 µl/lane). Lane 4 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 838 bp in length as indicated.
Figure 3.32 Sequencing results for β-actin RT-PCR product amplified by specific primers

Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (i). The β-actin PCR products from cultured dermal papilla cells showed 98% correlation with the known human sequence.
3.5.2 Human cultured dermal papilla cells express the gene for HGF:

The expression of the gene for HGF in human cultured dermal papilla cells was investigated using RT-PCR. The resulting PCR products for HGF were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. Both dermal papilla cDNA samples produced PCR products of the expected size 262 bp for the gene of HGF (figure 3.33).

The negative control, in which cDNA was omitted from the PCR reaction mix and replaced with nuclease free water, was clear of any bands. This demonstrated that all PCR products were originated from the amplification of the cDNA synthesis from the mRNA samples and demonstrated an absence of any contamination.

Sequence analysis was used to ascertain the identity of the PCR products for HGF. Bands of expected size of HGF gene were excised from the gel, purified and sent for sequencing. The homology of the sequenced HGF PCR products was compared to the known expected human sequence, obtained from the ensemble genome browser, using the NCBI BLAST program. The PCR products which produced from the experimental samples exhibited 99% homology to the known human sequence for HGF (figure 3.34).
Figure 3.33 Expression of HGF in human cultured dermal papilla cells.

Reverse transcriptase PCR demonstrated expression of HGF in mRNA from two human dermal papilla samples. The cDNA samples were taken from non-balding dermal papilla cells of two individuals. RT-PCR was performed using specific primers for HGF. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2 & 3 contained human cultured dermal papilla HGF PCR products (20 µl/lane). Lane 4 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 262 bp in length as indicated.
Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (|). The HGF PCR products from human cultured dermal papilla cells showed 99% correlation with the known human sequence.
3.5.3 Human cultured dermal papilla cells express the gene for MSP:

The expression of the gene for MSP in human cultured dermal papilla cells was investigated using RT-PCR. The resulting PCR products for MSP were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. Both dermal papilla cDNA samples produced PCR products of the expected size 415 bp for the gene of MSP (figure 3.35).

When nuclease free water was used as a template in the negative control instead of cDNA, no PCR products were detected. This demonstrated that there was no DNA contamination in the samples used for RT-PCR and that the MSP PCR products were the result of the amplification of cDNA synthesized from the mRNA samples.

Sequence analysis was used to ascertain the identity of the PCR products for MSP. Bands of expected size of MSP gene were excised from the gel, purified and sent for sequencing. The homology of the sequenced MSP PCR products was compared to the known expected human sequence, obtained from the ensemble genome browser, using the NCBI BLAST program. The PCR products which were produced from the experimental samples exhibited 96% homology to the known human sequence for MSP (figure 3.36).
Figure 3.35 Expression of MSP in human cultured dermal papilla cells.

Reverse transcriptase PCR demonstrated expression of MSP in mRNA from two human dermal papilla samples. The cDNA samples were taken from non-balding dermal papilla cells of two individuals. RT-PCR was performed using specific primers for MSP. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2 & 3 contained human cultured dermal papilla MSP PCR products (20 µl/lane). Lane 4 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 415 bp in length as indicated.
Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (|). The MSP PCR products from human cultured dermal papilla cells showed 96% correlation with the known human sequence.
3.5.4 Human cultured dermal papilla cells express the gene for RON:

The expression of the gene for the MSP receptor, RON, in human cultured dermal papilla cells was investigated using RT-PCR. The resulting PCR products for RON were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. Both dermal papilla cDNA samples produced PCR products of the expected size 217 bp for the gene of MSP (figure 3.37).

The negative control, in which cDNA was omitted from the PCR reaction mix and replaced with nuclease free water, was clear of any bands. This demonstrated that all PCR products were originated from the amplification of the cDNA synthesis from the mRNA samples and demonstrated an absence of any contamination.

Sequence analysis was used to ascertain the identity of the PCR products for RON. Bands of expected size of RON gene were excised from the gel, purified and sent for sequencing. The homology of the sequenced RON PCR products was compared to the known expected human sequence, obtained from the ensemble genome browser, using the NCBI BLAST program. The PCR products which were produced from the experimental samples exhibited 97% homology to the known human sequence for RON (figure 3.38).
Figure 3.37 Expression of RON in human cultured dermal papilla cells.

Reverse transcriptase PCR demonstrated expression of RON in mRNA from two human dermal papilla samples. The cDNA samples were taken from non-balding dermal papilla cells of two individuals. RT-PCR was performed using specific primers for RON. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100 bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2 & 3 contained human cultured dermal papilla RON PCR products (20 µl/lane). Lane 4 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 217 bp in length as indicated.
Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (|). The RON PCR products from human cultured dermal papilla cells showed 97% correlation with the known human sequence.

**Figure 3.38 Sequencing results for RON RT-PCR product amplified by specific primers**
3.5.5 Human cultured dermal papilla cells express the gene for c-Met:

The expression of the gene for the HGF receptor, c-Met, in human cultured dermal papilla cells was investigated using RT-PCR. The resulting PCR products for c-Met were separated using agarose gel electrophoresis and visualised with ethidium bromide staining. Both dermal papilla cDNA samples produced PCR products of the expected size 536 bp for the gene of c-Met (figure 3.39).

When nuclease free water was used as a template in the negative control instead of cDNA, no PCR products were detected. This demonstrated that there was no DNA contamination in the samples used for RT-PCR and that the c-Met PCR products were the result of the amplification of cDNA synthesized from the mRNA samples.

Sequence analysis was used to ascertain the identity of the PCR products for c-Met. Bands of expected size of c-Met gene were excised from the gel, purified and sent for sequencing. The homology of the sequenced c-Met PCR products was compared to the known expected human sequence, obtained from the ensemble genome browser, using the NCBI BLAST program. The PCR products which were produced from the experimental samples exhibited 94% homology to the known human sequence for c-Met (figure 3.40).
Figure 3.39 Expression of c-Met in human cultured dermal papilla cells.

Reverse transcriptase PCR demonstrated expression of c-Met in mRNA from two human dermal papilla samples. The cDNA samples were taken from non-balding dermal papilla cells of two individuals. RT-PCR was performed using specific primers for c-Met. PCR products were applied to 1.5% (w/v) agarose gel for electrophoresis with ethidium bromide staining. DNA was visualized with UV illumination. Lane 1 denotes 100bp DNA molecular size marker (range from 100-1,500) (10 µl was loaded), Lane 2 & 3 contained human dermal papilla c-Met PCR products (20 µl/lane). Lane 4 contained the negative control in which nuclease free water was used as a template instead of cDNA. The cDNA amplification product was predicted to be 536 bp in length as indicated.
Figure 3.40 Sequencing results for c-Met RT-PCR product amplified by specific primers

Following PCR products analysis by electrophoresis, the identity of DNA fragments were verified and confirmed by sequence analysis. The sequencing data (black) were compared to the previously identified sequence (red) using NCBI BLAST programme. The query displays expected known gene sequence and the subject is the sequenced PCR product. Matching bases between the two sequences are indicated (|). The c-Met PCR products from human cultured dermal papilla cells showed 94% correlation with the known human sequence.
3.6 Comparison of the gene expression of normal and balding scalp hair follicles from men with androgenetic alopecia using DNA microarray:

3.6.1 Checking the quality of total RNA:

The most important key for successful GeneChip microarray analysis is starting with high quality RNA. The total RNA quality and purity of the six RNA samples from the three matched pairs of samples of balding and non-balding anagen hair follicles (see section 2.2.1) were evaluated using the Agilent 2100 Bioanalyzer (Agilent technologies, USA). Intact total RNA should have sharp 28S and 18S rRNA bands (Skrypina et al., 2003), with the 28S rRNA band being approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is intact and not degraded. Furthermore, partially degraded RNA will not exhibit a 2:1 ratio, whereas completely degraded RNA will appear as a very low molecular weight smear (Ambion, USA). The results for all 6 samples showed clear bands with much stronger bands for 28S than 18S (figure 3.41). Non-balding samples gave stronger bands than those from balding follicles.

Further quality check on the RNA were carried out by using the Agilent Bioanalyzer to produce Electropherograms. High quality RNA electropherograms show several features: there should be clear and well defined 28S and 18S peaks, with a low background between the peaks, as well as minimal low molecular weight contamination. Moderately degraded RNA is characterised by the presence of a smaller 28S peak than that of 18S, high background between the 28S and 18S peaks and low molecular weight peaks. These features tend to interfere with the labelling and amplification which can lead to possibly poor array performance. Extreme degradation is characterized by the absence
of 28S and 18S peaks, and the presence of only low molecular weight peaks. This would badly affect in the labelling procedure and could not be used (Agilent, USA). Electropherograms (figures 3.42 and 3.43) showed high quality RNA had been prepared for all the follicle samples for the following reasons: there are clear 28S and 18S peaks, the noise between the peaks is very low with minimal low molecular weight noise and the ratios are close to two.

RNA from balding samples (n=3) and non-balding samples (n=3) were evaluated using the Agilent 2100 Bioanalyzer. The rRNA bands (28S and 18S) exhibited 2:1 ratio and are clearly visible in the total RNA gels.
Fig 3.42  Electropherograms of non-balding hair follicle RNA samples

RNA obtained from non-balding samples (n=3) were evaluated using the Agilent 2100 Bioanalyzer. The graphs show high quality RNA for the following reasons: there are clear 28S and 18S peaks, the noise between the peaks is very low with minimal low molecular weight noise and the ratios are close to 2.
Fig 3.43 Electropherograms of balding hair follicles RNA samples

RNA obtained from balding samples (n=3) were evaluated using the Agilent 2100 Bioanalyzer. The graphs show high quality RNA for the following reasons: there are clear 28S and 18S peaks, the noise between the peaks is very low, with minimal low molecular weight noise and the ratios are close to 2.
3.6.2 Microarray data analysis and data quality assessment:

The difference in gene expression between balding and non-balding hair follicles in individuals with androgenetic alopecia was investigated using DNA microarray analysis. Initially, the microarray data were assessed for quality and overall differences between the two groups of matched non-balding and balding follicles.

3.6.2.1 Data normalization and transformation:

The microarray fluorescence intensity values were normalized using the Robust Multi-array Analysis (RMA) method (Irizarry et al., 2003). These values were transformed to a logarithmic scale. The normalization process aims to remove certain systematic biases from microarray data (Smyth & Speed, 2003). The normalized intensity values were visualized using Box-and-Whiskers plots (figure 3.44), which displays the distribution of these values for the experimental groups, balding follicles (n=3) and non-balding follicles (n=3). To better display this large range of values and to ensure a more even representation of data, the box plot was generated by log signal intensities rather than absolute signal intensities (Quackenbush, 2002). The key features of the box plot are the smallest observation (minimum), the largest observation (maximum), the first quartile (Q1) or the 25\textsuperscript{th} percentile of the values, and the third quartile (Q3) or the 75\textsuperscript{th} percentile of the values. The band near the middle of the box represents the median or the second quartile (Q2) or the 50\textsuperscript{th} percentile of the values. Values not included between the whiskers represent values beyond 1.5 times IQR. It is clear from the graph that the variance and shape of distribution are different in both conditions. This indicates that the normalized values are appropriate to be taken to subsequent steps of analysis.
Fig 3.44 Box-and-Whiskers plot displaying the distribution of microarray intensity values in balding and non-balding follicle samples.

The diagram showing the distribution of the intensity values for non-balding samples (n=3) and balding samples (n=3) through their five-number summary including the minimum and maximum observations, the first quartile (Q1), the third quartile (Q3) and the median (Q2). The inter quartile range (IQR) is the range between Q1 and Q3. Values beyond 1.5 times IQR are shown in red. It is clear from the graph that the variance and shape of distribution are different in both conditions.
3.6.2.2 Correlation between grouped data:

Since the relationship between two variables is described by their correlation, correlation analysis of the normalized fluorescence intensity values of each gene by all balding and all non-balding follicles was carried out (figure 3.45). A scatter diagram has been used as one of the tools of quality control to check the quality of these data (Lee and Park, 2007). The graph in figure 3.45 gives a clue that the two variables are related and how they move together. This plot showed the correlation between the gene expression of balding samples (n=3) and non-balding ones (n=3). The results showed that the correlation coefficient equals -0.74 indicating there is a negative correlation (P<0.03) in gene expression between the balding and non-balding samples. The correlation curve on the plot indicates that the gene expression pattern follows a downward slope and it moves down and to the right from a high-value on the y-axis down to a high-value on the x-axis. In other word, when the balding sample has high fluorescence associated with expression of one gene, then the non-balding sample is tending to have a low amount of fluorescence, i.e binding or expression. In addition, the reverse pattern also occurs with high fluorescence in a non-balding sample corresponding to low expression in a balding one.
Fig 3.45 Scatter plot shows the correlation of gene expression between balding and non-balding follicles.

The diagram reveals the correlation between the gene expression of balding follicles (n=3) versus non-balding follicles (n=3). The correlation curve shows that gene expression follows a downward slope indicating that the pattern of gene expression is running in a band from upper left to lower right. The correlation coefficient equals -0.74 indicating there is a negative correlation (P<0.03) in gene expression between the balding group and non-balding one.
3.6.2.3 Principal Component Analysis (PCA):

The quality of the microarray data was investigated further using the principal component analysis (PCA) method. This test provides a roadmap for reducing a complex data set to a lower dimension (Komura et al., 2004; Shlens, 2009). The three dimensional graph (3D) in figure 3.46 shows each sample discrimination and shows clear differences between balding and non-balding follicles. Each sample demonstrated variations from each of the others. However, the balding group is separated distinctly from the non-balding group. The principal component 1 (PC1), principal component 2 (PC2) and principal component 3 (PC3) are plotted on the x, y and z axes respectively. PC1 and PC2 indicate 20.68% and 17.17% variations respectively within the same group, whereas PC3 indicate 55.36% variation when all samples are grouped together. This denotes that there are differences in gene expression between balding and non-balding follicle samples.
Fig 3.46 Principal components analysis (PCA) shows the discrimination of balding and non-balding samples.

Three-dimensional (3D) graph, PCA analysis, shows that the experimental samples separated into two distinct groups, balding (blue dots) and non-balding (red dots). Principal components (PC): PC1, PC2 and PC3 are plotted on the x, y and z axes respectively. PC1 and PC2 indicate 20.68% and 17.17% variation respectively within the same group, whereas PC3 indicates 55.36% variation in the total gene expression.
3.6.3 Comparison of overall differences in gene expression between balding and non-balding follicles in androgenetic alopecia:

3.6.3.1 Sample clustering:

To obtain an overall view of the similarities and differences between the non-balding and balding hair follicles, gene clusters were compared in each individual sample. Genes were organized into balding versus non-balding follicles by both individual samples as well as grouped samples. Genes that have the most similar expression profiles were clustered together and arranged based on similarity in pattern of gene expression (Eisen et al., 1998).

The hierarchical clustering analysis showed a clear variability in gene expression in each individual though there were obvious pattern of similarities between the three non-balding and three balding samples (figure 3.47). When the samples were combined into two groups, balding and non-balding (figure 3.48), the clustering analysis showed that the expression of genes were segregated into discrete clusters in the balding group compared to the control, non-balding one.
Fig 3.47 A comparison of the gene expression profile in individual balding and non-balding follicle samples from androgenetic alopecia men using a microarray profiling approach. Hierarchical clustering diagram showing individual expression patterns for balding samples (2, 4, 6) versus non-balding ones (1, 3, 5). The diagram shows clear differences in gene expression between the two conditions in each individual. The colour scale for gene expression is shown on the left.
Fig 3.48 A comparison of the gene expression profile in pooled balding and non-balding follicle samples from androgenetic alopecia men using a microarray profiling approach. Hierarchical clustering diagram showing pooled data obtained from balding samples (n=3) versus non-balding ones (n=3) when the individual samples were combined. The diagram shows clear differences in gene expression between the two conditions. The colour scale for gene expression is shown on the right.
3.6.3.2 **Statistical analysis:**

A statistically based method, the t-test, was used to analyse the meanings of the microarray experimental results to determine which genes were significantly differentially expressed, and to assess the associated uncertainty. It takes the variability of gene expression into account on a gene-per-gene basis. After applying the t-test, a total of 2,481 genes were differentially expressed in balding follicles, out of the 28,869 genes on the microarray gene chip, and showed a significant change in gene expression with p-values of less than 0.05 (figure 3.49 A). Analysis of the gene list showed that 1,462 genes were up-regulated and 1019 genes were down-regulated in balding scalp (figure 3.49 B). Table 3.2 summarizes the number of genes changed significantly in balding samples compared to non-balding ones when different levels of significant were used. Significantly differentially expressed genes, 2,481 genes in total, were visualized by drawing a volcano plot which highlighted the fold change between the conditions (figure 3.50).

<table>
<thead>
<tr>
<th>P value</th>
<th>&lt; 0.05</th>
<th>&lt; 0.01</th>
<th>&lt; 0.005</th>
<th>&lt; 0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes</td>
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<td>781</td>
<td>469</td>
<td>115</td>
</tr>
<tr>
<td>Up-regulated</td>
<td>1462</td>
<td>485</td>
<td>301</td>
<td>77</td>
</tr>
<tr>
<td>Down-regulated</td>
<td>1019</td>
<td>296</td>
<td>168</td>
<td>38</td>
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</tbody>
</table>

**Table 3.2** The number of genes changed significantly (P<0.05) in balding versus non-balding scalp follicles after performing the t-test with the data obtained from the DNA microarray analysis.
Figure 3.49 Changes in the number of genes expressed between non-balding and balding scalp follicles from men with androgenetic alopecia

Diagram (A) displays the total number of genes (2,481) which changed significantly (P<0.05) in balding versus matched non-balding scalp follicles after performing the t-test with the data obtained from the DNA microarray analysis.

Diagram (B) displays the number of genes whose expression was up and down regulated significantly (P<0.05) in balding follicles compared to matched non-balding follicles after performing the t-test with the data obtained from the DNA microarray analysis.
Figure 3.50 Volcano plot showing differentially expressed genes in balding follicles compared to non-balding follicles.

The diagram shows a volcano plot displaying differentially expressed genes in balding versus non-balding samples for the data obtained from the DNA microarray analysis. Significantly different genes, 2,481 out of 28,869 genes detected as changed, are highlighted in red (P<0.05). The y-axis is the negative log of the p-value at base 10, while the x-axis is the log₂ of the fold change.
3.6.3.3 Fold change (FC) analysis:

Fold change analysis was also used to identify genes with expression differences between balding samples and non-balding ones. There is no associated value of fold change that can indicate the level of confidence in the designation of genes as differentially expressed or not differentially expressed (Cui and Churchill, 2003), though it is frequently used as an easy measure of difference. Table 3.3 and figure 3.51 display the changes in the number of genes expressed significantly differently after the t-test in balding versus non-balding follicles with different fold change cut-offs. Twelve genes were expressed differently using a 2 fold cut-off which did not show significance using the t-test, whereas it was 276 genes in the case of using a 1.5 fold cut-off (see figure 3.50).

<table>
<thead>
<tr>
<th>Fold change</th>
<th>FC&gt; 1.0</th>
<th>FC&gt; 1.5</th>
<th>FC&gt; 2.0</th>
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<tr>
<td>Total number of genes</td>
<td>2481</td>
<td>370</td>
<td>68</td>
</tr>
<tr>
<td>Up-regulated</td>
<td>1462</td>
<td>203</td>
<td>41</td>
</tr>
<tr>
<td>Down-regulated</td>
<td>1019</td>
<td>167</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 3.3 The number of genes changed significantly (P<0.05) in balding versus non-balding scalp follicles after performing the t-test and fold change filters with the data obtained from the DNA microarray analysis.
Figure 3.51 Changes in the number of significantly differentially expressed genes between non-balding and balding scalp follicles from men with androgenetic alopecia expressed by fold change

Diagram (A) displays the total number of genes (2,481) which changed significantly (P<0.05) in balding versus matched non-balding scalp follicles after performing the t-test and fold change filter with the data obtained from the DNA microarray analysis.

Diagram (B) displays the number of genes whose expression was up and down regulated significantly (P<0.05) in balding follicles compared to matched non-balding follicles after performing the t-test and fold change filter with the data obtained from the DNA microarray analysis.
The number of genes changed significantly (P<0.05) in balding follicles was classified further. Table 3.4 and figure 3.52 show the changes in the number of genes expressed differentially in balding follicles compared to non-balding follicles at different levels of significance after applying the t-test and different fold change cut-offs. Sixty-eight genes out of 2481 which were significantly differentially expressed using the t-test, also differed by more than 2 fold; 17 of those genes were very highly significantly different.

<table>
<thead>
<tr>
<th>P-value</th>
<th>&lt; 0.05</th>
<th>&lt; 0.01</th>
<th>&lt; 0.005</th>
<th>&lt; 0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of genes (FC&gt; 1.0)</td>
<td>2481</td>
<td>781</td>
<td>469</td>
<td>115</td>
</tr>
<tr>
<td>Number of genes (FC&gt; 1.5)</td>
<td>370</td>
<td>180</td>
<td>130</td>
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</tr>
<tr>
<td>Number of genes (FC&gt; 2.0)</td>
<td>68</td>
<td>48</td>
<td>40</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 3.4 The number of genes changed significantly in balding versus non-balding scalp follicles by gene microarray analysis at different levels of significance and different fold change cut-offs.

Figure 3.52 Changes in the number of genes expressed between balding and non-balding scalp follicles from men with androgenetic alopecia. Diagram displays the total number of genes which changed significantly in balding versus matched non-balding follicles after performing the t-test and fold change filter at different levels of significance and different fold change cut-offs with the data obtained from the DNA microarray analysis.
3.6.4 Comparison of the expression of keratin and keratin associated protein genes in balding and non-balding scalp hair follicles in men with androgenetic alopecia:

Since non-balding scalp follicles are larger and produce more hair than those showing some miniaturization, the expression of the keratin genes in balding hair follicle samples from three men was compared to those in their non-balding follicles. The expression levels of ten keratin genes were significantly down-regulated in balding follicles compared to non-balding ones using Student’s t-test (P<0.05) by about 1.811-2.627 fold (figure 3.53).

The expression of the keratin associated protein genes was also compared. The expression levels of eight keratin associated protein genes were significantly down-regulated (P<0.05) by about 1.731-3.011 fold, whereas only KRTAP10-7 was up-regulated by 1.104 fold in balding follicles compared to non-balding ones (figure 3.54). Therefore, the majority of keratin associated protein genes showed a significant reduction in expression in balding follicles compared to non-balding follicles.
Figure 3.53 Comparison of the expression of keratin genes in balding versus non-balding scalp follicles from men with androgenetic alopecia using fold change analysis.

DNA microarray analysis of keratin gene expression showed that ten keratin genes were significantly down regulated in balding follicles compared to non-balding follicles, (*P<0.05, **P<0.01).
Figure 3.54 Comparison of the expression of keratin associated protein genes in balding versus non-balding scalp follicles from men with androgenetic alopecia using fold change analysis.

DNA microarray analysis showed that eight keratin associated protein genes were significantly down-regulated, whereas only KRTAP10-7 was up-regulated in balding follicles compared to non-balding follicles. Blue colour indicates down-regulated and green colour indicates up-regulated. (*P<0.05, **P<0.01, #P<0.001)
3.6.5 Comparison of the expression of androgen receptor gene and 5α-reductase gene in balding and non-balding follicles in men with androgenetic alopecia:

Since balding follicles are more responsive to androgens in vivo as this drives their miniaturization (Randall, 2008) and since cultured dermal papilla cells from balding follicles contain more androgen receptors than non-balding scalp follicles (Hibberts et al., 1998), the levels of androgen receptor gene expression were also compared. The gene expression level of androgen receptor was significantly up-regulated in balding follicles when compared to non-balding ones using Student’s t-test (P<0.03) by about 1.856 fold (figure 3.55).

Since 5α-reductase type II plays an important role in conversion of testosterone to dihydrotestosterone which is a more potent androgen and binds to the androgen receptors with a higher affinity than testosterone (Jenkins et al., 1992; Liu and Yamauchi, 2008) and is believed to play a role in androgenetic alopecia (Randall, 2007), the expression levels of 5α-reductase type II gene were also investigated. The gene expression level of 5α-reductase type II was significantly up-regulated (P<0.01) in balding follicles when compared to non-balding follicles by about 1.698 fold (figure 3.55).
Figure 3.55 Comparison of the expression of the androgen receptor gene and 5α-reductase type II gene in balding versus non-balding scalp follicles from men with androgenetic alopecia

DNA microarray analysis was performed to investigate the gene expression levels of androgen receptor and 5α-reductase type II in balding follicle samples (n=3) compared to matched non-balding follicle samples (n=3). Data are the means ± SEM from three different individuals. Androgen receptor expression and 5α-reductase type II expression were both significantly up-regulated in balding follicles compared to non-balding follicles (*P<0.03, **P<0.01).
3.6.6 Comparison of the expression of some potential paracrine factor genes in balding and non-balding scalp hair follicles in men with androgenetic alopecia:

The widely accepted mechanism of androgen action involves the stimulation of androgen receptors in the dermal papilla which alter the production of paracrine factors which regulate other cells in the follicle (Randall, 1994; 2008). Several paracrine factors have been implicated in androgen effects on hair follicles and in the regulation of hair growth. These include: TGF-ß1 (Inui et al., 2002; 2003), SCF (Randall et al., 2008), IGF (Itami et al., 1995b), IGF-IR (Liu et al., 1993), IGFBP3 (Conover and Powell, 1991), IL-1ß (Ruckert et al., 2000), IL-1α (Harman and Nevins, 1993), IL-6 (Kwack et al., 2010), and VEGF (Lachgar et al., 1996c; Aslan et al., 2005). Therefore, the expression of these factors was investigated.

The expression level of the TGF-ß1 gene was significantly up-regulated (P<0.01) in androgen-responsive follicles of balding scalp compared to normal follicles by about 1.848 fold (figure 3.56). In contrast, the expression level of the SCF gene was also significantly down-regulated (P<0.04) in balding follicles compared to non-balding ones by about 1.934 folds (figure 3.57).
**Figure 3.56 Comparison of the expression of the TGFβ-1 gene in balding versus non-balding scalp follicles from men with androgenetic alopecia**

DNA microarray analysis was performed to investigate the expression of TGFβ-1 in balding follicle samples (n=3) compared to matched non-balding follicle samples (n=3). Data are the means ± SEM from three different individuals. TGFβ-1 expression was significantly up-regulated in balding follicles compared to non-balding follicles (**P<0.01).
Figure 3.57 Comparison of the expression of the SCF gene in balding versus non-balding scalp follicles from men with androgenetic alopecia

DNA microarray analysis was performed to investigate the expression of SCF in balding follicle samples (n=3) compared to matched non-balding follicle samples (n=3). Data are the means ± SEM from three different individuals. SCF expression was significantly down-regulated in balding follicles compared to non-balding follicles (*P<0.04).
The gene expression levels of the IGF-I and its receptor, IGF-IR, were significantly down-regulated in balding follicles when compared to non-balding ones using Student’s t-test (P<0.02, P<0.002) by about 1.762 and 1.588 fold respectively (figure 3.58). In addition, the expression of the receptor, IGF-IR, was higher than that of IGF-I in both matched balding and normal follicles. The expression level of the IGFBP3 gene was also significantly reduced (P<0.001) in androgen-responsive follicles of balding scalp by about 1.914 (figure 3.59).

The gene expression levels of some other inhibitory factors including IL-1ß, IL-1α, and IL-6 were significantly up-regulated (P<0.04, P<0.01, and P<0.03 respectively) in balding follicles compared to normal follicles by about 1.993, 1.793 and 1.861 fold respectively (figures 3.60). In contrast, the expression level of the VEGF gene was significantly reduced (P<0.03) in androgen-responsive follicles of balding scalp by about 1.57 fold.
Figure 3.58 Comparison of the expression of the IGF-I gene and its receptor, IGF-IR, gene in balding versus non-balding scalp follicles from men with androgenetic alopecia

DNA microarray analysis was performed to investigate the gene expression of IGF-I and IGF-IR in balding follicle samples (n=3) compared to matched non-balding follicle samples (n=3). Data are the means ± SEM from three different individuals. The expression of both genes were significantly down-regulated in balding follicles compared to non-balding follicles (*P<0.02, ***P<0.002).
DNA microarray analysis was performed to investigate the expression of IGFBP3 in balding follicle samples (n=3) compared to matched non-balding follicle samples (n=3). Data are the means ± SEM from three different individuals. IGFBP3 expression was significantly down-regulated in balding follicles compared to non-balding follicles (#P<0.001).
Figure 3.60 Comparison of the expression of the interleukin family genes, IL-1β, IL-1α and IL-6 in balding versus non-balding scalp follicles from men with androgenetic alopecia

DNA microarray was performed to investigate the expression of IL-1β, IL-1α and IL-6 in balding follicle samples (n=3) compared to matched non-balding follicle samples (n=3). Data are the means ± SEM from three different individuals. The expression of all these genes was significantly up-regulated in balding follicles compared to non-balding follicles (*P<0.05, **P<0.01).
**Figure 3.61** DNA microarray analysis show significantly changes in gene expression (up- and down-regulated) for some potential paracrine factors and their receptors in balding follicles compared to matched non-balding follicles after performing fold change analysis. These factors have been implicated in the regulation of human hair growth. Blue colour indicates down-regulated and green colour indicates up-regulated. (*P<0.05, **P<0.01, ***P<0.005, #P<0.001).
3.6.7 Comparison of the expression of HGF family genes in balding and non-balding scalp hair follicles in men with androgenetic alopecia:

The expression levels of several members of the HGF family and their receptors in three balding hair follicle samples were compared to matched non-balding follicles from three men with androgenetic alopecia were investigated using DNA microarray analysis. The gene expression level of HGF was significantly down-regulated in balding follicles when compared to non-balding ones using Student’s t-test (P<0.04) by about 1.756 folds (figure 3.62). The expression level of the HGF receptor, c-Met, gene was lower than HGF and was also significantly reduced (P<0.03) in balding follicles when compared to non-balding follicles by about 1.584 folds (figure 3.62).

The gene expression level of MSP was significantly down-regulated (P<0.05) in androgen-responsive follicles of balding scalp by 1.803 folds (figure 3.63). The expression level of the MSP receptor, RON, gene was also significantly decreased in balding follicles when compared to non-balding ones using Student’s t-test (P<0.05) by about 1.644 folds (figure 3.63). In addition, the expression of the receptor, RON, was lower than MSP in both normal and balding follicles.
Figure 3.62 Comparison of the expression of the HGF gene and its receptor, c-Met, gene in balding versus non-balding scalp follicles from men with androgenetic alopecia

DNA microarray was performed to investigate the expression of HGF and c-Met in balding follicle samples (n=3) compared to non-balding follicle samples (n=3). Data are the means ± SEM from three different individuals. The expression of both genes were significantly down-regulated in balding follicles compared to non-balding follicles (*P<0.05).
Figure 3.63 Comparison of the expression of the MSP gene and its receptor, RON, gene in balding versus non-balding scalp follicles from men with androgenetic alopecia

DNA microarray was performed to investigate the expression of MSP and RON in balding follicle samples (n=3) compared to non-balding follicle samples (n=3). Data are the means ± SEM from three different individuals. The expression of both genes were significantly down-regulated in balding follicles compared to non-balding follicles (*P<0.05).
3.7 Comparison of the expression of the genes for HGF, MSP and their receptors, c-Met and RON, in non-balding and balding scalp hair follicles from men with androgenetic alopecia using real-time PCR analysis:

To complement the results from the DNA microarray analysis, quantitative real-time PCR was carried out to compare the levels of four genes involved in the mechanism of action of the hepatocyte growth factor family in matched normal and balding follicles in men with androgenetic alopecia. Initially, the method for this was developed using non-balding scalp follicles.

3.7.1 Annealing temperature optimization for each set of primers:

The annealing temperature for the GAPDH, HGF, MSP, c-Met, and RON primers was optimized using the gradient feature of the real-time PCR detection system. A range of different temperatures was examined to determine the optimal temperature for each set of primers to work, i.e. which would give the highest signals (figures 3.64 and 3.65). The optimization reaction mixture for each gene contained the following components: PCR master mix, DNA-binding dye (SYBR Green), cDNA template generated from human reference RNA (appendix II), and specific primer sequences for each gene as appropriate. Table 3.6 shows the optimal annealing temperature for each set of primers which gave the highest signals.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Optimization conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Annealing temperature (˚C)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Peak height</td>
</tr>
<tr>
<td></td>
<td>Annealing temperature (˚C)</td>
</tr>
<tr>
<td>HGF</td>
<td>Peak height</td>
</tr>
<tr>
<td></td>
<td>Annealing temperature (˚C)</td>
</tr>
<tr>
<td>c-Met</td>
<td>Peak height</td>
</tr>
<tr>
<td></td>
<td>Annealing temperature (˚C)</td>
</tr>
<tr>
<td>MSP</td>
<td>Peak height</td>
</tr>
<tr>
<td></td>
<td>Annealing temperature (˚C)</td>
</tr>
<tr>
<td>RON</td>
<td>Peak height</td>
</tr>
</tbody>
</table>

**Table 3.5** Annealing temperature optimization for each primer set which used for real-time PCR. * Optimal annealing temperature (˚C)
Figure 3.64 Annealing temperature optimization for HGF primers and c-Met primers. Different temperatures were examined to determine the optimal annealing temperatures for HGF primers (A) and c-Met primers (B) using the gradient feature of the real-time PCR detection system. Each reaction mixture contained a cDNA template generated from human reference RNA, SYBR Green, PCR master mix and HGF/c-Met primers as appropriate. The 58.3°C reaction yielded the highest signal for HGF, whereas it was 56.8°C for c-Met. Therefore, they were selected as the optimal annealing temperatures for HGF primers and c-Met primers respectively.
The optimal reaction at 58.3 °C

The optimal reaction at 60.5 °C

Figure 3.65 Annealing temperature optimization for MSP primers (A) and RON primers (B). Different temperatures were examined to determine the optimal annealing temperatures for MSP primers (A) and RON primers (B) using the gradient feature of the real-time PCR detection system. Each reaction mixture contained a cDNA template generated from human reference RNA, SYBR Green, PCR master mix and MSP/RON primers as appropriate. The 58.3°C reaction yielded the highest signal for HGF, whereas it was 60.5°C for c-Met. Therefore, they were selected as the optimal annealing temperatures for MSP primers and RON primers respectively.
3.7.2 The relative expression levels for HGF and its receptor c-Met in non-balding scalp hair follicles:

The relative expression levels of HGF and c-Met were investigated using relative quantitative real-time PCR. Since SYBR Green binds to any double stranded DNA, it is necessary to examine the specificity of HGF and c-Met PCR products. Melt-curve analysis allows the identification of any non-specific product which may be amplified with the HGF and c-Met such as genomic DNA contamination and primer-dimers as the presence of a non-specific product would show up as an additional peak in the melt-curve. The melt-curves for HGF and c-Met genes contained only single peaks indicating that these reactions generated only one product for each gene in each of the five samples used, and no contaminating products were present (figure 3.66). It is apparent from the graphs that the melting temperature (the inflection point) occurred around 80°C in both HGF and c-Met. The expression levels of HGF and c-Met relative to those of GAPDH in non-balding scalp hair follicles were analyzed using quantitative real-time PCR. Data from all five individual hair follicle samples were collected as cycle threshold (Ct) and the HGF and c-Met expression levels were calculated by normalizing the data against those of the endogenous control GAPDH in each sample. All five non-balding hair follicle samples expressed both HGF and c-Met, with the relative expression levels of HGF (7.282 ± 0.657) being higher than that of its receptor, c-Met (4.442 ± 0.537) (P<0.0001) (figure 3.67).
Figure 3.66 Melt-curve analysis for HGF and its receptor, c-Met.

Melt-curves were generated by real-time PCR for HGF (A) and c-Met (B) from five different non-balding hair follicle samples. The melt-curves for both genes contain only one peak indicating that no contaminating products are present in these reactions and the reactions in (A) and (B) both generated only one product. The melting temperature of amplified HGF and c-Met products or the points of inflection occurred at about 80°C in both cases.
Relative quantitative real-time PCR was performed to analyze the relative expression levels of HGF and c-Met in human non-balding scalp hair follicles. Expression levels were calculated by normalizing the value against those of the endogenous control (GAPDH). Data are the mean values ± SEM from five different individuals in both cases (#P<0.0001).

Figure 3.67 Relative expression levels of HGF & c-Met in non-balding follicles
3.7.3 The relative expression levels for MSP and its receptor RON in non-balding scalp hair follicles:

The relative gene expression levels of MSP and RON were also investigated using relative quantitative real-time PCR. The melt-curves for MSP and RON genes contained only single peaks indicating that these reactions generated only one product for each gene in each of the five samples used, and no contaminating products were present (figure 3.68). It is apparent from the graphs that the melting temperature (the inflection point) occurred around 80°C in both genes, MSP and RON. The relative expression levels of MSP and RON in non-balding scalp hair follicles were again analyzed using quantitative real-time PCR, normalized against the endogenous control GAPDH. All five non-balding hair follicle samples expressed both MSP and RON, with the relative expression levels of MSP (11.081 ± 0.560) being higher than that of its receptor, RON (6.814 ± 0.862) (P<0.002) (figure 3.69). In addition, the relative gene expression levels of MSP were significantly higher (P<0.0005) than that of HGF and the relative expression levels of RON were significantly higher (P<0.01) than that of c-Met.
Figure 3.68 Melt-curve analysis for MSP and its receptor, RON.

Melt-curves were generated by real-time PCR for MSP (A) and RON (B) from five different non-balding hair follicle samples. The melt-curves in both conditions contain only one peak indicating that no contaminating products are present in these reactions and the reactions in (A) and (B) both generated only one product. The melting temperature of amplified MSP and RON products or the points of inflection occurred at about 80°C in both cases.
Figure 3.69 Relative expression levels of MSP & RON in non-balding follicles

Relative quantitative real-time PCR was performed to analyze the relative expression levels of MSP and RON in human non-balding scalp hair follicles. Expression levels were calculated by normalizing the values against those of the endogenous control (GAPDH). Data are the mean values ± SEM from five different individuals in both cases (***P<0.002).
3.7.4 Comparison of the expression levels for hepatocyte growth factor family and their receptor genes in balding scalp follicles with non-balding follicles in men with androgenetic alopecia using real-time PCR:

3.7.4.1 Checking the quality of amplified RNA:

The total RNA quality and purity of each sample of amplified RNA was evaluated using the NanoDrop 2000 spectrophotometer (Thermo-scientific, UK). One single peak of absorbance occurred at the wavelength 260 nm for each sample (figures 3.70 and 3.71) indicating that the RNA is intact as degraded RNA shows more than one peak. The following table shows the concentrations of amplified RNA obtained from six different samples. All RNA samples exhibited a 260/280 ratio close to 2 indicating high quality resulting RNA.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sample</th>
<th>Sample type</th>
<th>Concentration (ng/µl)</th>
<th>260/280 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 (non-balding)</td>
<td>RNA</td>
<td>14680</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>2 (balding)</td>
<td>RNA</td>
<td>12718</td>
<td>2.10</td>
</tr>
<tr>
<td>B</td>
<td>3 (non-balding)</td>
<td>RNA</td>
<td>12321</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>4 (balding)</td>
<td>RNA</td>
<td>14589</td>
<td>1.99</td>
</tr>
<tr>
<td>C</td>
<td>5 (non-balding)</td>
<td>RNA</td>
<td>15170</td>
<td>2.11</td>
</tr>
<tr>
<td></td>
<td>6 (balding)</td>
<td>RNA</td>
<td>13821</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Table 3.6 The concentrations of cRNA obtained from six different samples of human hair follicles measured using the NanoDrop 2000.
Fig 3.70 Checking the quality of amplified RNA from non-balding follicles

RNA obtained from non-balding follicle samples (n=3) were evaluated using the NanoDrop 2000 spectrophotometer. The single peak in each sample at the wavelength 260 nm indicates that the RNA is intact.
Fig 3.71 Checking the quality of amplified RNA from balding follicles

RNA obtained from non-balding follicle samples (n=3) were evaluated using the NanoDrop 2000 spectrophotometer. The single peak in each sample at the wavelength 260 nm indicates that the RNA is intact.
3.7.4.2 The relative expression levels of HGF in balding and non-balding scalp hair follicles:

Melt-curves of all qRT-PCR products of both non-balding and balding hair follicles contained only single peaks indicating that these reactions generated only one product for HGF, and no contaminating products were present (Figure 3.72 A and B). The melting temperature (the inflection point) for HGF also occurred at 80°C in all three of both sets of follicles, non-balding and balding. To confirm the melt-curve results and check the size of the product, the HGF products for the same reactions were applied to a 1.5% agarose gel for electrophoresis (figure 3.72 C and D). There was only a single band of the anticipated size (144bp) for HGF in each sample. This demonstrated that there was no contamination in the samples and the HGF products originated from the amplified cDNA and suggested that the primers were identifying the correct gene sequence.

The relative expression levels of the HGF gene in three balding hair follicle samples compared to those three non-balding follicles were analyzed using relative quantitative real-time PCR against that of the endogenous control GAPDH (figure 3.73). The relative expression level of HGF was significantly reduced in balding follicles (2.452 ± 0.365) to less than half the expression in non-balding ones (5.498 ± 0.278) using Student’s t-test (P<0.01).
Figure 3.72 Melt-curve analysis for HGF in non-balding and balding follicles

Melt-curves for HGF were generated by real-time PCR for non-balding samples (A) and balding samples (B). The melt-curves in both conditions contain only one peak indicating that no contaminating products are present in these reactions and the reactions in (A) and (B) generated only one product. The melting temperature of amplified HGF products, the points of inflection, occurred at about 80°C.

To confirm the melt-curve results and check the size of the product, the HGF products for the same reactions were applied to 1.5% agarose gel for electrophoresis. There was only a single band of the anticipated size (144bp) for HGF in both non-balding follicles (C) and balding follicles (D). DNA ladder indicates 100bp DNA molecular size marker (range from 100-1,500).
Relative quantitative real-time PCR was performed to analyze the relative expression levels of HGF in balding follicle samples (n=3) compared to non-balding follicle samples (n=3). Expression levels were calculated by normalizing data against the endogenous control gene, GAPDH. Data are the mean values ± SEM from three different individuals in both conditions. HGF expression was significantly reduced in balding follicles compared to non-balding follicles (***P<0.01)

Figure 3.73 Comparison of the expression levels of the HGF gene in balding versus non-balding scalp follicles from men with androgenetic alopecia
3.7.4.3 *The relative expression levels of c-Met in balding and non-balding scalp hair follicles:*

Melt-curves of all qRT-PCR products of both non-balding and balding hair follicles contained only single peaks indicating that these reactions generated only one product for c-Met, and no contaminating products were present (Figure 3.74 A and B). The melting temperature (the inflection point) for c-Met also occurred at about 80°C in all three samples of both sets of follicles, non-balding and balding. To confirm the melt-curve results and check the size of the product, the c-Met products for the same reactions were applied to 1.5% agarose gel for electrophoresis (figure 3.74 C and D). There was only a single band of the anticipated size (152bp) for c-Met in each sample. This demonstrated that there was no contamination in the samples and the c-Met products originated from the amplified cDNA and suggested that the primers were identifying the correct gene sequence.

The relative expression levels of the c-Met gene in three balding hair follicle samples compared to those three non-balding follicles were analyzed using quantitative real-time PCR against that of the endogenous control GAPDH (figure 3.75). The relative expression level of c-Met was significantly reduced (P<0.01) in balding follicles (1.203 ± 0.202) to less than third of the non-balding follicles expression (3.895 ± 0.368).
Figure 3.74 Melt-curve analysis for c-Met in non-balding and balding follicles

Melt-curves for c-Met were generated by real-time PCR for non-balding samples (A) and balding samples (B). The melt-curves in both conditions contain only one peak indicating that no contaminating products are present in these reactions and the reactions in (A) and (B) generated only one product. The melting temperature of amplified c-Met products, the points of inflection, occurred at about 80°C.

To confirm the melt-curve results and check the size of the product, the c-Met products for the same reactions were applied to 1.5% agarose gel for electrophoresis. There was only a single band of the anticipated size (152bp) for HGF in both non-balding follicles (C) and balding follicles (D). DNA ladder indicates 100bp DNA molecular size marker (range from 100-1,500).
Figure 3.75 Comparison of the expression levels of the c-Met gene in balding versus non-balding scalp follicles from men with androgenetic alopecia

Relative quantitative real-time PCR was performed to analyze the relative expression levels of c-Met in balding follicle samples (n=3) compared to non-balding follicle samples (n=3). Expression levels were calculated by normalizing data against the endogenous control gene, GAPDH. Data are the mean values ± SEM from three different individuals in both conditions. c-Met expression was significantly reduced in balding follicles compared to non-balding follicles (**P<0.01).
3.7.4.4 The relative expression levels of MSP in balding and non-balding scalp hair follicles:

Melt-curves of all qRT-PCR products of both non-balding and balding hair follicles contained only single peaks indicating that these reactions generated only one product for MSP, and no contaminating products were present (Figure 3.76 A and B). The melting temperature (the inflection point) for MSP also occurred around 80°C in all three samples of both sets of follicles, non-balding and balding. To confirm the melt-curve results and check the size of the product, the MSP products for the same reactions were applied to 1.5% agarose gel for electrophoresis (figure 3.76 C and D). There was only a single band of the anticipated size (116bp) for MSP in each sample. This demonstrated that there was no contamination in the samples and the MSP products originated from the amplified cDNA and suggested that the primers were identifying the correct gene sequence.

The relative expression levels of the MSP gene in three balding hair follicle samples compared to those three non-balding follicles were analyzed using relative quantitative real-time PCR against that of the endogenous control GAPDH (figure 3.77). The relative expression level of MSP was significantly reduced in balding follicles (3.461 ± 0.744) to less than half the expression in non-balding follicles (9.851 ± 0.996) using Student’s t-test (P<0.03).
Figure 3.76 Melt-curve analysis for MSP in non-balding and balding follicles

Melt-curves for MSP were generated by real-time PCR from non-balding samples (A) and balding samples (B). The melt-curves in both conditions contain only one peak indicating that no contaminating products are present in these reactions and the reactions in (A) and (B) generated only one product. The melting temperature of amplified MSP products, the points of inflection, occurred at about 80°C.

To confirm the melt-curve results and check the size of the product, the MSP products for the same reactions were applied to 1.5% agarose gel for electrophoresis. There was only a single band of the anticipated size (116bp) for MSP in both non-balding and balding follicles (C). Lane1: DNA ladder, Lane 2, 3: MSP PCR products from non-balding and balding follicle samples respectively. DNA ladder indicates 100bp DNA molecular size marker (range from 100-1,500).
Figure 3.77 Comparison of the expression levels of the MSP gene in balding versus non-balding scalp follicles from men with androgenetic alopecia

Relative quantitative real-time PCR was performed to analyze the relative expression levels of MSP in balding follicle samples (n=3) compared to non-balding follicle samples (n=3). Expression levels were calculated by normalizing data against the endogenous control gene, GAPDH. Data are the mean values ± SEM from three different individuals in both conditions. MSP expression was significantly reduced in balding follicles compared to non-balding follicles (*P<0.03).
3.7.4.5 The relative expression levels of RON in balding and non-balding scalp hair follicles:

Melt-curves of all qRT-PCR products of both non-balding and balding hair follicles contained only single peaks indicating that these reactions generated only one product for RON, and no contaminating products were present (Figure 3.78 A and B). The melting temperature (the inflection point) for RON also occurred around 80°C in all three samples of both sets of follicles, non-balding and balding. To confirm the melt-curve results and check the size of the product, the RON products for the same reactions were applied to 1.5% agarose gel for electrophoresis (figure 3.78 C and D). There was only a single band of the anticipated size (117bp) for RON in each sample. This demonstrated that there was no contamination in the samples and the RON products originated from the amplified cDNA and suggested that the primers were identifying the correct gene sequence.

The relative expression levels of the RON gene in three balding hair follicle samples compared to those three non-balding follicles were analyzed using relative quantitative real-time PCR against that of the endogenous control GAPDH (figure 3.79). The relative expression level of RON was significantly reduced in balding follicles (1.596 ± 0.458) to less than half the expression in non-balding follicles (4.599 ± 0.359) using Student’s t-test (P<0.04).
Figure 3.78 Melt-curve analysis for RON in non-balding and balding follicles

Melt-curves for RON were generated by real-time PCR from non-balding samples (A) and balding samples (B). The melt-curves in both conditions contain only one peak indicating that no contaminating products are present in these reactions and the reactions in (A) and (B) generated only one product. The melting temperature of amplified RON products, the points of inflection, occurred at about 80°C in.

To confirm the melt-curve results and check the size of the product, the RON products for the same reactions were applied to 1.5% agarose gel for electrophoresis. There was only a single band of the anticipated size (117bp) for RON in both non-balding follicles (C) and balding follicles (D). DNA ladder indicates 100bp DNA molecular size marker (range from 100-1,500).
Figure 3.79 Comparison of the expression levels of the RON gene in balding versus non-balding scalp follicles from men with androgenetic alopecia

Relative quantitative real-time PCR was performed to analyze the relative expression levels of RON in balding follicle samples (n=3) compared to non-balding follicle samples (n=3). Expression levels were calculated by normalizing data against the endogenous control gene, GAPDH. Data are the mean values ± SEM from three different individuals in both conditions. RON expression was significantly reduced in balding follicles compared to non-balding follicles (*P<0.04).
4- Discussion
4. Discussion

Androgens are the major regulating hormones of human hair growth. It is believed that androgens act on hair follicles through the dermal papilla (Randall, 2008). In this model, circulating androgens enter the dermal papilla through its good blood supply and cause alteration in its production of paracrine regulatory factors, which influence the surrounding follicular components. These factors would play a role in altering the type of hair produced by androgen-dependent hair follicles. Paracrine factors may target the follicular melanocytes which control the amount of pigment produced, the keratinocytes which proliferate to produce the hair fibre, the follicular endothelial cells, and the cells of the connective tissue sheath, as well as the dermal papilla itself through an autocrine regulatory system. Investigating these factors could give us a better understanding of the mechanisms involved in androgen-dependent hair disorders. The aim of this study was to investigate the gene expression of some paracrine factors that may be involved in mediating androgen action in the human hair follicles, including members of hepatocyte growth factor family HGF, MSP, and their respective receptors, c-Met and RON, in human scalp hair follicles.

As an initial step of this study, the structure of red deer and human hair follicles were investigated using histochemical staining for the purpose of familiarisation of hair follicle structure and for histological comparisons between both species. Longitudinal and horizontal cross-cryosections were prepared from red deer and human skin. Making sections of deer skin was easier to deal with within the cryostat than human skin due to the large size of deer hair follicles compared to human hair follicles, which are smaller
and less densely packed. Prepared cryosections from both skin types were treated with histochemical staining using Sacpic (Nixon, 1993; Nutbrown and Randall, 1996) and haematoxylin and eosin staining techniques to clearly visualise the normal histology of the hair follicle and to differentiate the various structures of the skin and hair follicle. Different components can be clearly seen in the sections of human scalp skin stained with Sacpic, including a thin epidermis followed by a thick dermal layer (blue/green) composed of collagen bundles (bright blue) (figure 3.2). The hair follicles extend deep within a subcutaneous fat layer as highlighted by the staining; the hair fibre (yellow) is surrounded by the inner root sheath (red) and outer root sheath (blue). The hair bulb at its base contains the dermal papilla, surrounded by the epithelial hair matrix containing melanocytes, and attached to the connective tissue sheath at its base. The connective tissue sheath (pale blue) encases the entire follicle separating it from the dermis (figures 3.2, 3.3). These findings are similar to those previously observed in the human hair follicle using Sacpic staining (Nutbrown and Randall, 1996). Various layers of human hair follicles were also clearly seen in horizontal cross-sections (figures 3.4, 3.5), in which the hair follicles were sectioned in the upper part of the follicles, in which fully keratinised hair fibres are present and are stained yellow using Sacpic and pink using Haematoxylin and Eosin.

Generally speaking, the overall structure of both human and red deer hair follicles were similar. However, deer hair follicles are larger than those of human scalp, extending deep within the dermis, and the medulla is much larger and clearly visible compared to those in human scalp (figure 3.1). The medulla is well developed in these animals to provide the insulatory properties of animal hair.
4.1 Identification of hepatocyte growth factor family members and their receptors in isolated human scalp hair follicles and their components:

The investigation then moved on to investigate gene expression of specific molecules using RT-PCR. Human lower anagen hair follicles of normal (non-balding) scalp were isolated from five individuals by microdissection (figures 2.1, 3.6, 3.7, 3.8). The resulting whole follicles were collected from each sample for total RNA isolation, poly (A) RNA isolation and cDNA synthesis. The purity of total RNA was checked by agarose gel electrophoresis which exhibited the ratio 2:1 of rRNA (28S:18S) (figure 3.9). This indicated that the RNA was intact and suitable for use (Skrypina et al., 2003). RT-PCR was carried out to investigate the expression of genes for some potential paracrine factors in human hair follicles including HGF, MSP and their receptors c-met and RON. The presence or absence of specific gene sequences can be ascertained in cDNA produced from isolated poly (A) RNA by amplification using PCR.

The highly expressed cytoskeletal protein, β-actin, was used as a positive control; a negative control, in which the cDNA was excluded from the reaction mix, was also included in each experiment. The β-actin gene was amplified using specific forward and reverse primers to ensure that the RNA isolated from all five individuals was of sufficient quality to perform RT-PCR successfully (figure 3.10). The β-actin PCR products from all five individuals were similar in size to that anticipated from the human sequence, which is 838bp (figure 3.10) (Shorter et al., 2008). In all the negative controls, no PCR products were found when the template cDNA was replaced with nuclease-free water in the reaction mix, indicating an absence of any contaminating DNA in the poly (A) RNA samples used for RT-PCR and confirming that the PCR products from all experimental
samples were a result of amplification of the cDNA produced from the poly (A) RNA of each sample.

Following analysis of the β-actin PCR product by electrophoresis, the identity of the β-actin PCR product was confirmed using sequence analysis. The sequencing data were compared to the previously identified human sequence, obtained from the ensemble genome browser, using the NCBI BLAST programme. β-actin PCR products from human hair follicles exhibited 96% correlation with the known human sequence (figure 3.11). The successful amplification of β-actin with all cDNA samples confirmed that the cDNA samples were of sufficient quality for further RT-PCR analysis to be performed.

4.1.1 Investigating the expression of HGF and its receptor, c-Met, in isolated non-balding scalp hair follicles and their components:

The investigation then moved on to examine the suggestion that hepatocyte growth factor (HGF) is a possible paracrine factor involved in hair growth. It has been revealed that HGF may act as a powerful modulator of hair growth and be involved in morphogenesis and cycling (Jindo et al., 1995; Lindner et al., 2000), (section 1.11.3). In a variety of organs, HGF is known to exert its effect as a paracrine factor secreted by mesenchyme-derived cells acting on neighbouring epithelial or endothelial cells (Peus and Pittelkow, 1996; Young-Ran et al., 2001).

In this study, the expression of HGF and its receptor, c-Met, were investigated in human anagen hair follicles of non-balding scalp using the RT-PCR technique. The primer sequences used for HGF and c-Met had been previously optimised by Shimaoka et al. (1995) and by Imaizumi et al. (2000) respectively. cDNA was prepared from five
individual samples of isolated human hair follicles of non-balding scalp. When the resulting PCR products for each gene were separated using agarose gel electrophoresis and visualised with ethidium bromide staining, those from all five individuals corresponded to the anticipated size of HGF product 262 bp, when the HGF primers were used (figure 3.12). With the c-Met primers the bands also corresponded to the anticipated size 563 bp (figure 3.18). No bands were detected in the negative control lanes, indicating no contaminating DNA in the samples.

The identity of HGF and its receptor, c-Met, genes were verified by sequencing and comparing the human hair follicle PCR products of both HGF and c-Met with the known human sequences (figures 3.13 and 3.19). Thus, the results showed that the mRNA of both HGF and its receptor, c-Met, were expressed in anagen hair follicles from non-balding scalp of all five individuals (figures 3.12, and 3.18) demonstrating a possible role for HGF in the maintenance and control of human anagen hair follicles and supporting the suggestion that HGF may act as a powerful modulator of hair growth.

Following detection of the expression of HGF and its receptor c-Met in whole isolated human hair follicles, the next stage was to determine the location of each molecule within the hair follicles. The three components of the human hair bulb, comprising the dermal sheath, the epithelial bulb matrix, which also contains melanocytes, and the dermal papilla, were microdissected individually. This was a very time consuming procedure (figures 3.20, 3.21). PCR products of predicted size (838 bp) were produced from all three hair bulb components for the β-actin; however, the expression of β-actin had much greater intensity in the epithelial matrix than the dermal sheath and the dermal papilla in
the components from each of the three individuals examined. The dermal papilla bands had least intensity (figure 3.22). This concurs with previous studies showing that the volume of the matrix was about ten times that of the dermal papilla, and the ratio between the number of cells in the dermal papilla and those dividing in the matrix was nine to one (Van Scott and Ekel, 1958; Elliott et al., 1991).

The expression of HGF and c-Met were further investigated to refine their location in the three lower follicle components using RT-PCR. The results showed that gene expression of HGF was found in both the dermal sheath and dermal papilla, but not in the matrix (figure 3.24). The dermal papilla bands were less strong than those of the dermal sheath. This parallels the differences in the cDNA quantity observed in the case of β-actin. No bands were seen in the matrix lanes in either HGF gels. As the matrix cDNA was the strongest in β-actin analysis, if HGF is expressed in the matrix cells, bands should have been visible. The results indicated that the dermal papilla and dermal sheath have the capacity to synthesise HGF. The results also revealed that the gene for the HGF receptor, c-Met, was expressed in both the dermal sheath and dermal papilla (figure 3.25) indicating their ability to produce c-Met which allow them to respond to HGF. It has been observed that the gene for HGF was strongly expressed in rat anagen, and slightly in telogen, follicles using cDNA extracted from skin sections (Yamazaki et al., 1999) and localised to the dermal papilla cells of mouse pelage anagen follicles using immunohistochemistry (Lindner et al., 2000). Yu et al. (2004) reported c-Met expression in human scalp cultured dermal papilla cells using RT-PCR and also in rat follicular matrix keratinocytes using in situ hybridisation.
Since the dermal papilla is believed to be the main regulator of the hair follicle and can determine what sort of hair is produced (Jahoda et al., 2001; Randall, 2008), cultured dermal papilla cells are used extensively as a model system (Itami et al., 1995a; Jahoda et al., 2001; Inamatsu et al., 2006; Hamada and Randall, 2006). Therefore, the expression of HGF and its receptor, c-Met, was examined also in human cultured dermal papilla cells of non-balding scalp hair follicles isolated from two different individuals using RT-PCR. The dermal papilla cells in culture increased in number gradually to give multi-polar shapes and formed small clumps (figure 3.30). When confluent, they were collected for RNA extraction, cDNA synthesis and gene expression investigation. The resulting PCR products for HGF and its receptor, c-Met, appeared on the agarose gel as distinct bands of the anticipated sizes of 262 bp and 563 bp respectively, and no bands were present in the negative controls indicating that the primer pair was binding specifically to the cDNA template (figures 3.33 and 3.39). Sequence analysis confirmed the identity of HGF and c-Met PCR products (figures 3.34 and 3.40). This agrees with observations of prior studies which determined the gene expression of HGF in cultured dermal papilla cells derived from human hair follicles (Shimaoka et al., 1995; Merrick, 2000). In addition, Yu et al. (2004) showed that the gene for c-Met was expressed in cultured dermal papilla cells from human scalp hair follicles and rat vibrissae follicles using RT-PCR, concurring with the results reported here. This indicates that the dermal papilla cells can synthesise HGF and its receptor, c-Met, which would enable them to react to HGF in culture; this correspond to the results mentioned earlier using freshly isolated dermal papilla, i.e. as close to in vivo as possible. It was important to confirm that cultured dermal papilla cells are behaving the same as in vivo if the cells are to be useful as a model system in this
area. There is a possibility of loss of normal gene expression as soon as the cultured cells are established (O’Shaughnessy et al., 2004).

Immunohistochemistry was also used to locate the protein for c-Met in the human hair follicle. Several different concentrations of the reaction solutions and the blocking serum solutions were used to optimise the procedure until the optimum staining was obtained. Three negative controls were included in each experiment to detect any non-specific binding to ensure that the colour detection system was not activated alone, to ensure that the secondary antibody is not binding non-specifically to the tissue, and that the primary antibody was not activating the red chromogen AEC solution without the presence of the secondary antibody.

Initially, to learn the technique of immunohistochemistry, the location of cytokeratin 6, a highly expressed cytoskeletal protein, was examined in human hair follicles using a goat polyclonal anti-cytokeratin 6 antibody (Novocastra Laboratories, UK) with mouse serum for blocking and in the diluting procedure. Expression of cytokeratin 6 was detected in the outer root sheath of human hair follicles (figures 3.26, 3.27). These observations were consistent with the findings of previous studies using human hair follicles (Stark et al., 1987), sheep wool follicles (Bond et al., 1996), and red deer follicles (Croft, 2002).

The presence of c-Met within non-balding scalp hair follicles was then examined using immunohistochemistry using a mouse monoclonal anti-human c-Met antibody (Novocastra Laboratories, UK). The protein expression was detected in the outer root sheath and the dermal papilla cells of human anagen hair follicles (figures 3.28, 3.29). This concurs with previous reports detecting c-Met in the outer and inner root sheath in
anagen mouse follicles using immunohistochemistry (Lindner et al., 2000). From the results reported here, both RT-PCR and immunohistochemistry revealed the presence of HGF receptor c-Met in both dermal sheath and dermal papilla cells of human hair follicles.

The detection of genes for both HGF and its receptor, c-Met, in the dermal papilla cells would enable this signalling system to work as an autocrine regulator. Dermal papilla cells have been shown to secrete autocrine regulatory factors which alter the growth of the dermal papilla cells *in vitro* (Thornton et al., 1998; Hamada and Randall, 2006). Since the size of the dermal papilla and number of cells it contains are directly related to the size of the hair produced by the follicles (Van Scott and Ekel, 1958; Elliott et al., 1999), alteration in such factors could be important in androgen-stimulated alteration in hair follicle and hair size.

### 4.1.2 Investigating the expression of MSP and its receptor, RON, in isolated non-balding scalp hair follicles and their components:

Another member of the hepatocyte growth factor family, macrophage stimulating protein (MSP), has also been implicated in hair growth control. McElwee et al. (2004) examined the effect of MSP on hair growth due to the similarity and close relationship between MSP and HGF. They observed that exposure to different concentrations of MSP led to increases in human hair follicle length when non-balding scalp follicles were cultured *in vitro*. In addition, MSP stimulated mouse telogen follicles to enter anagen *in vivo* (McElwee et al., 2004). These findings suggest MSP may be a possible paracrine factor produced by the dermal papilla cells.
In order to investigate the hypothesis that MSP may play a role as a potential paracrine factor affecting hair growth, the expression of MSP and its receptor RON were also investigated in human anagen hair follicles of non-balding scalp using RT-PCR as described previously. All five human hair follicle cDNA samples produced PCR products of the expected size: 415 bp for the gene of MSP (figure 3.14) and 217 bp for the gene of RON (figure 3.16) when separated using agarose gel electrophoresis. Sequence analysis also verified the identity of the DNA fragments for both MSP and RON.

The results of this investigation agree with the finding of MSP in cultured cells derived from human occipital scalp dermal papilla and dermal sheath using western blotting, and the MSP receptor, RON, in the matrix, the dermal papilla and the dermal sheath using immunohistochemistry (McElwee et. al., 2004). In addition, Shorter (2007) showed that the gene for MSP was expressed in the three tissue types, the dermal sheath, the matrix and the dermal papilla, were microdissected from the human hair bulb of non-balding scalp follicles using RT-PCR.

To localise the MSP receptor, RON, within human hair follicles using RT-PCR, cDNA was extracted from the three components of the hair bulb: the dermal sheath, matrix and dermal papilla. Gene expression of RON was found in all three tissue types of the human hair bulb. The expression had much greater intensity in the epithelial matrix than in the dermal sheath and the dermal papilla in the components from each of the three individuals examined. The dermal papilla bands had least intensity (figure 3.23). This parallels the differences in the cDNA quantity observed in the β-actin gels and shows interesting differences from the location of HGF and its receptor c-Met.
To confirm the expression of MSP and RON in the dermal papilla using RT-PCR, total RNA was extracted from human cultured dermal papilla cells of two individuals. It is apparent from the results that the PCR products from both samples corresponded to the expected size of MSP product 415 bp when the MSP primers were used (figure 3.35) whereas those with RON primers were 217 bp (figure 3.37). When the identity of MSP and RON were ascertained via sequencing, with the cultured human scalp dermal papilla cells’ PCR products of both MSP and RON exhibited homology with the known human sequences respectively (figures 3.36 and 3.38).

The findings of this investigation indicated that the dermal papilla, the matrix and the dermal sheath have the capacity to synthesise the MSP receptor, RON, which allows them to respond to MSP. Since the earlier studies detected MSP in the dermal papilla and dermal sheath of human occipital scalp follicles (McElwee et al., 2004; Shorter, 2007) and also the bulb matrix (Shorter et al., 2007), this would suggests that this signalling pathway works as paracrine signalling system. This could be an autocrine response, as suggested earlier in the discussion of HGF and its receptor, since the dermal papilla can express both MSP and the receptor RON. On the other hand it could be the more normal paracrine route with the signal being produced by either the dermal papilla or the dermal sheath or the matrix cells and received by the receptors in one of the other tissues. Since all the cells in the bulb are potential targets of androgens (Randall, 2008) either directly, or indirectly via a signal from a direct target such as the dermal papilla, MSP and its receptor RON seem good candidate for a paracrine role in androgen-stimulated alteration of hair growth.
4.2 Comparison of the gene expression of some potential paracrine factors in normal and balding scalp hair follicles from males with androgenetic alopecia:

The role of paracrine factors produced by dermal papilla cells in androgen-dependent hair follicles has been the focus of much research since they are believed to be important in determining the type of hair produced (Jahoda et al., 2001; Randall, 2008). The alteration of the size of the hair produced by a hair follicle, while at the same time maintaining many processes involved in hair growth and the hair cycle, probably requires a number of paracrine factors, some of them are inhibitory and others stimulatory. Androgens are the main known key regulator responsible for changing the type of hair produced by a hair follicle (Randall et al., 2008), stimulating enlarged follicles producing larger terminal hair in many areas after puberty, while causing reduction in follicle size on the scalp resulting in androgenetic alopecia. Therefore, having established a molecular biological approach to investigate paracrine factors in non-balding scalp follicles, this was extended to investigate how androgens alter gene expression in target follicles to change the type of hair produced. In this study, the focus was on comparing the gene expression in isolated non-balding and androgenetic alopecia hair follicles and determining whether there are significant differences between them using two comparative methods, DNA microarray analysis and quantitative real-time PCR. To maximize the likelihood of obtaining meaningful results, the comparisons were carried out using matched pairs of non-balding and balding follicles from the same men with androgenetic alopecia.

The skin samples used were obtained from three healthy men undergoing cosmetic dermatological surgeries, aged 51, 56 and 55, with androgenetic alopecia (male pattern baldness), which was classified as class IV according to the Hamilton classification.
The hair follicles were microdissected from two areas of the scalp from each individual: frontal scalp for the balding follicles and occipital scalp for the control, non-balding ones. The non-balding and balding scalp hair follicles showed morphological differences which distinguished them from each other. The vast majority of the balding hair follicles were vellus follicles, though there were a few intermediate follicles. The balding vellus follicles were tiny in length and diameter compared to the matched terminal follicles of the non-balding scalp. They produced hairs which were mainly pale in colour, being less pigmented. Furthermore, the blood supply system in balding follicles was considerably reduced, concurring with a finding which was also recorded by Crovato, Moretti and Bertamino (1968) and Hibbert et al. (1996). The balding intermediate follicles were characterised by their intermediate length, their diameters being smaller than the terminal ones and wider than the vellus follicles, closer to those of vellus ones than those in non-balding follicles, and their bulbs being situated in the interface between the dermis and subcutaneous fat tissue compared to non-balding follicle bulbs, which were deep within the fat tissue. These observations concur with a recent study reported by Miranda et al., (2010). These differences in hair follicle size, shape, diameter and colour between the follicles from the two sites supports the use of these follicles to investigate changes in gene expression promoted by androgens.

Isolation of balding follicles was very time-consuming and needed much care and patience due to their small size and pale colour. Following hair follicle isolation, the total RNA was extracted and later cDNA was synthesised. It was noticeable that the total RNA extracted from balding follicles was less than that extracted from the similar number of non-balding follicles, reflecting the difference in size between the balding and normal
folicles. Nevertheless, the electrophoretic separation of total RNA showed the rRNA bands (28S and 18S) exhibited a 2:1 ratio for all balding and non-balding samples indicating that the total RNA was intact (Skrypina et al., 2003), (see figure 3.41). The electropherograms also showed high quality RNA for all samples characterised by the presence of clear defined 28S and 18S peaks and little noise between the peaks with minimal low molecular weight noise (figures 3.42 and 3.43).

DNA microarray analysis was carried out to investigate the changes in gene expression in matched non-balding and balding scalp hair follicles. The Affymetrix GeneChip human exon 1.0 ST array containing 28,869 genes was used in this study; six chips were used, one chip per sample. Following scanning of the hybridized arrays, DNA microarray data were analyzed using GeneSpring GX 11 software (Agilent).

The robust multi-array analysis (RMA) method was used to normalize the experimental data to standard microarray data as per-gene normalization. This method aims to avoid and remove any systematic errors or biases from the data which may cause negative effects on the data analysis afterward (Irizarry et al., 2003). To stabilize the variation across the expression range and to treat up- and down-regulated genes in a similar fashion, the data were transformed to a log scale (Quackenbush, 2002; Cui and Churchill, 2003). A box and whiskers plot (figure 3.44) showed the variation in the intensity values distribution in the balding and non-balding groups indicating that these results are appropriate to be taken forward to the next stage of analysis.
To check the quality of the microarray intensity values further, a scatter plot (figure 3.45) was used (Lee and Park, 2007). This revealed that there was a negative correlation in the gene expression between the balding and non-balding group ($r = -0.74$, $P<0.03$). In other words, when the balding sample has high fluorescence associated with the expression of one gene, then the non-balding sample is tending to have a low amount of fluorescence, i.e. binding or expression. In addition, the reverse pattern also occurs with high fluorescence in a non-balding sample corresponding to low expression in a balding one.

The principal component analysis (PCA) method (Komura et al., 2004; Shlens, 2009) was used to clarify the quality of the microarray intensity values further. This type of test can reduce a huge number of complex data to a lower dimension. It is evident from the three-dimensional (3D) graph (figure 3.46) that the six experimental samples are separated into two distinct groups; the non-balding group and the balding group. The principal component 1 (PC1) and the principal component 2 (PC2), indicate 20.68% and 17.7% variation respectively within the same group, whereas the principal component 3 (PC3) on the z-axis indicates 55.36% variation in the total gene expression between the balding and non-balding groups. Thus, the PCA test revealed that there are clear differences in gene expression between the balding and non-balding samples and that there was much less difference within the three different samples in each type of follicles.

As the checks on the quality of the microarray data had shown that they were appropriate for further analysis, gene clustering comparisons were performed. An overall view of DNA microarray experiment performance was gained by comparing gene clustering in each individual sample. The hierarchical clustering analysis showed a clear variability in
gene expression, though there were obvious similarities within the set of balding follicles and also within the set of non-balding ones (figure 3.47). When the samples were combined into two groups (figure 3.48), the clustering analysis showed that the overall gene expressions were separated into discrete clusters where genes that have the most similar expression profiles were clustered together (Eisen et al., 1998).

The microarray data analysis was continued by applying a statistical method, Student’s t-test, to determine which genes are significantly differentially expressed and to extract the meanings of microarray experimental results. The t-test takes the variability of gene expression into account on a gene-per-gene basis. The result revealed that a total of 2,481 genes were significantly differentially expressed in balding follicles, out of the 28,869 genes on the microarray gene chip, showing a significant change in gene expression with probability values of less than 0.05. Of these 1,462 genes were up-regulated in balding follicles whereas 1,019 genes were down-regulated. For further investigation, the fold change filter was performed to determine differences in gene expression in both groups. Table 3.3 shows the number of genes changed significantly (P<0.05) in balding versus non-balding with different fold change cut-offs. The traditional approach for comparing analysis is to look for genes with two fold differences in expression i.e. increases or decreases of a hundred percent. However, a more biologically relevant approach is to look at genes whose expression is significantly altered as changes in levels of an enzyme in a cell for example would not need to be a hundred percent to have far reaching effects.
The hair keratins are the cytoplasmic intermediate filaments which form the main structural proteins of mammalian hair. They belong to a multi-gene family which consists of genes that are expressed in different type of epithelia (Powell et al., 1997). Since non-balding scalp follicles are larger and produce more hair than those of balding scalp, the expression of the keratin genes were investigated. The data obtained from DNA microarray analysis showed that ten keratin genes were significantly (P<0.05) down-regulated in balding follicles compared to non-balding follicles by about 1.811-2.627 fold (figure 3.53). This would be expected as balding follicles produce much smaller hairs than non-balding ones and suggest that these decreased levels of keratin genes in androgen-sensitive follicles of balding scalp may occur under the influence of androgen.

Whether this is direct regulation or indirect via paracrine factors from the dermal papilla is not known as yet. Java-Suarez et al., (2004) observed the that keratin hHa7 was expressed in medullary cells of all types of male and female sexual hairs including beard, axillary and pubic hairs, but not in occipital terminal hairs using western blot analysis. They also reported its expression in beard and temple vellus hair follicles using immunohistochemistry. In addition, they demonstrated the expression of androgen receptor in beard hair follicles, and demonstrated the specific binding of the androgen receptor to all three putative hHa7 androgen receptor-binding elements, suggesting this type of keratin may be directly regulated by androgens. It has been reported that mouse endodermal cells that are deficient in keratin 8 and keratin 18 are more sensitive to TNF which induces cell death suggesting a functional link between these keratin and TNF signalling through their binding to the cytoplasmic domain of TNFR while moderates the
influence of TNF (Caulin et al., 2000), concurring with the observations reported by Inada et al., (2001) in human liver cells.

Since balding follicles are more responsive to androgens in vivo as this drives their miniaturization (Randall, 2008), the gene expression levels of androgen receptor was also investigated in this thesis using DNA microarray analysis. The results revealed that the expression of androgen receptors was significantly up-regulated (P<0.03) with a 1.856 fold increase in balding scalp hair follicles compared with the control follicles (figure 3.55), supporting the original hypothesis. These results concur with earlier in vitro studies demonstrating that cultured dermal papilla cells derived from follicles from androgen-sensitive regions, both balding scalp and beard, contain a higher level of androgen receptors than those derived from androgen-insensitive regions such as non-balding scalp (Randall et al., 1992; Hibberts et al., 1998). Another group also demonstrated that cultured dermal papilla cells derived from beard and auxillary follicles expressed the gene for androgen receptor, whereas this was only present at a low level in dermal papilla cells derived from occipital scalp follicles (Ando et al., 1999). These parallels support the microarray analysis and the difference in androgen receptor levels in balding follicles from normal men indicates that androgen should be able to regulate gene expression in balding follicles as the activated androgen receptor is a transcriptional regulator (Narayanan et al., 2008).

Since the enzyme 5α-reductase plays an important role in conversion of testosterone to 5α-dihydrotestosterone, a more potent androgen which binds to the androgen receptors with a higher affinity than testosterone (Jenkins et al., 1992; Randall et al., 2008) and
since it is expressed in cultured dermal papilla cells derived from both beard and scalp hair follicles (Liu and Yamauchi, 2008), the expression level of 5α-reductase type II gene was also investigated. Individuals deficient in 5α-reductase type II do not show male patterns of body hair, in spite of their circulating androgen. They only produce female patterns of pubic and axillary hair and have little or no beard (Wilson et al., 1993). This suggests that axillary and pubic follicles respond to testosterone, whereas the male secondary sexual hair demands metabolism of testosterone to 5α-dihydrotestosterone. In addition, the 5α-reductase type II inhibitor, finasteride, can restore hair growth in men with androgenetic alopecia (Kaufman et al., 1998). These observations support the microarray analysis which revealed that the gene expression level of 5α-reductase type II was significantly up-regulated (P<0.01) in androgen-responsive follicles of balding scalp when compared to matched non-balding follicles by 1.698 fold (figure 3.55) suggesting 5α-dihydrotestosterone is the active androgen in these follicles.

The investigation was continued using DNA microarray to examine the hypothesis that transforming growth factor-β1 (TGF-β1) is a paracrine factor produced by dermal papilla cells and is involved in altering the size of hair follicles during androgenetic alopecia. TGF-β1 has been observed in organ culture as a strong inhibitor factor of human hair growth (Philpott et al., 1990). Androgens induced the production of TGF-β1 by balding dermal papilla cells in vitro (Inui et al., 2002; 2003), and TGF-β1 delayed catagen progression in mice in vivo (Tsuji et al., 2003). Androgens also stimulated balding cultured dermal papilla cells to produce TGF-β2 (Hibino and Nishiyama, 2004) which is associated with the transition from anagen to catagen in human hair follicles in vitro (Soma et al., 2002). Another study showed that cultured balding dermal papilla cells have
the ability to produce inhibitory autocrine factors (Hamada and Randall, 2006). The microarray analysis in this thesis showed that the expression level of TGF-β1 was significantly greater (P<0.01) by 1.848 fold in androgen-inhibited scalp hair follicles than in those from non-balding scalp (figure 3.56). This supports the earlier view that androgen may induce the production of TGF-β1 in balding scalp follicles which may work as an inhibitory factor during androgenetic alopecia causing gradual formation of miniaturized follicles. Importantly, this also validates the microarray results.

Stem cell factor (SCF) is known to play a role in the development of epidermal (Williams et al., 1992; Grichnik et al., 1998) and hair pigmentation (Geissler et al., 1988; Fleischman et al., 1991). Cultured dermal papilla cells derived from beard, balding and non-balding scalp secrete SCF (Hibberts et al., 1996b, Randall et al., 2008). Since the SCF receptor, c-kit, is expressed in scalp hair follicle melanocytes (Randall et al., 2008), this suggests that dermal papilla cells produce SCF to regulate follicular melanocytes and therefore alter hair pigmentation. Dermal papilla cells from beard follicles, which respond to androgen by producing darker hair in vivo, produce more SCF than those derived from non-balding scalp, which is androgen-insensitive (Hibberts et al., 1996b). In contrast, cultured cells from balding follicles, which respond to androgens by forming smaller paler hairs, secreted less SCF (Randall et al., 2008). This suggests that the production of SCF may be increased in beard hair follicles and reduced in balding ones under the influence of androgen, leading to formation of differently coloured hairs. In the DNA microarray results in this investigation there was a significant decrease (P<0.04) in the expression level of SCF in balding scalp follicles compared to the control, non-balding follicles (figure 3.57). These observations make SCF a good candidate as a
paracrine factor produced by dermal papilla cells and controlled by androgens. In addition, the strong parallels between the SCF ELISA results from the cultured cells and the microarray analysis support the biological relevance of the microarray results.

Insulin-like growth factor-I (IGF-I) was also investigated in this study as a possible paracrine factor implicated in androgen-regulation of hair follicle growth, using DNA microarray. IGF-I is a potent stimulator mitogen that has been shown to maintain anagen scalp hair follicles at different physiological concentrations in vitro (Philpott et al., 1994) and stimulate the proliferation of human skin keratinocytes in vitro (Barreca et al., 1992; Tavakkol et al., 1992). Previously, Itami et al., (1995b) showed that the production of IGF-I was increased in response to androgens in cultured beard dermal papilla cells in vitro. They also observed that androgens induced the proliferation of outer root sheath cells when co-cultured with dermal papilla cells, but this stimulatory effect was stopped when IGF-I was blocked using specific antibodies, indicating IGF-I as an androgen-regulated stimulator of hair growth in beard follicles. The microarray data analysis of androgenetic alopecia follicles revealed that the expression level of IGF-I was significantly down-regulated (P<0.02) by 1.762 fold in balding scalp follicles compared to the normal follicles (figure 3.58), suggesting that this reduction may occur under the effect of androgens. We also found that the expression of insulin-like growth factor-I receptor (IGF-IR) was significantly reduced (P<0.002) in isolated balding follicles compared with the control follicles by 1.588 fold.

The stimulatory action of IGF-I can be mediated by specific binding protein such as IGFBP3, which induces the cellular response to IGF-I, as its binding to the cell surface
leading to increased chances of receptor binding (Conover and Powell, 1991; Tesch et al., 1993). In this study, a dramatic decrease of 1.914 fold in IGFBP3 expression in balding follicles at a high level of significance (P<0.001) was also observed (figure 3.59). Overall, although these results need further investigation, they do suggest that androgens may stimulate the growth of beard follicles by increasing the expression of IGF-I in beard dermal papilla cells to produce large follicles, and inhibit scalp hair follicle growth by decreasing the production of IGF-I and IGFBP3 in balding scalp follicles compounding the reduction of IGF-I and resulting in smaller follicles.

Interleukin-1β (IL-1β) is a highly potent inhibitor of the growth of human hair follicles in organ culture in vitro (Xiong and Harmon, 1997). When IL-1β was injected intradermally into the back skin of mice, this resulted in stimulation of the apoptosis of hair bulb keratinocytes in vivo (Ruckert et al., 2000). In organ culture, IL-1β induced inhibition of human scalp hair follicles in vitro (Hoffmann et al., 1997). When human hair follicles were incubated with interleukin-1α (IL-1α), this also led to inhibition of hair follicles growth and hair fibre production (Harman and Nevins, 1993). The DNA microarray analysis in this thesis revealed that IL-1β, and IL-1α were significantly up-regulated (P<0.04 and P<0.01 respectively) in androgen-responsive follicles of balding scalp compared to androgen-insensitive follicles of normal scalp by 1.993 and 1.793 fold respectively (figure 3.60). Recently, Kwack and colleagues (2010) found that the level of interleukin-6 (IL-6) was higher in conditioned media of balding dermal papilla cells than in those from non-balding dermal papilla cells. Following treatment with 5α-dihydrotestosterone, it was observed that the expression level of IL-6 was up-regulated. In addition, injection of IL-6 into the back skin of mice resulted in the
induction of the catagen phase, whilst onset of the anagen phase was delayed by implantation with agarose beads containing IL-6. The microarray analysis also showed that the expression of interleukin-6 (IL-6) was also significantly up-regulated (P<0.03) in balding follicles compared to non-balding follicles by 1.861 fold (figure 3.60). These observations suggest that these inhibitory factors may be produced by androgen-sensitive follicles under the influence of androgen causing inhibition of hair growth.

Vascular endothelial growth factor (VEGF) was also analyzed in the DNA microarray results due to its possible role as a modulator of angiogenesis during the hair growth cycle and during hair size changes. Anagen hair follicles have a well-developed vascular system. Cultured dermal papilla cells were observed to express and secrete VEGF (Lachgar et al., 1996c; Hibberts et al., 1996a; Merrick et al., 1999). The expression of VEGF has been found at low levels in catagen and telogen scalp dermal papilla cells using fluorescent in situ hybridization and confocal laser microscopy (Lachgar et al., 1996b). This suggests that microvascular angiogenesis may occur at the beginning of every new anagen stage in the presence of VEGF. In the DNA microarray analysis, the expression of VEGF was significantly reduced (P<0.03) in androgen-responsive follicles of balding scalp with a decrease of 1.57 fold. This fits with the gradual changes in the size of androgen-responsive hair follicles, for instance during puberty, being reflected in the different size of their blood vessels (Montagna and Ellis, 1958). The secretion of VEGF was stimulated by androgens in both rodent and human androgen-sensitive prostate cancer (Joseph et al., 1997). In patients undergoing androgen deprivation therapy for prostate cancer, the levels of VEGF were decreased compared to its level prior to treatment (Aslan et al., 2005). Based on these observations, VEGF secretion seems to be
regulated by androgen in prostate, suggesting VEGF to be a potential paracrine factor involved in androgen-modulation of hair follicles.

Since HGF may play a role as a powerful modulator of hair growth and be involved in morphogenesis and cycling (Jindo et al., 1995; Lindner et al., 2000), the expression of HGF and its receptor, c-Met, were focused on in the DNA microarray analysis to see if their expression was decreased or absent in balding scalp hair follicles compared to non-balding ones. Following data analysis using the t-test, it was observed that the gene expression levels for HGF and its receptor, c-Met, were both significantly down-regulated (P<0.04 and P<0.03 respectively) in androgen-dependent hair follicles of balding scalp compared to normal follicles of non-balding scalp (figures 3.62). The expression level of HGF in was suppressed by 1.756 fold whereas the expression of c-Met had a decrease of 1.584 fold.

These observations were then validated using another comparative method, quantitative real-time PCR. This method was initially developed using another set of non-balding follicles as matched balding and non-balding samples are too valuable to be used in new methods. Initially, specific forward and reverse primers for each gene were designed by selecting them from the exon part of the genomic sequence of the target gene to ensure that the correct-sized product would only be obtained from cDNA and not from any contaminating genomic DNA sequence that might be present in the PCR mixture. The designed primers for each gene were checked by comparing them with the specific human gene sequence of the target gene using the NCBI BLAST programme. The housekeeping protein, GAPDH, was used as an endogenous control. SYBR Green was
used in each reaction as a DNA binding dye to give the fluorescence signal. Initially, the designed primers for GAPDH, HGF and c-Met were optimised using the gradient feature of the real-time PCR detection system. The cDNA template used for the optimisation reactions was generated from human reference RNA because it is readily available and easy to prepare compared to the human hair follicle cDNA, which is very valuable, due to the limited sample availability and time consuming to prepare, because of the difficult hair follicles isolation prior to cDNA preparation and synthesis. The optimal temperatures for GAPDH, HGF and c-Met primers, which gave the highest signals and yielded the lowest cycle threshold values, were 60.5°C, 58.3°C and 56.8°C respectively (table 3.64).

It is evident from figure 3.66 that the melt curves for both HGF and c-Met only contained a single peak, indicating that these reactions generated only one product for each target gene and no contaminating products were present. Thus, this demonstrated that the genes for both HGF and its receptor, c-Met, were expressed in isolated anagen hair follicles from non-balding scalp from all five experimental samples as seen in figure 3.66.

Due to the limited sample availability from the androgenetic alopecia patients and the lower yield of total RNA, the extracted total RNA from all six samples was amplified to produce high-quality RNA for use in quantitative real-time PCR. The melt curves and agarose gel electrophoresis for HGF and c-Met in both balding and non-balding samples (figures 3.72 and 3.74) confirmed the absence of any contaminating products and confirmed there was only one band of the expected size (144bp) for HGF and (152bp) for c-Met confirming that the PCR products for both genes were produced from the amplification of cDNA. The relative gene expression levels of HGF were significantly (P<0.01) much lower in the small follicles from androgen-responsive balding scalp
than those from the larger androgen-insensitive normal scalp follicles (5.498 \pm 0.278) (figures 3.73 and 3.75), supporting the DNA microarray results. The relative expression level of c-Met was also significantly down-regulated (P<0.01) in balding follicles (1.203 \pm 0.202) when compared to its expression non-balding follicles (3.895 \pm 0.368), confirming the observations of DNA microarray.

Thus, both the DNA microarray and quantitative real-time PCR demonstrated that the levels of expression of both HGF and c-Met genes are reduced in miniaturised scalp follicles in men with androgenetic alopecia. These findings support the original hypothesis and suggest that androgens may have an inhibitory effect on the gene expression of HGF and its receptor, c-Met, in androgen-responsive balding scalp follicles leading to a reduction in the stimulatory action of HGF as a hair-growth-promoting factor. These results in combination with the identification of HGF and c-Met gene expression only in the dermal papilla suggest that reducing the production of HGF by dermal papilla scalp follicle cells under the influence of androgens may result in the formation of smaller or miniaturised follicles by an autocrine mechanism, whereas HGF in normal scalp may have a role in maintaining large hair follicles. Thus, HGF is a good candidate for involvement in androgen-modulated hair growth as a paracrine factor produced by the dermal papilla cells. Interestingly, HGF was also differently expressed in cultured dermal papilla cells derived from human non-balding and balding scalp and beard skin (Merrick, 2000). HGF expression was very low in balding scalp dermal papilla cells, whereas beard cells showed a greater HGF expression than a non-balding scalp cells in line with this study.
The investigation then moved on to examine whether another hepatocyte growth factor family member, MSP, a possible hair growth regulating paracrine factor, and its receptor, RON, could be reduced in androgen-sensitive follicles of androgenetic alopecia scalp compared to normal follicles, using the DNA microarray method. Exposure to different concentrations of MSP has resulted in increased human hair follicle length in vitro, and stimulated telogen follicles of mouse skin to enter anagen phase in vivo (McElwee et al., 2004). Another study observed that MSP stimulated mouse epithelial cell growth in vitro (Wang et al., 1996b). The microarray analysis showed that the expression of both MSP and its receptor, RON, were considerably reduced (P<0.05) in balding scalp hair follicles compared to non-balding scalp hair follicles by about 1.803 and 1.644 fold respectively (figure 3.63).

The findings of gene microarray analysis were then validated using the quantitative real-time PCR in which MSP and its receptor, RON, were amplified using specific primer sequences for each gene which optimised initially to obtain the optimal annealing temperatures which were 58.3°C and 60.5°C respectively (figure 3.65). The melt curves and agarose gel electrophoresis for MSP and RON in both balding and non-balding samples confirmed the absence of any contaminating products and confirmed there was only one band of the expected size for MSP (166bp) and RON (117bp) confirming that the PCR products for both genes were produced from the amplification of cDNA (figures 3.76 and 3.78). The relative expression levels of both MSP and its receptor, RON, were significantly reduced (P<0.03 and P<0.04 respectively) in androgen-dependent hair follicles of balding scalp (3.461 ± 0.744 and 1.596 ± 0.458 respectively) compared to androgen-insensitive follicles of non-balding scalp (9.851 ± 0.996 and 4.599 ± 0.359)
respectively) (figures 3.77 and 3.79) supporting the microarray observations. This is similar to an earlier report by Shorter (2007) who was unable to detect MSP in isolated human hair follicles of balding scalp using RT-PCR.

The differential expression of MSP and its receptor, RON, between small androgen-responsive follicles of balding scalp and large androgen-insensitive follicles of normal scalp, suggests that androgens have suppressed the expression of these genes \textit{in vivo}. Therefore, these results support the original hypothesis that androgens may also inhibit the gene expression for MSP and its receptor, RON, in androgen-dependent follicles of balding scalp, resulting in a decrease in the stimulatory effect of MSP as a hair growth promoter. Therefore, decreasing the production of MSP in androgenetic alopecia under the effect of androgens may lead to the formation of smaller hair follicles. However, this mechanism appears to be more complex than HGF and c-Met, because in contrast to HGF and c-Met whose expression was restricted to the dermal papilla, MSP and RON were found in both dermal papilla and epithelial cells of the follicle.

Further work could be done to confirm that HGF and MSP have an effect on hair follicle growth using silencing RNA (siRNA) approach (Chen and Zhaori, 2010; Atkinson et al., 2010) in isolated human scalp hair follicle organ culture (Shorter et al., 2008). This would aim to block the synthesis of HGF or MSP taking place by binding the mRNA with a complementary sequence. If HGF or MSP are necessary for hair follicle growth, it would be expected that hair growth in organ culture would be inhibited.
Another experiment could be applied to examine the role of hepatocyte growth factor family members, HGF and MSP, in androgen-regulation of hair growth by measuring the gene expression levels of these molecules in human cultured dermal papilla cells in the presence, and absence, of testosterone \textit{in vitro}. The influence of testosterone on the expression of these molecules can be intercepted using anti-androgens for example cyproterone acetate. This is a difficult experiment to carry out because the cells would need to be derived from follicles which are sensitive to androgens for example beard or androgenetic alopecia follicles. These are now very difficult to obtain.

Another experimental method could be done to confirm the effect of HGF and MSP on hair follicle growth \textit{in vitro} using different concentrations of recombinant human HGF or MSP in the growth media, whereas control follicles should receive PBS instead. Hair follicle length should be measured everyday to see if there is any difference in their length comparing to control ones. The effect of these molecules on hair follicle length could be examined further by blocking their effects using specific antibodies.

From the work in this thesis, it is clear that androgenetic alopecia involves reduction in the expression of a range of genes including HGF family members. Whether these genes are directly regulated by the androgen-androgen-receptor complex or altered as a result of an earlier response gene such as a transcription factor is currently unclear. Chromatin immunoprecipitation (CHIP) technique (Nelson et al., 2006) could be used to identify androgen binding sites in androgen regulated genes. It is based on precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. CHIP is used to investigate the interaction between protein and DNA in the cells through
determining whether specific proteins are associated with specific genomic regions such as transcription factors on promoters or other DNA binding sites. Using this method, the androgen receptor would be selectively immunoprecipitated from a chromatin preparation using anti-AR antibodies to identify the DNA sequences associated with it. This should establish whether HGF family members or other genes identified in this thesis are directly regulated by androgens. Again the difficulty will be in obtaining sufficient androgen-sensitive human material to carry out these exciting studies.

To conclude, the data provided in this thesis support the view that androgens may exert their effect on the hair follicle via its dermal papilla cells altering their production of paracrine factors, some of which are stimulatory and some of which are inhibitory. It is clear from the microarray analysis that the changes are not due to a simple switch on or off of a single factor or factors, but rather involve modulation of a range of factors. It is not surprising that this mechanism is complex, since the hair follicle is a complicated organ involving a range of cell types and regressive and regenerative processes during the hair cycle. All these processes need to be maintained to keep producing hairs even if the type, size and colour of the hair produced is altered due to androgen influence. These factors may target different follicular components resulting in changes in the type of hair produced. An equilibration between these stimulatory and inhibitory factors is required for hair follicle maintenance and normal hair production. Disruption of this balance may lead to disorders of hair growth such as androgenetic alopecia and hirsutism. Better understanding of the roles of these factors in androgen-regulating hair growth could lead to better treatments of hair disorders.
References
References


and sweat gland genetic programs in response to embryonic dermal stimuli. Development 127, 5487-5495.


Randall, V. A., Thornton, M. J., and Messenger, A. G. (1992). Cultured dermal papilla cells from androgen-dependent human hair follicles (e.g. beard) contain more androgen receptors than those from non-balding areas of scalp. J Endocrinol 133, 141-147.


Shorter, K. (2007) Investigation into the mechanisms of factors which alter hair growth, PhD thesis, Department of Biomedical Sciences, University of Bradford


Appendix I

Preparation of solutions for Sacpic staining:

Celestine blue:
Celestine blue (2.5g) was added to 5% aqueous solution of Ferric ammonium sulphate (500ml), this was boiled for 3 minutes, cooled and filtered. Glycerol (70ml) was then added and mixed well.

Safranin:
Safranin (6g) was added to Ethanol/H2O (1:1) (300ml), the solution was mixed thoroughly and filtered before use.

Picric acid/ethanol:
Saturated picric acid alcoholic solution (5ml) was added to absolute ethanol (300ml) and mixed thoroughly.

Picro-indigo carmine:
Indigo carmine (1g) was added to saturated picric acid aqueous solution (300ml) and mixed thoroughly.

Scott's tap water:
Potassium bicarbonate (2g) and magnesium sulphate (20g) were added to distilled water (1L) and mixed thoroughly.

Gills haematoxylin:
Haematoxylin (2g), sodium iodate (0.2g), aluminium sulphate (17.6g), and ethylene glycol (250ml), were added in order to distilled water (370ml) with glacial acetic acid (20ml). This was mixed on a magnetic stirrer for 1 hour at room temperature.
**Appendix II**

**Human reference total RNA:**

The universal human reference RNA (Stratagene, UK) is comprised a collection of total RNA pooled from ten different cell lines for an optimal broad coverage of human genes. Quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance.

Human reference total RNA cell line derivations:

1- Adenocarcinoma, mammary gland  
2- Hepatoblastoma, liver  
3- Adenocarcinoma, cervix  
4- Embryonal carcinoma, testis  
5- Glioblastoma  
6- Melanoma, skin  
7- Liposarcoma  
8- Histiocytic lymphoma, histocyte  
9- Lymphoblastic leukemia, T lymphocyte  
10- Plasmacytoma, myeloma, B lymphocyte