Hair Follicle Transplantation: Implications for Cell Migration and Wound Healing

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Abstract

Chronic wounds are a major problem in the health care system. The increasing incidence of obesity and diabetes and longer life expectancy are adding to the burden. Treatment options are currently limited indicating needs for new treatment modalities.

Impaired innate healing response is the main characteristic of chronic wounds. In recent years, there has been a focus on stem cell therapy and tissue engineering as an alternative treatment for chronic wounds to replace the damaged tissue with cells that possess a normal injury response.

The hair follicle’s continuous cycling and their response to trauma stimulation, indicate a strong regenerative potential. It has been shown that hair follicles participate in wound re-epithelialization and respond to skin injury by promoting skin innervation and vascularization.

This dissertation explores the potential of hair follicle transplantation to enhance wound healing. It was hypothesized that hair follicle transplantation can promote healing in skin wounds. Whole hair follicle were isolated and re-established in the wound site where they preserved their structure and function. This approach exploits the normal endogenous function of hair follicle cells to enhance skin wound healing.

First, a vibrissae follicle transplantation technique was established and optimised in nude mice. Using donor follicles from GFP transgenic mice made it possible to investigate cell trafficking from transplanted follicles during epidermal and dermal repair. Transplanted vibrissae follicles integrated normally into the host epithelium and ninety percent of grafted vibrissae produced a hair shaft. The GFP labelled cells were confined to the transplanted hair follicles and were not found in the interfollicular epidermis. It was then possible to identify migrating hair follicle cells from recipient inter-follicular epidermis in lineage tracing wound experiments.

Healing responses were then compared in excisional wounds with and without adjacent transplanted follicles. My findings re-confirmed that hair follicle cells participated in
wound re-epithelialisation. Neighbouring transplanted follicles accelerated wound closure. Wounds adjacent to transplanted follicles also showed four times higher nerve density in the granulation tissue than the wounds without adjacent follicular transplants. There was no significant difference in capillary density and macrophage density in wounds with and without hair transplants.

I also conducted a preliminary investigation of incisional wounds perpendicular or parallel to transplanted follicles, to compare hair follicle cell migration from damaged and intact hair follicles. In these experiments, intact hair follicles participated only in the epidermal component of wound healing and cells migrated from transplanted hair follicles to the healing dermis only when the hair follicles were cross-sectioned.

The results of this dissertation indicated that re-epithelialization and promotion of innervation were possible mechanisms by which transplanted follicles contributed to wound healing. These findings lay the foundations for using follicular transplantation to promote healing in chronic or neuropathic wounds and peripheral nerve injuries. In addition, damage to hair follicles may be a starting signal for migration and participation of hair follicle cells in the repair of dermis, which may subsequently influence the healing outcome.
Declaration

This is to certify that

i. The thesis comprises only my original work towards the Doctor of Philosophy,

ii. Due acknowledgment has been made in the text to all other material used;

iii. The thesis is less than 100,000 words in length, exclusive of tables and bibliographies.

Azar Asgari
Acknowledgements

Without the support of many this project would have never come to fruition.

Foremost, I would like to express my sincere gratitude to my supervisors, Prof. Rodney Sinclair, Dr. Nicholas Rufaut, Prof. Wayne Morrison, Dr. Rodney Dilley and Ass/Prof. Lesley Jones.

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List of Publications and Presentations

Publications

An intraepithelial route for metastasis: a possible mechanism for distant metastasis arising from in situ carcinoma.

Hair transplantation in mice: Challenges and solutions.

Comparative Analysis of Paracrine Factor Expression in Human Adult Mesenchymal Stem Cells Derived from Bone Marrow, Adipose, and Dermal Tissue.

Male pattern androgenic alopecia
Asgari AZ, R Sinclair
Medicine Today, 2011

Presentations

Hisao ST, Dusting GJ, Asgari AZ & Dilley RJ. Characteristics of cellular paracrine activity in human adipose mesenchymal stem cells. Poster presentation at International Vascular Biology Meeting16th scientific conference. Los Angeles, California ,USA, June 2010

Asgari AZ, Dilley RJ, Rufaut NW & Sinclair RD. Hair follicle transplantation improves wound healing. Poster presentation at 41st Annual ESDR Meeting, Barcelona, Spain. September 2011, Award: St Vincent's Hospital, Department of Medicine travel award.


Asgari AZ, Rufaut NW, Jones LNN & Sinclair RD. A simple hair transplantation model to investigate follicular cells and their interaction with surrounding skin. Poster presentation at Epworth research week, Epworth Research Institute, Richmond, Victoria, Australia, July 2016

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### Abbreviations

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<th>Full Form</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>CNPase</td>
<td>2',3'-cyclic nucleotide 3'-phosphodiesterase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Dermal papilla</td>
</tr>
<tr>
<td>DS</td>
<td>Dermal sheath</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGA</td>
<td>Estimated gestational age</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EPCs</td>
<td>Endothelial progenitor cells</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGFb</td>
<td>basic Fibroblast growth factor</td>
</tr>
<tr>
<td>GAD67</td>
<td>Glutamate decarboxylase 67</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HF</td>
<td>Hair follicle</td>
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<td>HT</td>
<td>Hair transplant</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>iPS cell</td>
<td>Induced pluripotent stem cell</td>
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<tr>
<td>LRC</td>
<td>Label retaining cells</td>
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<tr>
<td>microRNA</td>
<td>miRNA</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
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<td>Room temperature</td>
</tr>
<tr>
<td>SKPs</td>
<td>Skin-derived precursors</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
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<td>Tg</td>
<td>Transgenic mice</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
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<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
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### Measurement and statistical symbols

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<tbody>
<tr>
<td>×</td>
<td>times</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>cm</td>
<td>centimeter</td>
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<td>g</td>
<td>gram</td>
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<td>IU</td>
<td>international units</td>
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<td>ng</td>
<td>nanogram</td>
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<td>nm</td>
<td>nano meter</td>
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<tr>
<td>P</td>
<td>probability</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<td>U</td>
<td>units</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<td>µl</td>
<td>microliter</td>
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<td>µm</td>
<td>micrometer</td>
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Chapter 1
General Introduction and Review

1.1 Problem statement

Skin is the largest organ in the body, accounting for 15% of the total body weight of human adults. Protection against dehydration, physical and biological damage, and the wound healing response are the main functions of skin, and vital biological processes [1].

The complex process of wound repair has evolved to prevent bleeding, and rapidly close the wound gap. Repair mechanisms involve skin contraction and replacement of the lost tissue by re-epithelialization and formation of a new dermal matrix.

The ability to regenerate damaged tissue varies in different organisms [2]. Primitive multicellular organisms like sponges [3] and primitive vertebrates such as small amphibians and reptiles [4] are capable of complete wound healing. However, in larger and more complex animals regeneration is more difficult. With the exception of injury to foetal skin [5], wound healing in adult humans and other mammals is associated with scar formation. This rapid formation of scar tissue in adult mammals presumably provides a survival advantage by preventing infection and mechanical deformation in larger tissues [2].

The healing process in all tissues consists of overlapping stages of coagulation, haemostasis, inflammation, proliferation, including dermal fibrosis and re-epithelialization and tissue remodelling. When these processes are altered, a chronic wound may develop. Chronic wounds associated with an impaired healing response due to systemic diseases, vascularity and innervation defects, or an abnormal microenvironment such as one caused by inflammatory cytokines/growth factors.

Chronic wound most commonly occur with systemic diseases and classified based on their underlying cause. Vascular insufficiency, diabetes mellitus, and local-pressure are the
major causes of non-healing wounds. Advanced age and compromised nutritional or immunological status are also associated with chronic wounds.

Chronic wounds are a major health and economic burden for individuals and society and cause a significant degree of global morbidity and mortality [6]. American figures show that more than 6.5 million patients suffered from chronic wounds, which cost more than US$25 billion per year (Sen et al, 2009) [7]. In the United Kingdom, the costs of chronic wounds to the National Health Service (NHS) were estimated at £2.3 to £3.1 billion per year [8]. In Australia treating wounds costs $2.85 billion annually which is approximately 2% of the total national health care budget (Australian Wound Management Association 2014) [9]. This figure is rising with increases in life expectancy and the prevalence of diabetes and obesity in the population.

Despite the availability of several treatment options, the treatment of chronic wounds is still far from satisfactory. In recent years, there has been a focus on stem cell therapy and tissue engineering as an attractive alternative treatment for chronic wounds, with the hope that they will not only repair the physical defect, but also provide cytokines and growth factors, and replace the wound with a tissue that evinces normal response to injury.

Over the last decade, hair follicles have been a focus of regenerative medicine and stem cell research. The follicles’ continuous cycling and repair response to damage stimulation suggests a strong regenerative capacity. The onset of the catagen phase of the hair cycle is associated with involution of the entire hair follicle below the insertion of the arrector pili muscle through apoptosis. Activation of stem cell populations in the hair bulge, induce regerantion of the hair buld and the onset of the anagen growth phase. Various additional populations of epithelial, mesenchymal and neural crest stem cells have been identified in the follicle bulb, suprabulbar region, sebaceous duct and dermal papilla (will be discussed in 1.5). Hair follicles are not only involved in hair regeneration, but also participate in wound re-epithelialization and respond to skin injury by promoting skin innervation and vascularization [10, 11].
This thesis explores the potential of hair follicle transplantation in wound management. It was hypothesized that hair follicle transplantation may promote healing in skin wounds. This approach exploits the normal endogenous function of hair follicle cells to enhance skin wound healing. Transplantation of hair follicles may promote healing by providing a tissue rich in epidermal and dermal stem cells, and an organ responsive to injuries in the skin wounds.

Chapter 1 provides an up to date review of the existing literature relevant to this thesis. This literature review begins with an introduction to skin development and structure, hair follicle anatomy, embryology and physiology, and passes on to a review of the discrete stem cell populations identified within the hair follicle. This in turn is followed by a review of cutaneous stem cells, the normal wound healing process and the physiopathology of chronic wounds in humans. The last part of the review is focused on the response of hair follicles during wound healing and the potential applications of hair follicle transplant in wound healing.

Chapter 2 describes the methodologies used in the series of experiments conducted. Chapter 3 describes the development of the hair transplantation technique in nude mice. This technique was then used to investigate the contribution of cells from hair follicles in excisional and incisional wounds described in Chapters 4 and 5 respectively.

Chapter 6 is a general discussion and overview of the significance of my research findings and outlines future possible avenues for research on this topic.
1.2 Skin anatomy and development

1.2.1 Skin anatomy

Skin is the largest organ in the body and consists of three distinct layers: the outermost stratified cellular epidermis, the underlying connective tissue of the dermis, and a layer of fatty tissue beneath the dermis that separates the skin from the fascia that covers the rest of the body (Figure 1-1). In rodents, there is a layer of striated muscle deep in the sub-dermal adipose tissue, called the *panniculus carnosus muscle*. The anatomical structure of human skin is similar in most areas of the body; however, this structure is modified in specialized areas such as the palms, soles of the feet and the scalp.

![Anatomy of skin and its appendages](image)

*Rook’s Text Book of Dermatology, 2010*

*Figure 1-1  Anatomy of skin and its appendages [12]*

**Epidermis**

The epidermis is formed by proliferation of keratinocytes in a basal layer named the *stratum basale*. Cells move outward and become flattened as they differentiate. The differentiating keratinocytes form several layers of cells that can be divided into four well-defined layers: *stratum basale, stratum spinulosum, stratum granulosum* and *stratum corneum* (Figure 1-1). The process of differentiation whereby a cell travels from the
stratum basale to reach the stratum corneum takes about 28 days in humans and 8-10 days in mice.

The stratum basale consists of small cuboid cells with large nuclei and a cytoplasm rich in ribosomes [12] (Figure 1-1). Generally the stratum basale is one layer of cells; however, in glabrous skin and in hyper-proliferative skin it may be two or three layers of cells. Keratin 5 and Keratin 14 are expressed in the mitotically active basal layer keratinocytes and this expression is down-regulated as cells differentiate [13, 14]. Basal layer keratinocytes are also characterised by abundant intra-cellular actin filaments, which are connected to the extra-cellular matrix by α- and β-cadherin mediated adherent molecules and integrin mediated connections (desmosomes, see below) (reviewed in [15] and [16]). The basal layer of epidermis contains epithelial stem cell populations responsible for skin maintenance and repair. This will be discussed in 1.5.2.

The stratum spinulosum is located immediately above the stratum basale and is composed of several layers of post mitotic, non-proliferative cells. They produce more robust intermediate keratin filaments (Keratin 1 and Keratin 10) connected with cell membrane adhesion molecules (see below) which accumulate as the cells move upward (reviewed in [17]). The spinous or prickled appearance of the cells is due to numerous intercellular connections that tent the cell membrane following the cell dehydration that occurs during in vitro fixation.

The stratum granulosum is the layer above the stratum spinulosum, which is characterised by intra-cellular granules containing keratohyalin proteins that give the cells a granular appearance. The cytoplasm of the granular cell layer also contains smaller lamellate granules containing lipid compounds, which are known as lamellar granules or bodies. In a late stage of differentiation at the uppermost layer of stratum granulosum, intracellular lamellar granules discharge their lipid components into the extracellular space between squames (dead flattened stratum corneum cells), and act as a waterproof barrier (reviewed in [12, 17]).
The outer layer of epidermis, the stratum corneum, consists of flattened cells that have lost their nucleus and cytoplasmic organelles. The individual corneocytes and the extracellular lipids extruded from the lamellar granules, act together to form the outermost waterproof barrier.

Stratum corneum is predominately comprised of α-keratin intermediate filaments (Keratin 1 and Keratin 10) and a matrix formed from keratohyalin proteins, including filaggrin, cross-linked together with the plasma membrane proteins, including involucrin and loricrin. This layer also contains several additional lipids produced by cells in deeper layers and forms an insoluble cornified envelope (reviewed in [18] and [17]). Filaggrin is an interfilamentous protein, which has an important role in aggregating keratin intermediate filaments into disulfide cross-linked macrofibrils. Mutation in the filaggrin gene impairs the barrier function of skin and is a major risk factor for the development of atopic dermatitis, and systemic allergies (reviewed in [19]).

**Cell membrane adhesion complexes**

Cellular membrane adhesion molecules exist between keratinocytes, and serve to bind these cells together. These molecules also exist between cells and basement membranes and serve to maintain the integrity of the dermal-epidermal junction. Adhesive molecules are also involved in communication between cells, and regulate barrier integrity in skin. Alterations in the cell membrane adhesion complex influence cell adhesiveness and play an important role in cell migration during embryonic development [20], wound repair and malignancies (reviewed in [12, 21, 22]). The role of adhesion molecules in wound re-epithelialization will be discussed in 1.6.1.

There are various types of adhesion molecules. Adhesion molecules may anchor to intracellular keratin (desmosomes) or actin filaments (adherens junction), which provide structural integrity in epidermis [12]. Adhesion molecules may connect the cytoplasm of adjacent cells and allow molecules to pass between cells (gap junctions), or seal adjacent cells together and regulate the passage of water and other molecules through the epithelial
cell layer (tight junctions). Adhesion molecules may also connect cells to the extra-cellular matrix (hemi-desmosome) (Figure 1-1, reviewed in [12, 22]).

**Desmosomes**

Desmosomes are complex cellular structures that contain a number of adhesion molecules. These molecules bind cell membranes from neighbouring cells together and connect the cell membrane to the keratin intermediate filaments in the cytoplasm [23]. The intra- and inter-cellular connections of keratin filaments through desmosomes produce a durable structure that allows epidermal cells to withstand trauma and protect the underlying dermis and subcutis [12].

![Molecular blueprint of the desmosome](image)

**Figure 1-2** Molecular blueprint of the desmosome. Transmembrane desmosomal cadherins, desmoglein and desmocollin, bind the armadillo family protein plakoglobin, which in turn anchors the plakin family member desmoplakin and plakophilin. The cytoplasmic plaque anchors the intermediate filament cytoskeleton to the desmosome [24].

Over twenty structural proteins have been identified in desmosomes. The trans-membrane components of the desmosome complex are glycoproteins that belong to the desmoglein (Dsg) and desmocollin (Dsc) subfamilies of cadherins (Figure 1-2). Cadherins are Ca^{2+}-dependent cell adhesion proteins [20]. Transmembrane proteins are connected to the keratin intermediate filament via a complex of several proteins, including desmoplakin, plakoglobin, plakophilin and additional accessory proteins (Figure 1 2) [20, 25]. There are several isoforms for each protein encoded from multigene families, and several isoforms of
Dsg or Dsc can be found in the same cell or even the same desmosome [22], which adds to the complexity of desmosomes.

**Adherens junctions**

Adherens junctions are trans-membrane structures that anchor to intra-cellular actin microfilaments and contribute to tissue integrity and tensile strength in the epidermis. [26] Adherens junctions consist of cadherin molecules (E- and P-cadherin) combined with cytoplasmic plaque proteins (α-catenin, β-catenin, γ-catenin), connected to cytoskeletal actin microfilament (reviewed in [12, 22]).

Adherens junctions influence cell migration during morphogenesis, wound healing and carcinogenesis. E-cadherin is down-regulated in keratinocytes that invade the underlying dermis during skin appendage formation. Failure to down-regulate cadherin-based adhesion suppresses invasive growth of keratinocytes and thus appendage formation [27]. Using an *in vitro* human skin model Alt-Holland *et al.* [28] showed that down-regulation of E-cadherin in squamous cell carcinoma increases cell migration and invasion through the basement membrane.

**Hemi-desmosomes**

Hemi-desmosomes in the cell membrane connect intra-cellular keratin filaments to extra-cellular matrix and play a critical role in attaching keratinocytes to the basement membrane and maintaining the function and integrity of the skin (Figure 1-1) [29].

Hemidesmosomes are visualized by electron microscopy as electron-dense attachment complexes that extend from the intra-cellular compartment of basal keratinocytes to the dermo-epidermal basement membrane [12]. Several molecules have been characterised in the structure of hemi-desmosomes through studies of inherited or autoimmune blistering diseases in humans such as Bullous pemphigoid (BP) or Epidermolysis Bullosa (EB) [22].

In the inherited blistering skin diseases, a mutation in the gene responsible for formation of a structural protein leads to reduced or non-functional protein. In the immunobullous diseases, autoimmunity leads to either antibodies or cell-mediated immunity directed at
structural proteins. If the absent, altered or immunologically attacked structural protein is within the hemidesmosomes, this will lead to fragility at the dermal epidermal junction and tense blister formation with trauma. In contrast, if the absent, altered or immunologically attacked structural protein is within desmosomes, this will lead to fragility within the epidermis and flaccid blister formation with trauma [22].

Intra-cellular K5 or K14 intermediate filaments are connected to hemi-desmosomal plaque components. Molecules such as integrin and type XVII collagen in keratinocyte cell membrane form the trans-membrane component of hemi-desmosomes, which are connected to proteins in the basement membrane such as Laminin and type IV collagen (reviewed in [12, 22]).

The specific composition of desmosomes and hemi-desmosomes determine their structure, which in turn, influences the adhesive properties of cells and regulates cell migration (reviewed in [24] [30]). Desmosomes are thought to function as signalling molecules that participate in fundamental processes such as cell proliferation, migration, differentiation and morphogenesis (reviewed in [20]).

Tight junction

Tight junctions are believed to “seal” the intercellular space and are crucial for maintaining a barrier between two compartments (reviewed in [22]). In the granular layer of the epidermis, tight junctions maintain the integrity of the water barrier in skin and prevent water loss.

Gap junction

Gap junctions are channels within the cell membrane that connect the cytoplasm of adjacent cells. Gap junctions allow molecules to pass between cells. Communication between gap junctions plays an important role in cell synchronization and differentiation (reviewed in [22]).
**The dermal–epidermal basement membrane**

The interface between the lowest part of the epidermis and the uppermost layer of the dermis is the basement membrane. The basement membrane consists of the extracellular matrix macromolecules, including type IV collagen and collagenous glycoproteins, such as laminin [12, 31], which are synthesized by both basal keratinocytes and dermal fibroblasts [32].

Electron microscopy has shown that the dermo-epidermal basement membrane consists of two distinct layers with different optical densities [12]. The top layer is the lamina lucida. The lamina lucida has a lower electron density and is in contact with basal keratinocytes. The lower layer has a higher electron-density and is called the lamina densa (reviewed in [12]). Electron microscopy also enables visualization of the hemidesmosomes (see above) that connect the keratin filaments from basal keratinocytes to the upper papillary dermis. As mentioned previously, an acquired autoimmune attack or an inherited absence or alteration to components of the hemidesmosomes, anchoring filaments or anchoring fibrils, produces fragility at dermal epidermal junction and bullae formation with minor trauma [33] (reviewed in [12]).

**Dermis**

The dermis provides structural and nutritional support to the epidermis. In adult skin, dermis has two anatomically distinguishable layers. The upper papillary dermis comprises loose connective tissue in contact with rete ridges of the epidermis, while the deeper layer, reticular dermis is composed of dense connective tissue [34].

In contrast to the epidermis, dermis is vascularised, has few cells and is mainly composed of an extracellular matrix (ECM) of connective tissue. The ECM is the collection of extracellular molecules secreted by cells and is composed of proteoglycan and glycosaminoglycan macromolecules, collagen and elastin fibres. These molecules contribute to tissue integrity and provide mechanical stability in response to biomechanical forces. In addition, ECM macromolecules regulate cell properties and function, through interactions between ECM components and cell-surface receptors, growth factors and
cytokines [35].

The main cellular components of dermis are fibroblasts. Fibroblasts are a primary source of proteoglycan/glycosaminoglycan macromolecules, collagen and elastic fibres in the dermis and basement membrane [12, 36]. Fibroblasts also produce protease enzymes such as metalloproteinases that are involved in ECM homeostasis and turn-over [37, 38].

**Collagen fibres**

Collagen comprises about 80% of the total dry weight of the dermis [12]. Several types of collagen are recognised in vertebrates.

All collagens consist of three helical monomer chains, which form a triple helical structure. The three helical monomer chains may be the same (homotrimeric) as in Type II collagen, or different (heterotrimeric), as in Type I collagen [39].

The main dermal collagens are types I, II, and III, which are the fibril-forming collagen triple helical structures that bind together and form fibrils and fibres [12]. While type I collagen is the major component of the fibrils in adult dermis, during embryogenesis and wound repair, the content of type III collagen is prominent [40].

The collagen type IV that forms sheets or networks, is the main collagen found in the basement membrane [12]. In addition, nests of type IV collagen found within the dermis bind to the anchoring fibrils that loop through the basement membrane and attach the basement membrane to the dermal ECM [40].

The anchoring fibrils are made of type VII collagen, (which attaches the basement membrane to the dermal ECM,) while the anchoring filaments that attach the type IV collagen in the basement membrane to the basal keratinocytes, is made up of type XVII collagen [40]. Mutations of collagen VII and XVII genes produce skin fragility at the dermal-epidermal junction and sub-epidermal blistering disease [22].

The biochemical structure of various collagen molecules determines their physical characteristics. Collagen fibrils are triple helix polymers. The triple helix is formed from
three helical monomer chains. In each monomer chain, every third amino acid is glycine, so the sequence of an α-chain can be expressed as (Gly-X-Y). [40]. A high number of hydroxyproline residues are in Y positions which contributes to the stability of the helix, due to hydrogen bonds between the hydroxyl groups of hydroxyproline [40]. Triple helical collagen fibrils cross-link to form collagen fibres. Multiple intra- and inter-molecular cross-links provide collagen with great tensile strength [18]. In some types of collagen, an uninterrupted Gly-X-Y repeated sequence provides a rigid, rod-like structure. When the sequence is interrupted by one or more amino acids, the collagen molecule acquires more flexibility [40].

**Elastic fibres**

Elastic fibres comprise about 2-4% of the total dry proteins in the dermis. In the reticular dermis, horizontally oriented elastic fibres with interconnections form a network structure. There are also vertical extensions of fine elastic fibres with fewer cross-linkages [12].

Elastic fibres have two main components: highly cross-linked elastin monomers surrounded by elastin-associated micro-fibril structures. Elastin-associated micro-fibrils are less characterised compared to elastin fibres and their precise physiological roles are currently unknown [41].

The amino acid composition of elastin is similar to that of collagen fibrils, and glycine comprises about one third of the total amino acid contents; however, glycine is not evenly distributed in elastin in every third position as it is in collagen fibrils [12].

During embryonic development and wound repair, the first elements of the elastic fibres to form are the micro-fibrils, which form a scaffold for the alignment of elastin molecules [42]. As fibres grow, the elastin proteins come to predominate over micro-fibrils. Elastin protein makes up 90% of fully developed elastic fibres, while the microfibres that surround the core elastin molecule, comprise a small percentage of the total content of mature elastic fibres (reviewed in [43]).
Elastic fibres are highly cross-linked and have significant elasticity. They can be stretched by more than 100% and still return to their original form [40].

**Proteoglycan / glycosaminoglycan macromolecules.**

In addition to collagen and elastin fibres, the ECM also contains proteoglycan and glycosaminoglycan macromolecules. Glycosaminoglycans (GAG) are polysaccharides composed of sulfated and acetylated sugars with negative charges that can bind large amounts of ions and water.

Hyaluronic acid is the most prominent protein-free GAG, which has long filaments (500 nm–10 mm). This macromolecule binds to water providing the hydrated consistency and viscoelasticity of skin [35]. Hyaluronic acid also interacts with cell surface receptors, which influence inflammation, chemotaxis, cell migration, collagen secretion and angiogenesis [44-46].

Multiple chains of glycosaminoglycans (GAG) can bind to core proteins and form proteoglycans [12]. They are able to bind to collagen in the ECM. Glycosaminoglycans are considered biologically active molecules as they are able to bind to several growth factors, cytokines and cell adhesion molecules or growth factor binding proteins and work as antiproteases [12].

<table>
<thead>
<tr>
<th>Proteoglycan</th>
<th>Size of the core protein (kDa)</th>
<th>Glycosaminoglycan (GAG) side chains (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versican</td>
<td>265–370, splice variants</td>
<td>Chondroitin/dermatan sulfate (10–30)</td>
</tr>
<tr>
<td>Perlecan</td>
<td>400–467</td>
<td>Heparan/chondroitin sulfate (3)</td>
</tr>
<tr>
<td>Decorin</td>
<td>40</td>
<td>Chondroitin/dermatan sulfate (1)</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>42</td>
<td>Keratan sulfate (2–3)</td>
</tr>
<tr>
<td>Lumican</td>
<td>38</td>
<td>Keratan sulfate (3–4)</td>
</tr>
<tr>
<td>Keratocan</td>
<td>38</td>
<td>Keratan sulfate (3–5)</td>
</tr>
<tr>
<td>Biglycan</td>
<td>40</td>
<td>Chondroitin/dermatan sulfate (2)</td>
</tr>
<tr>
<td>Syndecans 1, 2, 4</td>
<td>35–120</td>
<td>Heparan/chondroitin sulfate (3–</td>
</tr>
</tbody>
</table>

Proteoglycans can vary by the number, type and length of their glycosaminoglycans, and by their protein content (Table 1-1). The most important proteoglycan in dermis is
versican, which binds with elastic fibres and forms huge complexes with hyaluronic acid, providing structural integrity in the dermis [40].

**Fibroblasts**

Fibroblasts are the main cellular component of the dermis and the primary source of proteoglycan/glycosaminoglycan macromolecules, collagen and probably elastic fibres in the dermis and basement membrane [12, 36]. They are involved in ECM homeostasis and turnover by providing protease enzymes such as metalloproteinases [37, 38].

The embryonic mesoderm is the origin of fibroblasts in the dermis, except in the craniofacial area, in which both embryonic neural crest and mesoderm contribute to formation of dermis [47-49]. Diversity in the developmental origin of dermis will be discussed in 1.2.2.

In addition to this diversity, functional heterogeneity is also observed in two populations of dermal fibroblasts in the upper and lower dermis. Using transplantation assays and lineage analysis, Driskell et al. [50] revealed that cells originating from the upper dermis at early stages of embryogenesis provide signalling for hair follicle formation and participate in formation of hair follicle dermal papilla, the dermal sheath and arrector pili muscles. The lower embryonic dermis; however, is involved in the formation of the reticular dermis, adipocytes of the hypodermis, and synthesis of the bulk of the extra cellular matrix [50]. They also showed that during wound healing it is mainly the lower dermal fibroblasts that participate in dermal repair. Upper dermal fibroblasts are recruited only in the later stage and appear only underneath the fully re-epithelialised epidermis [50]. This will be discussed in more detail in 1.6.1.

**Subcutaneous fat**

Adult men and women have at least 3.0–4.5 kg of adipose tissue. 80% of total fat is contained in subcutaneous tissue [12]. Subcutaneous adipose tissue has diverse biological properties compared to abdominal adipose tissue. Distinct white and brown subcutaneous adipose tissues have been characterised (reviewed in [12, 51]).
Brown adipose tissue is found more in newborn humans and rodents. It is characterised by a large number of mitochondria and multilocular lipid droplets (as opposed to unilocular lipid droplets found in white adipose tissue). Further characteristics include a high degree of vascularization and sympathetic innervation, and most importantly, expression of uncoupling protein 1 (UCP1). UCP1 is a mitochondrial protein that uncouples oxidative phosphorylation. The uncoupling of oxidative phosphorylation results in inefficient production of ATP and release of energy in the form of heat as part of the process of non-shivering thermogenesis (reviewed in [51]).

The known functions of subcutaneous fat are temperature insulation, mechanical cushioning, energy storage, and heat production (in the case of brown adipose tissue in newborns) (reviewed in [51]). In addition, subcutaneous adipose tissue is a source of hormone and signalling molecules, and is involved in metabolic regulation, the pathogenesis of type II diabetes, and atherosclerosis (reviewed in [51]). Leptin is an example of a hormone synthesized within the subcutis that regulates energy turn over and appetite [52]. Adiponectin is another hormone produced in the subcutis that has insulin-sensitizing, anti-atherosclerotic, and anti-inflammatory properties (reviewed in [51]). All these components make subcutaneous fat a diverse and complex biologically active tissue.

**Lymphatic and blood vessels**

Networks of blood vessels in the dermis and subcutis form the skin vasculature, while the epidermis is free of blood vessels. Dermal vasculature comprises an interconnected network of paired arterioles and venules that form a superficial and deep vascular plexus, with additional vascular networks surrounding sweat glands and hair follicles [53] (reviewed in [54]).

The superficial horizontal vascular network located between the papillary and reticular dermis. A lower horizontal network is located on the border between the dermis and subcutis. The two horizontal networks are connected by vertical vessels. There are additional vertical capillaries raised from the arterioles in a superficial network which
extends to the papillary dermis between the epidermal rete ridges which then loops back down to the venules, forming arcade-like structures [55].

In addition to providing oxygen and nutrients, there are anastomosing arteries and veins within the dermis that regulate heat loss. This system includes arteriovenous anastomoses and extensive subcutaneous venous plexus, which can hold large quantities of blood (to dissipate heat from the surface of the skin). These anastomoses are prominent in areas often exposed to maximal cooling, such as the volar surfaces of the hands and feet, lips, nose and ears [55].

Cutaneous lymphatic drainage forms microscopic blind-ended dermal vessels called lymphatic capillaries, which collect both cells and interstitial substances that are then directed toward regional lymph nodes. Lymphocytes, macrophages and dendritic cells are able to exit the skin via lymphatic drainage [34].

1.2.2 Skin development

During early embryonic differentiation three germ layers of endoderm, mesoderm and ectoderm are formed. The ectoderm or neuroectoderm is a single cell layer on the surface of the embryo and has the potential to differentiate to either nervous system or skin epithelium depending on Wnt signaling [56]. Activation of Wnt signaling blocks the fibroblast growth factor (FGF) response and promotes epidermal differentiation, while lack of Wnt signaling, increases the response of the neuroectoderm to FGFs, and leads to neural differentiation and formation of the neural tube [56]. Neural crest cells separate from the dorsal portion of the neural tube immediately after its closure and migrate to different regions of the embryo [57].

Skin derives from the ectoderm, mesoderm and neural crest. The epidermis derives from the ectoderm (reviewed in [22]). The mesoderm is the origin of dermis in the dorsal trunk [58], while dermis in the face and anterior scalp is derived from neural crest ectoderm [59]. Melanocytes also originate from the neural crest.
The mesoderm provides signaling essential for morphogenesis of epidermal structures such as the hair follicle [12]. It is also the origin of endothelial cells and haematopoietic cells such as Langerhans cells (reviewed in [22]).

**Development of epidermis**

In human embryonic development, the epidermis initially consists of a single layer of undifferentiated cuboid epithelial cells. Stratification in the embryo starts at around 8 weeks of gestation. The first stratification produces a layer of cells known as the periderm, which covers the developing epithelium throughout embryogenesis and is shed once the epidermis is stratified [60]. In addition to protection from the uterine environment [61], the proposed functions of the periderm include regulation of underlying mesenchyme [62] and the prevention of pathological epithelial adhesions during embryogenesis [63].

During the development of the epidermis, an intermediate cell layer is formed between the basal layer and periderm. Unlike the adult’s supra-basal layer, the embryonic intermediate layer is proliferative, which makes the skin able to expand during the rapid growth of the embryo (reviewed in [22]). The proliferative intermediate layer is gradually replaced by differentiated post-mitotic keratinizing cells, which form spinous and granular cells in the second trimester. Keratinization initially starts in the hair canal around 15 weeks of estimated gestational age (EGA) and then in interfollicular epidermis around 22-24 weeks of EGA. By 24 -26 weeks of EGA, a cornified cell layer is formed, and by the mid-third trimester the epidermis is morphologically similar to adult epidermis (reviewed in [22]). Melanocytes and Langerhans cells migrate into the epidermis during early stages of embryonic development (reviewed in [22]).

**Development of specialized cells in epidermis**

**Melanocytes**

Melanoblasts are a precursor of melanocytes and migrate from the trunk neural crest to appear in the epidermis by the middle of the first trimester, around day 50 of EGA in humans [22]. Melanoblast density is high in the early stage of embryonic development and
increases to 3000 cells/mm² by the end of the first trimester, after which time it decreases as the embryo grows and becomes similar to an adult (800-1500/mm²) [22].

Melanin production begins in humans only around 3-4 months of EGA and melanin granules appear in the keratinocytes around the fifth month EGA. Newborn skin is not fully pigmented. It darkens over the first few weeks after birth [22].

Melanocytes also appear in dermis during embryonic development; however, by the time of birth most dermal melanocytes have either migrated to the epidermis or undergone apoptosis and disappeared from the dermis. Exceptions where dermal melanocytes persist are the head, neck, pre-sacral area and distal extremities (reviewed in [22] and [64]).

During the early postnatal period in mice, melanoblasts are found both in the hair follicle and in the interfollicular epidermis; however, the interfollicular population of melanoblasts disappears a few days after birth. In adult mice, there are no melanocytes in the interfollicular epidermis of the hairy skin [65]. In contrast, in humans the interfollicular melanocytes persist and produce melanin that pigments the skin [64].

**Langerhans cells**

Langerhans cells are dendritic antigen-presenting cells of the epidermis. Their main function is to initiate an immune response against microorganisms. They contribute to immune tolerance in the skin and proliferate in allergic diseases such as atopic dermatitis [66]. These immune cells capture and process antigens, and then combine the antigen with major histocompatibility complexes for presentation to naive T-cells in secondary lymphoid tissue [12]. Langerhans cells appear in the epidermis in the first trimester and are characterised by their dendritic morphology, expression of CD45 HLA DR CD1c and a high level of ATPase activity and expression of Langerin protein [22]. Their density gradually increases during the embryonic period, followed by extensive proliferation between days 2-7 after birth [67]. After this period, Langerhans cells have a low rate of proliferation. Although Langerhans cells are myeloid in origin, they maintain their number in adult skin without any replenishment from bone marrow [67].
Merkel cells

Merkel cells are well innervated mechanoreceptors found in the basal layer of the epidermis in hairy skin as well as tactile areas of glabrous skin [12]. Merkel cells appear in the epidermis between the 8th and 12th week of EGA. They appear first in glabrous skin and later in hairy skin. The origin of these neuroendocrine cells has been the subject of long-standing controversy [22]; however, recent studies suggest that Merkel cells are probably derived from the epidermis rather than neural crest [68].

Development of dermis

The origin of the dermis varies and depends on the anatomical location in question. The dermis of the face and anterior scalp is derived from neural crest ectoderm [59], while the dermis in the dorsal trunk [58] and ventral trunk [69] are derived from the somites, and lateral plate respectively. The somites and lateral plate both derived from the mesoderm (reviewed in [70]). Jinno et al. (2010) confirmed the diversity in the developmental origin of dermis by lineage tracing in several Cre-reporter mouse lines [71].

Dermis appears as a homogenous structure in the early stages of embryonic development (E12.5 in mouse); however, by E18.5 weeks, the papillary dermis is morphologically distinguishable from the thicker reticular dermis [72] when collagen fibres start to form. Collagen fibres gradually increase in density and more mature and complex fibres gradually assemble during the second and third trimester [43]. By 6-8 weeks of EGA in humans, mesenchymal cells under the epidermis are able to synthesize collagen I, II, and III; however, the ratio of collagen III to I is 3:1, which is the reverse of the ratio in adult dermis [43].

By the end of the second trimester, elastic fibres can be detected in electron microscope studies and at the same time the fetus skin shifts from non-scarring to scarring wound repair [43].

Recent comprehensive transplantation assays and lineage analysis done by Driskell et al. [50] revealed that the fibroblasts of skin connective tissue arise from two lineages with distinct anatomical and functional characteristics. Cells originating from the upper dermis
at an early stage of embryogenesis participate in hair follicle dermal papilla and dermal sheath formation, and the arrector pili muscles. The lower embryonic dermis, on the other hand, is involved in reticular dermis formation, synthesizing the bulk of the ECM, and adipocytes of the hypodermis [50].

Specific patterns of the vascular network of the dermis are detected as early as six weeks of EGA [73]. Endothelial cells form tube-like structures which themselves form initially one and then, after eight weeks, a second plexus parallel to the epidermis [73]. Prenatal vasculature undergoes remodeling during the embryonic period, and the structure of blood vessels is not mature until after birth. Dermal vessels in the embryo and fetus express less α-smooth muscle actin (α-SMA) protein (an actin protein isoform found in smooth muscle cells around the blood vessels) and have fewer perivascular leukocytes than adult skin [74]. At eight to ten weeks of EGA, the vessel size is significantly smaller compared with those in adults, but by eleven weeks, they reach adult size [75]. Prenatal skin has; however, a significantly higher vessel density [74] to provide nutrient requirement for growing skin [74]. It has also been suggested that already formed vessels move apart after birth in proportion as the body and skin grow [74].

Networks of nerve fibres in skin appear in the first trimester and follow the pattern of blood vessels. The development of subcutaneous fat appears in the second trimester and lobules separated by fibrous septae are formed during the third trimester [43].
1.3 Hair follicle anatomy, physiology and development

During the last decade, hair has been a focus of biological research. Repetitive cyclic regeneration of the follicles [76] together with their repair response to damage stimulation make the follicles a suitable model for tissue regeneration. In fact, hair follicle research has revealed the basic mechanism, signals and growth factors involved in regeneration and repair of other tissues (reviewed in [77]).

The presence of hair is characteristic for mammals, where it covers the entire surface of the body except the palms of the hands and soles of the feet. The hair follicle is one of the most functionally diverse organs [78]. Hair provides the first defence against environmental trauma, aids thermal homeostasis, camouflage, the dispersion of sweat and sebum, as well as biosynthesis of structural proteins and hormones (reviewed in [77]). The rich nerve supply of hair follicles performs sensory and tactile functions [77] and is considered a sensory receptor [79, 80]. Human hair has a significant impact on social and sexual communication, and disorders such as hair loss or excess hair can lead to significant emotional and psychological distress.

Formation of hair follicles largely takes place during perinatal skin development from two embryonic layers of ectoderm and mesoderm. After birth, hair follicles undergo cyclic transformations from a period of rapid growth to apoptosis and regression over their entire life-time [76, 81]. During the hair cycle, initiation of the active growth phase of the follicle is accompanied by an increase in vascularity [10] and innervation [11]. Also, in rodent experiments in which the hair cycle is synchronised, active hair growth is associated with better wound healing [78]. Furthermore, cells from hair follicles migrate and participate in regeneration of new epidermis (will be discussed in 1.5.4).

Hair follicles have been considered a reservoir of stem cells analogous to “the bone marrow” of skin [81]. Various populations of epithelial and mesenchymal stem cells have been identified in hair follicles, making them an ideal accessible source in regenerative medicine.
1.3.1 Hair follicle anatomy

Hair follicles can be conceptualised as an epidermal fold extending deep into the dermis and the hair fibre is the keratinized product of rapidly proliferating matrix cells in the hair bulb. Longer hair follicles extend into the subcutaneous layer. The follicle is in an oblique position to the skin surface and the arrector pili muscles run from the mid region of hair follicles to the papillary dermis. In the human scalp, the arrector pili muscles surround up to four hair follicles together. Above the muscles is the opening of the sebaceous gland. One follicular unit is considered as a single arrector muscle, which splits to encircle each follicle within the follicular unit [82].

The hair follicle is composed of dermal (mesenchymal) and epidermal (epithelial) compartments which are separated by a basement membrane. The dermal part of the hair follicle originates from the mesoderm and is the continuation of the dermis in skin. It is comprised of two parts, the dermal sheath and dermal papilla. The dermal sheath, or the connective tissue sheath, surrounds the epithelial portion of the hair follicle and is separated from it by a basement membrane.

Cells within DP and DS are fibroblasts of mesodermal origin. As discussed in 1.2.2 the dermis of the face and anterior scalp is derived from neural crest ectoderm [59]. Diversity in embryonic origin of skin dermis also applies to dermal components of hair follicle. The neural crest derived cells in hair follicles will be discussed in 1.5.9.

The epidermal part of the hair follicle originates from the ectoderm and is the continuation of skin epidermis. It gives rise to all epithelial components of the pilosebaceous unit such as the sebaceous and apocrine glands, as well as to several axial layers in the hair follicle. The axial layers in the hair follicle, from outermost to innermost are: the outer root sheath, inner root sheath and hair shaft.

The hair follicle is conventionally divided into different regions in longitudinal sections (Figure 1-3): Infundibulum, Isthmus, supra bulbar region and bulb [83]. The infundibulum is the most proximal part relative to the epidermis, extending from the sebaceous duct to the epidermal surface. The isthmus is the area between the opening of the sebaceous duct...
and the insertion of arrector pili muscles in the bulge. The suprabulbar region lies between the isthmus and the bulb. The deepest part of the hair follicle is the bulb, which, depending on the size of the hair follicle, can be located as deep as subdermal fat. The infundibulum and isthmus are the permanent parts of the hair follicle, while the lower portion degenerates and regenerates with each hair cycle.

![Hair follicle anatomy](image)

**Figure 1-3 Hair follicle anatomy**

The hair follicle also is divided into different layers, which appear as several concentric rings in cross section. The layers are (from innermost to outermost): hair shaft, inner root sheath, outer root sheath, and dermal sheath.

**The hair bulb**

The hair bulb is the deepest part of the hair follicle and in terminal follicles is located in the subcutaneous fat [84]. It consists of bulb matrix or germinative epithelium, which surrounds the dermal papilla at the base of the follicle. Cells in the matrix are highly proliferative, with a rate of cell turnover similar to that of bone marrow [84]. Matrix cells in the hair bulb proliferate and move upward as they differentiate and give rise to the hair shaft and the inner root sheath, which surrounds the hair fibre. The inner root sheath disintegrates before the hair emerges from the skin. The melanocytes in the matrix produce the pigment of hair shaft.
**The hair shaft**

The hair shaft is the product of rapidly proliferating matrix keratinocytes, located at the apex of the dermal papilla and proliferating in response to dermal papilla signals. It consists of three concentric layers of central lineages: an outer **cuticle**, which surrounds an intermediate **cortex** and central **medulla**.

The **cortex** is the main structural component of the hair shaft. The basic components of the hair cortex are alpha helical keratin molecules [85]. These molecules self-assemble in the presumptive cortical cells to form tetramers and intermediate filaments with their axes oriented parallel with the hair axis. These filaments are aggregated to form macrofibrils appearing as large cable-like structures [85, 86]. Keratin filaments are cross-linked to keratin-associated proteins, which form a matrix between the filaments [84]. Melanin granules are dispersed throughout the cortex and determine hair colour [84].

The **cuticle** surrounds the cortex and consists of flattened (tile-like) cells which anchor the hair shaft in the follicle and also protect the interior of hair fibres [87]. The properties of the cuticle contribute to the appearance of the hair fibre. The cuticle can be damaged or destroyed by chemical or mechanical injuries and is generally less intact at the distal end of long hair fibres [84, 87]. The **medulla** is the variable structure of hair follicle and exists at the centre of larger hairs, where it may be continuous, discontinuous or absent [84].

**The inner root sheath**

The inner root sheath (IRS) is the second innermost layer of the hair follicle and encircles the hair shaft. The IRS arises from hair matrix cells at the base of the bulb. The IRS continues from the bulb up to the level of the sebaceous glands, where it is separated from developing hair shaft and degraded by proteolytic enzymes within the hair follicle [88]. Consequently, the hair shaft emerges from the skin surface without an association with the IRS. Cells in the IRS are keratinised and undergo desmosomal remodelling during terminal differentiation as they migrate up the hair follicle [89].

The IRS consists of separate layers, from inner- to outermost: IRS cuticle, Huxley's layer, Henle's layer, and the companion layer. Henle’s layer consists of only a single layer of
cells [84]. Keratinisation of cells in the hair follicle occurs first in the outermost Henle’s layer, then in the innermost cuticle and lastly in Huxley’s layer, which is situated between these layers [84]. Keratinization of the inner root sheath precedes keratinization of the hair shaft. The IRS acts as a scaffold to shape the developing hair shaft [90].

Cells of the inner root sheath cuticle become flattened and overlap and interlock with the scales in the shaft cuticle which anchor the hair shaft within the follicle [84].

Huxley's layer is the most bulky layer of the inner root sheath. It is composed of two to four layers of cells. The cytoplasmic process cells called Flügelzellen in Huxley’s layer pass through the fully keratinised Henle's layer and link to the companion layer via numerous desmosomes [91].

The companion layer of IRS consists of a flattened layer of cells between Henle’s layer and the outer root sheath. It was traditionally considered part of the outer root sheath; however, the ultra-structure of desmosomes between the companion layer and inner root sheath shows that the companion layer is more connected to the inner root sheath [91]. This tight layering of inner root sheath and companion layer provides an optimal moulding for the geometric shape of the growing hair shaft [91].

**The outer root sheath (ORS)**

The outer root sheath is the continuation of the epidermis that extends from the infundibulum to the bulb of the hair follicle, providing a slippage plane, nutrition, regulatory molecules, and stem cells [22]. In contrast to the hair shaft and inner root sheath, the outer root sheath does not originate from the upward growth of matrix cells, but rather directly from the bulge (Figure 1-3) [92], which is the epithelial stem cell niche in the permanent portion of the follicle. This will be discussed in detail in 1.5.3.

The cells of the outer root sheath change considerably throughout the follicle [83]. ORS forms a single flattened layer of cells around the bulb, while in the supra bulbar area ORS cells are larger and contain glycogen. At the infundibulum, the ORS is keratinised and similar to the epithelium and contains a granular layer. ORS remains non-keratinized all
over the entire lower portion [93] and only begins to keratinize in the upper isthmus, immediately above the attachment of arrector pili muscles [92-94]. The pattern of sudden keratinization of outer root sheath without formation of a granular layer in this area, is called “trichilemmal keratinization” [92, 94].

**The bulge**

Outer root sheath cells form a “bulge” at the level of the isthmus, where arrector pili muscles loop around the hair follicle. As described by Cotsarelis *et al.* in 1990 [95], outer root sheath cells at the bulge area in contrast to other parts of the hair follicle have undifferentiated or stem cell characteristics, such as a higher nuclear/cytoplasmic ratio, the relative absence of keratin filament bundles, and the cytoplasm filled with ribosomes, [95]. This area contains slow-cycling epithelial stem cells [96] and melanocyte stem cells [97]. The bulge is believed to be the reservoir of epithelial stem cells for the entire outer root sheath [95, 98], which will be discussed in detail in 1.5.3.

**The dermal sheath (DS)**

The dermal sheath is the collagenous layer that surrounds the hair follicle in the dermis. Dermal sheath is separated from the outer root sheath by a basement membrane. The dermal sheath and dermal papilla are the mesenchymal or dermal component of the hair follicle. Cells in the dermal sheath are specialized fibroblasts and able to reconstitute dermal papilla and, like dermal papilla cells (see the sub-section below: The dermal papilla) have the capacity to induce hair follicle formation in adult human skin [99, 100]. Like dermal papilla cells, dermal sheath cells are believed to have mesenchymal stem cell characteristics, and are able to repopulate the haematopoietic system in lethally irradiated recipient mice [101].

The exact role of the DS in stem cell physiology and hair regeneration is not well understood [102]. The DS has been considered as a reservoir of DP cells during the resting phase of the hair follicle cycle [103]. The similarity between dermal papilla and dermal sheath cells suggests that cell trafficking occurs between these two mesenchymal components during the hair cycle that is similar to epithelial cell trafficking between the
bulge and outer root sheath [100, 102-105]. The details and evidence of cell trafficking in the dermal component of the hair follicle will be discussed in ‘Cutaneous stem cells’ section in 1.5.7.

**The dermal papilla (DP)**

The dermal papilla is located at the base of the hair follicle, and is separated from the dermal sheath by a narrow stalk. The extracellular matrix in dermal papillae is rich in basement membrane proteins and proteoglycan [84]. The dermal papilla often contains a nerve bundle and a capillary loop in larger hair follicles [84]. The fibroblasts in the dermal papilla originate from mesoderm, and have mesenchymal stem cell characteristics that will be discussed in 1.5 and 1.5.5.

Hair follicle dermal papilla cells also express neuronal markers suggesting a neural crest origin [106-108] As was discussed in 1.2.1 all of the mesenchymal derivatives of the facial skin, including dermis, are derived from the neural crest [59, 109]. Hair follicle dermal papilla and dermal sheath are derived from skin dermis and comprehensive lineage analysis has confirmed that facial dermal papilla and dermal sheath are of neural crest origin while truncal dermal papilla and dermal sheath are of mesenchymal origin [50]. This will be discussed in detail in this chapter in “Hair follicle development” (1.3.2) and “Neural crest stem cells” (1.5.9).

The volume of the dermal papilla increases in the anagen phase of the hair cycle. During this phase there is an increase in the number of cells as well as the extracellular matrix volume; however, there is no change in cell proliferation during anagen [103]. It is believed that the increased cell count may be a result of replenishment from the dermal sheath [103, 110]. The volume of the dermal papilla also varies between different types of hair [111]. The dermal papilla is known to be the source of signals responsible for keratinocyte proliferation during the hair cycle, and hair induction. [112-117]. DP cells control hair bulb size, hair shaft diameter and anagen duration [76, 108, 118, 119] and are able to induce and determine the type of hair in hair induction experiments [114, 120]. The
induction capacity of dermal papilla cells reduces in vitro after several passages. This indicates complex in-vivo interactions during hair follicular neogenesis [121].

Several molecules and signalling pathways are involved in communication between dermal papilla cells and hair follicle epithelial cells. These molecules regulate morphogenesis and induction ability of dermal papilla, as well as the hair cycle. Both the hair bulb and DP are rich in bone morphogenetic proteins (BMP) and BMP receptors [122]. Activation of Wnt signalling is important for the initiation and maintenance of hair morphogenesis [123], hair induction [124] and trichogenesis [125].

Another factor found in dermal papilla fibroblasts is Keratinocyte Growth Factor-2 (KGF-2). KGF-2 stimulates human hair-follicle keratinocytes’ proliferation in organ culture [126]. Other factors possibly involved in the communication between dermal papilla and epithelial cells are Corin, TBX18, SOX2, CD133 and fibroblast growth factors 10 (FGF10). These molecules are expressed in dermal papilla cells, during different stages of epidermal development or hair cycle [107, 127-129].

**The sebaceous glands**

The sebaceous glands are a part of the pilosebaceous unit, and secrete sebum, which consists of squalene (a biochemical precursor to the whole family of steroids), cholesterol, wax esters and triglycerides [130]. The sebaceous glands connect to the hair follicle canal at the level of the infundibulum and isthmus. The outermost basal layer cells of the sebaceous glands proliferate and differentiate. As cells differentiate and move towards the center of the gland, they begin to produce lipids and finally extrude lipids by disintegration of entire cells. This process is known as holocrine secretion. The function of the sebum is to maintain stratum corneum hydration [131] and protect skin from bacterial and fungal infection [132]. Oxidation of secreted lipids such as squalene may have a role in pathogenesis of acne [133] and the inflammatory responses of keratinocytes to UVA+UVB, which could be relevant for skin inflammation in the sun-exposed oily skin [134].
The arrector pili muscle (APM)

The arrector pili muscle (APM) is composed of bundles of smooth muscle cells, which are under the control of the sympathetic nervous system. The APM attaches to hair follicles at the bulge region and extends into the upper dermis or under the surface of the epidermis. The contraction of APM results in piloerection (goose bumps), which increases air trapping and prevents temperature loss in most mammals; however, the APM does not have this thermoregulatory function in humans [135].

The APM may play a further functional role in hair and skin regeneration. The insertion of APM in hair follicle is a repository of follicular epithelial stem cells [95, 136] (will be discussed in 1.5). The keratinocytes in the distal arrector pili muscle attachment site also express epithelial stem cell markers, as recently showed by Torkamani et al. [137] using histological examination in mice (will be discussed in 1.5.2). These studies suggest a close relationship between the APM and the epithelial stem cell population in the bulge and in the interfollicular epidermis.

Furthermore, irreversible miniaturization in advanced stages of androgenic alopecia is associated with loss of contact between APM and hair follicles, while in alopecia areata, the arrector pili muscles maintain their attachment with the reversible miniaturised follicles [138]. These findings imply an important role for APM in the follicular unit and the epithelial stem cells in skin, which will be discussed in 1.3.3, 1.5.2. and 1.5.5.

1.3.2 Hair fibre types

Hair types in human

Human hair follicles can produce three different types of fibres; lanugo, vellus and terminal hairs [139]. Lanugo hair is the first fine silky hair that forms in the uterus around eight to nine weeks of gestation [140]. Lanugo hair appears first in the region of the eyebrows, upper lip and chin [141] (Lavker et al. 2003), then in other regions around the fourth month. These hairs are fine, soft and non-pigmented, and characterised by absence of central medulla. Lanugo hairs are shed before birth and replaced by either vellus hairs on the face, trunk and limbs or progress to terminal hairs on the scalp.
Vellus hairs are less than two centimetres long, less than 30μm in diameter, non-medullated and occasionally pigmented. Terminal hairs are typically 60 μm in diameter and can grow to over one metre in length. Terminal hairs are pigmented and have a central medulla, and occur on the scalp, eyebrows and eyelashes [142]. Development of different types of hairs within the follicular unit will be discussed in 1.3.3.

**Hair types in mice**

At least eight different major hair types can be distinguished in mice [143]. The hair coat or pelage alone contains four separate hair subtypes with distinct morphologies: guard, awl, auchene and zigzag [144]. ‘Zigzag’ hair follicles have two kinks in the shaft and account for 76% of follicles in the coat. ‘Awl’ and ‘auchene’ hair follicles have longer shafts that are either straight or have a single kink and make up roughly 23% of the coat follicles. Finally, ‘guard’ hairs are the longest, but they are the least abundant hair type in mice. These follicles represent 1% of coat hair follicles (reviewed in [144]).

Guard hairs develop around E14.5, during the first wave of hair follicle morphogenesis. Awl and auchene hairs form during the second wave around E16.5, and zigzag hairs during the third wave starting at 18.5 [144, 145]. The sequence of development of various follicles will be discussed in 1.3.3.

Other types of hair in mice include vibrissae or whiskers, cilia or eyelashes, tail hairs, ear hairs, and hairs around the feet, the genitals and perianal area. These types of hairs can be unequivocally distinguished by length, number of medulla columns, and the presence of hair shaft bends [144].

**Mouse vibrissae**

Vibrissae (or whiskers) have attracted attention in hair biology studies due to their size, and specific position pattern [146], accessibility and ease of micro-dissection. Anatomically they are identified by their large size of hair fibre and large follicle, which has a capsule of blood sinus that is well innervated by sensory nerves [147, 148]. Because of the size and ease of micro-dissection, mouse vibrissae were used in animal experiments...
in this project. However, they are specialized hair follicles and an important proprioceptive sensory organ in mice, and their analogous organ in humans has not been identified.

Vibrissae are considered proprioceptive sensory receptors. In mice, vibrissae are arranged in five horizontal rows. Each vibrissae projects to a cylindrical cluster of cells in the primary somatosensory cortex \([149]\) and the brainstem \([150]\). Labial follicles in the mystical region are smaller than vibrissae follicles but bigger than pelage follicles. Labial follicles differ from vibrissae follicles, in not having anatomical characteristics such as rich innervation and muscle attachment.

The muscles in the mystical region are divided into two categories of extrinsic and intrinsic muscles. The extrinsic muscles belong to the facial muscles and originate outside the mystical region and insert into the corium between the mystical vibrissae. The intrinsic follicular muscles are connected only to vibrissae follicles and have no bony attachments \([151]\). The motor innervation of vibrissae is from the facial nerve to the intrinsic and extrinsic muscles, and sensory innervation supplied by branches of all three divisions of the trigeminal nerve \([151]\).

The hair cycle in mouse vibrissae is not synchronised like that of pelage hair follicles. The new hair cycle begins before the hair shaft of the previous cycle is shed, which prevents loss of vibrissae – an important proprioceptive sensory organ – during the hair cycle \([152]\).

1.3.3 Development of follicular unit

The follicular unit (FU) or pilosebaceous unit was first described by Headington in 1984. In transverse (horizontal) histology sections of adult human scalp, Headington showed that individual follicles are not distributed randomly at the level of reticular dermis, but arranged in groups of follicles bounded together by coarse collagen fibres \([153]\). In the human scalp, each follicular unit is composed of two to four terminal follicles and one or occasionally two vellus follicles associated with a sebaceous gland and insertion of the arrector pili (AP) muscle \([153]\).
The concept of development of primary and secondary follicles within the follicular unit is well described in sheep and mouse (reviewed in [154] and [155]). In sheep, the first hair follicles to be formed are the primary hair follicles, which start to develop by day 70 in the foetus. The ‘primary’ hair follicles are distinguished by their trio pattern, their attachment to AP muscle and by inclusion of sweat glands in the trio. The ‘secondary’ follicles form in the foetus by day 85, and are closely associated with the primary follicles. In some breeds, the ‘secondary derived follicles’ which are branches of the secondary follicles, appear by day 105 and eventually form the bulk of the fleece (reviewed in [154]).

In mouse, pelage follicles (1.3.2) develop in three distinct waves during the late embryonic period. The first wave forms guard hairs, while the second and third waves preferentially form awl or auchene hairs and zigzag hairs, respectively (reviewed in [155]). Signalling molecules expressed in dermal papilla at different stages of development have been shown to be involved in the formation of different types of follicles (reviewed in [155] and [156]).

The concept of primary and secondary follicles within the follicular unit has not been clearly shown in human. In adult human scalp, a follicular unit typically consists of a larger, central terminal follicle surrounded by smaller terminal follicles and the arrector pili muscle is predominantly attached to the large follicle with variable attachment to smaller follicles [138, 157]. Montagna and Carlisle also observed groups of large and small follicles within each follicular unit in 6 month old human foetus scalp [158], however the sequence of development of primary and secondary follicles has not been shown in human foetus (reviewed in [157]).

After birth, hair follicles can switch from the production of one type of hair fibre to another type. For example, the majority of auchene hairs in mice switch to awl, while up to 20% of zigzags switch to either awl or auchene between the first and second growth cycles after birth (reviewed in [159]). Several genetic regulators such as Eda/Edar, BMP, Igfbp5, FGF and Sox2/Sox18 have been found to differentially affect the development and differentiation of follicles formed at different times (reviewed in [155]).
In humans no additional follicular units are formed after birth; however the size of the hair shaft and follicle can change. Lanugo hairs are shed before birth and replaced by either vellus hairs from the same follicles on the face, trunk and limbs or progress to terminal hairs on the scalp. During puberty, sex hormones influence hair production and this leads to sexual dimorphism. At puberty, vellus hairs change to terminal hairs in the beards of males and in the genitalia and axilla in both sexes. Paradoxically in male pattern hair loss (MPHL) and female pattern hair loss (FPHL) in humans, the same sex hormones convert terminal hairs of the scalp into miniaturized vellus hair follicles.

Yazdabadi et al. showed that hair loss in FPHL is due to reduction in the number of terminal hairs per follicular unit rather than miniaturization of entire follicular units [157]. They hypothesized a hierarchy of androgen sensitivity within follicular units that resulted in secondary follicles having an increased susceptibility to androgen-sensitive miniaturization into vellus hairs [157, 160].

### 1.3.4 Hair follicle development

Hair follicle formation largely takes place during perinatal skin development. Skin has the highest density of hair follicles in the foetus and this density decreases as the skin surface expands [161, 162]. In humans, no further hair follicle production normally occurs after birth [163], except after skin wounding where *de novo* hair follicle formation has been observed in adult mouse [164, 165], rabbit [165, 166] and human skin [167].

The hair follicle comprises cell populations that are of ectodermal, mesodermal and neural crest origin (reviewed in [168]). The epithelial components of the hair follicle including the sebaceous gland and apocrine gland, originate from ectoderm. As discussed in 1.2.2, melanocytes originate from the neural crest [168, 169] and the dermal papilla and dermal sheath are derived from mesoderm and neuroectoderm [59, 109].

Molecular communication between the epidermal and underlying mesenchymal tissue plays an important role in hair follicle development [115, 170]. Similar signalling pathways also determine hair follicle cycling [81, 171, 172]. These molecular signals found through characterisation of human counterparts (homologues), were originally
discovered in the normal development of Drosophila (fruit fly). The signalling molecules include secreted molecules of the Wnt/wingless family, the hedgehog family, and members of the TGF-β/BMP (transforming growth factor-β/bone morphogenetic protein), FGF (fibroblast growth factor) and TNF (tumour necrosis factor) families (reviewed in [81, 123, 173]). Different combinations of these signals may determine whether a tooth or a hair follicle will develop [56, 81].

After gastrulation, neuroectoderm forms on the surface of the embryo, which either undergoes neural differentiation through FGF+ BMP inhibition signalling or forms the epidermis under the influence of Wnt +BMP signalling [174]. At this stage, there is a single layer of embryo epidermis only, and no microscopic sign of hair follicle formation. Soon after the formation of single layer epidermal cells at the surface of the embryo, mesenchymal cells from dermomyotomes in dorsal skin and migrate to skin under Wnt signalling [175] (reviewed in [77]).

Figure 1-4  **Embryonic pelage hair development in mice [77]**

<table>
<thead>
<tr>
<th>Induction</th>
<th>Organogenesis</th>
<th>Cytodifferentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Stage 2 (germ)</td>
<td>Stage 6-8 (bulbous peg)</td>
</tr>
<tr>
<td>Stage 1 (placode)</td>
<td>Stage 3-5 (peg)</td>
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</table>

As discussed in 1.3.3 and 1.3.4 the development of pelage hair follicles in mice takes place in two waves and leads to formation of primary (guard) hairs and secondary (awl, auchene and zigzag) hairs. The signalling molecules involved in hair follicle development vary between the different hair types [81] (reviewed in [77]).

Overall, the formation of hair follicles can be divided into three stages of induction, organogenesis and differentiation (reviewed in [81], Figure 1-4). During these stages, the developing hair is named successively placode, hair germ, peg and bulbous peg, according to its morphology.
Induction

The first ‘microscopic sign’ of hair follicle development appears around embryonic day (E) 14.5 in mice (around 10 weeks’ gestation in humans) and is characterised by formation of regularly spaced thickenings of the epidermis called placodes [176].

Early studies in epithelial mesenchymal tissue recombination in chicks and mice, revealed that early signals from mesenchyme determine the formation and spacing of the placodes [176], and FGFs [177, 178] and BMP inhibitory factors (BMP inhibitory protein Noggin) [179] are key component of these signals.

Organogenesis

Following the appearance of placode epidermal-mesenchymal signals induce the formation of the derma papilla [176] and proliferation of the placode. An epidermal signal induces a condensation of underlying mesenchymal fibroblasts to eventually form a dermal papilla. The dermal papilla feeds back signals to the placode to induce proliferation and growth downward into the underlying dermis to form the hair germ initially and then the hair peg subsequently. Proximally located keratinocytes enwrap the dermal papilla in the hair peg stage.

Sonic Hedgehog (Shh) is one of the main identified molecular signals expressed in the placode. Shh induces the formation of dermal papilla by condensing the mesenchymal cells [180, 181]. Wnt signals also influence the massive proliferation of epidermal cells down to the mesenchymal component [182, 183].

Cytodifferentiation

At this stage, when proximal keratinocytes proliferate even further down and enwrap the dermal papilla, the developing hair follicle is called a bulbus peg. Gradually epithelial differentiation within the hair follicle becomes morphologically noticeable and simultaneously sebaceous gland precursor cells appear in the upper part of hair follicle. Maturation of the hair follicle continues by separation and development of the outer, inner root sheath and hair shaft (reviewed in [56]). BMP signalling is involved and is essential
for both IRS and hair shaft differentiation and Wnt signalling has a crucial role in the
differentiation of matrix cells to hair shaft. Other transcriptional regulatory proteins
important in maturation of hair shaft are HOXC13 and FOXL1, both of which cause hair
defects when mutated (reviewed in [56]).

At about embryonic day 18.5 in mice, a central core of hair shaft cells emerges. At the
same time, sebaceous gland precursor cells appear in the upper part of the hair follicle [56].

Pigmentation of the hair follicle occurs by differentiation of melanoblasts that originate
from the neural crest and have migrated along the basement membrane to the dermis [64].
Follicle development is completed by migration of haematopoietic cells, and its
innervation and vascularisation.

1.3.5 Hair cycle

The hair follicle undergoes cyclic transformations, from the period of rapid growth
(anagen), regression (catagen), the resting phase (telogen), and shedding of the hair fibre
(exogen) [76, 81]. Hair follicle morphology changes tremendously with the lower portion
of the follicle undergoing cyclical destruction and regeneration. Only the upper
infundibulum and isthmus remain intact throughout the entire hair cycle.

In many animals, all hairs synchronously progress from one phase of the cycle to the next.
In mice, for example, hair follicles cycle in a wave along the anterior to posterior axis
[184, 185]. In humans however, the hair cycle is asynchronous except during pregnancy
and infancy. Pregnancy induces a synchronous anagen in the scalp hair follicles of both
mother and foetus. [186, 187]. The hair cycle becomes progressively asynchronous after
birth. Each follicle cycle is completely independent of its neighbouring follicle [184, 188,
189]. As each hair follicle is at a different stage of the cycle at any one time, scalp hairs do
not shed all at once.

The underlying mechanism of the hair cycle is complex and many different cytokines,
hormones, neurotransmitters, transcription factors and enzymes are involved [119]. Similar
signalling pathways are involved in both organogenesis of the hair follicle and hair cycling
The signalling molecules include secreted molecules of the Wnt/wingless family (reviewed in [190]), the hedgehog family, and members of the TGF-β/BMP (transforming growth factor-β/bone morphogenetic protein), FGF (fibroblast growth factor) and TNF (tumour necrosis factor) families (reviewed in [81, 123, 173]).

Subcutaneous adipose tissues are also source of cytokines and signals involved in hair cycle. During the early telogen, subcutaneous fat expresses BMP2, and makes hair follicles insensitive to anagen inducing signals [191]. Adipocytes also secrete platelet-derived growth factor-A (PDGFA) which promotes hair follicle cells to enter anagen [192].

An interesting group of genes found to regulate the hair follicle cycle are “circadian clock” genes. Comparing the mRNA (messenger RNA) expression at a genome-wide level in different stages of the hair cycle Lin et al. found more than six thousand genes correlated with a specific stage of the cycle [193, 194]. Interestingly genes which change their expression during the transition from telogen to anagen were also found to be involved in circadian clock regulation which may help to explain the seasonal cycling of hair follicles in animals, which enables them to change their coat according to the seasons [193].

Plikus et al. [195] showed that the hair follicles respond to the same signals differently at different times of the hair cycle and suggested this as a mechanism of cyclic regeneration of hair follicles. They showed that the bone morphogenetic proteins (BMPs) in the surrounding tissue at the beginning of telogen, keep the hair follicles in the refractory phase and maintain resistance to anagen signals, while in the second half of telogen, reduction in BMPs signals in the surrounding tissue makes the follicles responsive to anagen signals [195].

The anagen signals are produced as the result of follicle stem cells and dermal papilla interaction (intrinsic activators), or come from the surrounding tissue and neighbouring anagen HFs (extrinsic activators). Therefore hair follicles can enter anagen in two ways: autonomously, depending on the level of intrinsic activation, or non-autonomously, when activators are delivered by the surrounding tissue (reviewed in [191]).
Anagen

The duration of anagen determines the final length of the hair shaft. Anagen duration varies between species and different anatomical regions [196, 197].

Transcriptional activities in dermal papilla and secondary hair germ, which is a cluster of epithelial cells at the base of the telogen hair (Figure 1-5) are the earliest signals observed at the beginning of anagen [76, 198]. Subsequently mitotic activity in the secondary hair germ (SHG) begins and the SHG grows downward. During anagen the capillary network begins to develop around the growing follicle [10]. Fibroblasts in the dermal papilla show no mitotic activity at the beginning of anagen and dermal papilla growth during this period is solely due to an increase in the extracellular matrix and basement membrane protein [199] and recruitment of cells from dermal sheath into the DP [103, 110].

Figure 1-5  Schematic picture of different stages of hair cycle

The anagen phase is associated with an increase in vascularity [10, 200] and innervation [11] which will be discussed in 1.3.6. At the end of the anagen phase, proliferation of epithelial cells decreases, eventually ceases, and the hair enters the next stage of the cycle called catagen.
Catagen

Catagen is the regression phase of the hair cycle and is associated with apoptosis and the cessation of epithelial proliferation and differentiation in the bulb and outer root sheath together with apoptosis of ORS below the bulge. A spherical structure forms at the base of the hair shaft, called the club. The club anchors the resting hair fibre in the follicle (reviewed in [119]). The base of the follicle gradually moves upward to the level of the non-cycling upper part of the hair follicle while remaining connected to the dermal papilla by an epithelial strand (as shown in Figure 1-5). This epithelial strand, which is a temporary structure, disappears completely by the time the dermal papilla moves upward to the level of the hair club (reviewed in [119]).

In mouse vibrissae, the bulb shrinks in size during catagen; the follicle however, does not regress as much. The dermal papilla remains in the same place and does not move up to the bulge level [152]. In vibrissae follicles the new hair cycle starts before the exogen of the previous hair. This prevents loss of vibrissae, which is an important proprioceptive sensory organ, during the hair cycle [152].

Telogen

Telogen is the resting part of the hair cycle. During telogen, the hair follicle becomes quiescent without any significant proliferation, apoptosis or cell differentiation. The hair club remains anchored during telogen and the dermal papilla reaches to the level of bulge stem cells (reviewed in [77]), except in whisker follicles where the dermal papilla does not move up during the catagen and telogen [152].

As discussed earlier the bone morphogenetic proteins (BMPs) expressed in subcutaneous adipose tissue at the beginning of telogen make the follicles irresponsive to anagen signals (refractory telogen period), while a reduction of BMPs signals at late telogen (competent telogen phase), makes follicles responsive to anagen signals [195]. Other factors involved in the regulation of the refractory versus competent functional state of telogen are the fibroblast growth factors (FGFs) and the wingless (Wnt) stimulatory signals (reviewed in [201]).
**Exogen**

With the recommencement of anagen, epithelial cells in the bulb differentiate to produce the newly emerging inner root sheath and hair shaft that moves upwards, and pushes the resting telogen hair from the follicle [202]. This process of club fibre shedding has been described as a specific phase of the hair cycle known as exogen (128). There are two hypotheses about the process of hair shedding. A previous hypothesis described shedding as a passive process caused by a physical force from the new growing fibre [203, 204]. The new hypothesis considers hair shedding to be an active process in which specific molecular signals affect the adhesion molecules, leading to the release of the club fibre [204, 205]. The absence of club fibres in telogen follicle seen in humans, several breeds of domestic sheep, and in the back skin of mice confirms that exogen is an active process that occurs independently of the anagen phase of the hair cycle (reviewed in [204]).

The actual mechanisms involved in hair shedding are not fully understood [206]. Understanding factors that control exogen may provide therapeutic strategies for hair loss due to premature exogen and loss of telogen hair or club hair in human, which is called telogen effluvium (reviewed in [204] and [206]).

The club fibre is anchored within an outer root sheath (reviewed in [206]). Cell adhesion molecules have an important role in anchoring the telogen club hair in hair follicle and loss of their function leads to club hair shedding and exogen. Patients with pemphigus vulgaris (PV) or pemphigus foliaceus develop auto-antibodies against Dsg3 or Dsg1, respectively (reviewed in [206]). Patients with PV develop hair loss of club fibre. Koch et al. also demonstrated that desmoglein 3 knockout mice prematurely lose their club fibre [207].

Other factors that may be involved in the process of exogen are proteases and/or protease inhibitor activity. Examples are transglutaminases, plasminogen activator inhibitor type 2 and tissue inhibitor of metalloprotease 3 (reviewed in [206]).

In many species, exogen does not happen with every cycle, which means that the follicle re-enters the anagen phase before shedding the club hair. In mice, for example, the old hair shaft (club hair) may remain in the follicle for several cycles and contribute to the density
of the coat, together with the new anagen hair follicle merging from the same hair orifice [77].

**Kenogen**

In humans exogen generally occurs during the transition from telogen to anagen [208]; however, phototrichogram studies have revealed another cycle stage called kenogen or teloptosis, in which the follicle is empty and exogen has occurred before anagen commences [204, 209]. This finding confirms that exogen is an active process, and that hair shedding is not always a result of an exclusionary force [204].

Although kenogen follicles can be observed in healthy human skin, the frequency and duration may increase in patients with hair loss conditions such as male and female pattern hair loss (reviewed in [210]).

1.3.6 **Changes in skin vascularity and innervation during the hair cycle**

The hair cycle is associated not only with massive anatomical and physiological changes in the hair follicle itself, but also with constant changes within the skin which justify the use of the term “skin cycle” [211]. In rodents, with synchronous hair cycle the early stage of anagen is associated with epidermal hypertrophy [212]. As anagen progresses, the thickness of the epidermis decreases and instead there occurs an increase in dermal volume due to the increase of fat volume in adipocytes [212] and interstitial fluid [213].

As mentioned section 1.3.5 (Hair cycle Anagen), the anagen phase is associated with an increase in vascularity [10, 200] and innervation [11]. Mecklenburg et al. in 2000 found that active hair growth (anagen) is associated with angiogenesis in mice [10]. In this study mean vessel diameter and mean vessel length were significantly greater during anagen than telogen. Also, inhibition of angiogenesis (by fumagillin derivative TPN-470) retards anagen development in black 6 mice [10].

The hair follicle cycle also influences skin innervation [11]. The hair follicle is a sensory organ that participates in the sense of touch perception of hairy skin (discussed in the next section, 1.4). It constitutes a major component in the skin nerve network; in fact, the hair
follicle bulge is the most innervated area in skin [11]. This innervation changes during the hair cycle. By inducing anagen by hair pluck in mice, Botchkarev et al. in 1997 found a dramatic increase in single nerve fibres and nerve anastomosis within the dermis and subcutis during early anagen [11].

The connection between the hair cycle and the innervation, vascularity and healing of skin has not been reported in humans, as hair cycling is asynchronous [184, 188, 189] (discussed in 1.3.5) and the influence of the hair cycle in skin is less prominent [76]. If these findings were confirmed in human studies, there would be a potential clinical application, and manipulating hair follicle cycling might be found to accelerate skin innervation, vascularity and wound healing [214]. Plucking the hair, for example, induces anagen, which may accelerate wound healing in chronic wounds and surgical wounds [214].

The hair cycle also influences the healing properties of skin. The relationship between the hair cycle and wounds will be discussed in more detail in 1.8.
1.4 **Hair follicle as a sensory organ**

1.4.1 **Hair follicle innervation**

The hair follicle represents one of the most densely innervated organs in mammals [215-217]. This makes the hair follicle a sensory organ in skin, which detects very delicate touch sensations brought about by movements of the hair shaft [218], thus allowing the extension of the sense of touch beyond the skin surface itself [219]. As mentioned in 1.3.6 and 1.3.5, the hair follicle undergoes dramatic changes in innervation during the hair cycle (Figure 1-6, A). Increase in innervation during anagen also appears within the skin, in the dermis and subcutis, as is shown in rodent experiments [11, 220].

![Figure 1-6](image)

**Figure 1-6**  Hair follicle innervation. A) Changes in nerve fibre density around hair follicle during hair cycle [11] B) Various nerve fibres characterised around the hair follicle [221].

Similar to the dermal nerve network, nerves that supply the hair follicles consist of sensory afferent and autonomic sympathetic nerves [222]. Autonomic sympathetic nerves innervate the vasculature and the arrector pili muscles that are responsible for the goose bump reflex.

Complex sensory nerves and mechanoreceptors are developed in glabrous (hairless) and hairy skin, which are responsible for qualitative and quantitative detection of various stimulations. The perception of innocuous (painless) and noxious (hurtful) touch depends on special mechanoreceptors. The low-threshold mechanoreceptors (LTMRs) react to
innocuous mechanical stimulation, and high-threshold mechanoreceptors (HTMRs) respond to harmful mechanical stimuli.

Various cutaneous sensations also depend on nerve fibre characteristics. Nerve fibres are classified according to their thickness, myelination and conductive velocity. The thick and myelinated nerve fibres have higher nerve conductive velocity that provides rapid information about the location and physical characteristics of skin contact. The narrow unmyelinated nerves (C fibres) have lower conductive velocity that provides information in the case of noxious touch such as pain. C fibres are also activated by innocuous mechanical stimulation in hair follicle, such as those caused by changes of temperature [221].

Free nerve endings with HTMR found in the epidermis of both glabrous and hairy skin are responsible for pain and noxious touch detection, while complex and specialized combinations of LTMR receptors and nerve fibres have been identified in glabrous and hairy skin as detecting various mechanical stimulations, differentiating their position and velocity, and finally reacting differently to each stimulus [221].

In humans, glabrous rather than hairy skin has been the focus of research into cutaneous receptors and innervation, as the sense of touch in hairy skin is far less acute than in the glabrous skin of hand. For this reason most of what is known about hair follicle innervation has been inferred from animal studies [221].

Nerves around the hair follicle include longitudinal nerves running parallel to the follicle itself, and the smaller circular nerve branches around its epithelial stem cell in the bulge area [223, 224] (reviewed in [217]) (Figure 1-6, A). The nerve endings around the bulge are arranged as small palisades of so-called lanceolate endings parallel to the hair shaft. There are also circumferential endings (without lanceolate) around the bulge [225] (FIG B); however, the function and physiologic characteristics of these nerves have not yet been identified [221].

Sensory nerve fibres around hair follicle fall into three different categories: Aβ and Aδ nerves, which are myelinated sensory nerves, and un-myelinated C fibres [221, 225].
**Aβ nerve fibres**

The sense of movement is mediated by thick myelinated Aβ nerve fibres and rapidly adapting mechanoreceptors (RAMs). Aβ nerve fibres have specific sensitivity to movement but are unresponsive to static stimuli. Aβ nerve fibres are recognised as longitudinal lanceolate endings in rodents and primates (including humans) [226]. Interestingly the vibration sensitivity of RAMs is “tuned” to a narrow range of frequencies in hair follicle, being limited to frequencies between 10 and 50 Hz (reviewed in [225]). This means that these nerve fibres in hair follicle are excited by low frequency vibrotactile stimulations that evoke the sense of flutter vibration. This is similar to Meissner’s mechanoreceptors in human glabrous skin, which has a similar ‘tuning’ to low frequency vibration [225, 227]; however, the Pacinian corpuscle mechanoreceptors in human glabrous skin are most sensitive to vibrations above 100 Hz [225, 228, 229].

There are Aβ nerve fibres also associated with Merkel cells, which were first histologically described by Merkel in 1875 [230] as a cluster of epidermal cells in contact with nerve fibres. The Merkel cell-neurite complex is a known slow-adapting mechanoreceptor, which is responsible for transmission of a highly acute spatial image of tactile stimuli [231-233]. In humans, Merkel cells are found in the highest densities in the glabrous skin of the fingers and lips, allowing humans to discern the locations of stimulations separated by distances as small as 0.5 mm. Merkel complexes also present with a lower density in human hairy skin at the level of the basal layer of the epidermis. [221]. In rodents Merkel cell complexes are found in the highest density in vibrissae follicles, but are also associated with guard hair in hairy skin and the glabrous skin of the paws [221].

**Aδ nerve fibre**

Another group of myelinated nerves are the Aδ nerve fibres, which are narrower than Aβ nerve fibres, and have lower conductive velocity (about half of the conduction velocity of Aβ nerve fibres). Aδ nerve fibre receptors around the hair follicle (named D hair receptors after the down), or ‘zigzag’ hair in cats- are the most sensitive mechanoreceptors of hairy skin, with a threshold 10 times lower than the minimum forces
needed to activate classical Aβ mechanoreceptors [234]. Aδ-D receptors are also responsible for the ability to sense rapid cooling (but not warming) of skin [235, 236].

D hair receptors have been found in all mammals and non-human primates [225]. Although Aδ nerve fibres are found in human hairy skin, they are not always correlated with hair follicle, and there is no direct evidence of the presence of D hair receptors in human hair follicles [221, 235].

**Un-myelinated C fibres**

Un-myelinated C fibres are thin un-myelinated nerve fibres around the hair follicle. The conduction delay of C fibres suggests that it is unlikely that C fibres are involved in discriminative touch [225], but they may have a role in the pleasant sensation often associated with gentle touch [237, 238].

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Conduction velocity</th>
<th>Ending type</th>
<th>Location</th>
<th>Optimal stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAI-LMTR</td>
<td>Aβ(16-96 m/s)</td>
<td>Merkel cell</td>
<td>Around Guard hair and vibrissa follicle</td>
<td>Indentation pressure</td>
</tr>
<tr>
<td>SAII-LMTR</td>
<td>Aβ(20-100m/s)</td>
<td>Unclear</td>
<td>Unclear</td>
<td>Stretch</td>
</tr>
<tr>
<td>RAI-LMTR</td>
<td>Aβ(26-91m/s)</td>
<td>Longitudinal lanceolate ending</td>
<td>Guard/Awl - Auchene hair follicle</td>
<td>Hair follicle deflection</td>
</tr>
<tr>
<td>Aδ-LMTR</td>
<td>Aδ(15-30m/s)</td>
<td>Longitudinal lanceolate ending</td>
<td>Awl -Auchene Zigzag hair follicle</td>
<td>Hair follicle deflection</td>
</tr>
<tr>
<td>C-LMTR</td>
<td>C(0.2-2m/s)</td>
<td>Longitudinal lanceolate ending</td>
<td>Awl -Auchene Zigzag hair follicle</td>
<td>Hair follicle deflection</td>
</tr>
</tbody>
</table>

LTMRs: low-threshold mechanoreceptors, SAI and SAII: Slowly adapting receptor type I and II, RAI: Rapidly adapting receptor type I.

There is a substantial difference in innervation of the various hair-types. In humans vellus hairs have fewer nerve fibres than do terminal hairs [239]. In rodents, the innervation of sensory vibrissae hair follicles is more complex and denser than that of guard, pelage and vellus hair follicles. There are also different types of nerve fibres in the hair follicle [221], which are summarised in Table 1-2.
1.4.2 **Regulatory effect of follicular nerve on hair biology**

There is a close relationship between the nervous system and hair follicle biology. Hair follicles express receptors for all major stress response-mediators such as corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), cortisol, prolactin, neuropeptide Y, catecholamine, nerve growth factor (NGF), and substance P [240]. Merkel cells found around the bulge area, (as shown in Figure 1-6) produce cytokines and other regulatory factors, and are involved in neural control of the hair cycle [241]. It has been shown that the release of the neuropeptides substance P (SP) and Calcitonin gene-related peptide (CGRP) during stress influences keratinocytes, alters the hair cycle and can induce premature hair follicle regression [11, 242, 243].

The hair cycle is not, however, entirely dependent on innervations. As shown by Maurer et al. [244], skin denervation has no influence on the spontaneous hair cycle in mice, and only marginally delays the anagen development in plucked hair.

The regulatory effect of peripheral nerves on hair follicle development in neonatal mice was investigated by Peters et al. [243], who showed that nerve fibres appear in skin before the development of the hair follicle, but these nerve fibres were found in the locations where the follicle will subsequently develop [243].

Using Genetic Inducible Fate Mapping (GIFM) to mark and follow Gli1-expressing cells in hair follicle bulge, Brownell et al. [224] studied the detail of neural signals’ influence on hair follicle stem cells. They discovered that the neurons in the dorsal root ganglion (sensory neurons) were the source of Sonic Hedgehog (Shh) signalling to Gli1-positive cells in the upper bulge area. Gli1-positive cells are involved in the hair cycle as well as epithelialization during wound healing [224].

1.4.3 **Neurotrophic effect of hair follicle on peripheral nerves**

Hair follicles may also have an important trophic influence on nerve fibres. In an animal experiment, when skin from hairy occipital scalp was transplanted into the frontal scalp of stump-tailed macaques (*Macaca arctoides*), hair follicles in the skin graft were active
targets for nerves [245]. In another experimental study in which hairy foetal skin was transplanted into the anterior eye chamber of adult rats, autonomic and sensory nerve fibres grew through the anterior chamber of the eye and innervated the transplanted hair follicle [246]. In this experiment tyrosine hydroxylase (TH)-, substance P (SP)-, and CGRP-immunoreactive fibres were observed in association with the blood vessels in the graft two weeks after transplantation, and by four weeks TH-immunoreactive fibres were distributed in the arrector pili muscles, whereas SP- and CGRP-immunoreactive fibres were present around the hair follicles [246].

The follicular keratinocytes have been found to produce neurotrophins and influence nerves around the follicle [11, 247]. Furthermore, both in vitro and in vivo experiments adding dissociated hair follicle keratinocytes to tissue-engineered skin enhance nerve migration within the constructed tissue [248].

The neurotrophic and neuroprotective effect of hair follicle cells has also been shown by transplantation of cultured hair follicle nestin-positive cells, which improve recovery and nerve regeneration following peripheral nerve [249] and spinal cord injury [250] in mice. Even direct transplantation of the upper part of mice vibrissae promotes recovery in mice with peripheral nerve injury [251]. These studies suggest that hair follicle has potential clinical applications in nerve repair and regeneration.
1.5 Cutaneous stem cells

Various populations of epithelial and mesenchymal stem cells have been identified in skin based on their slow cycling, expression of stem cell markers or multipotency. The hair follicle and interfollicular epidermis are two distinct anatomical locations for epithelial stem cells (reviewed in [117]).

1.5.1 Slow cycling ‘label retaining’ stem cells

Initial identification of epithelial stem cells was based on early 'label retaining' studies. In 1988, Potten et al. identified slow-cycling cells in the interfollicular epidermis [252].

In label retaining studies, labelled thymidines such as tritiated thymidine, and thymidine analogs such as bromo-deoxyuridine (BrdU), are incorporated into the genome during DNA synthesis and cells, which retain their label for longer than the average. Label–retaining cells, (LRC) are assumed to have divided less frequently. Label-retention and slow cycling have thus been considered ‘stemness’ characteristics [253], present in certain populations of stem cells. This view has been supported by cell kinetic analysis and modelling studies [95, 252], and also expression of stem cell markers. It has been shown that the more quiescent label-retaining cell populations located in the basal layer in rodent epidermis express higher levels of stem cell markers such as α6 and β1-integrin, while committed progenitor cells with higher proliferation rates express lower levels of stem cell markers [254]. Similar heterogeneity has also been identified in human epidermis [255].

In 1990 Cotsarelis et al. discovered LRCs in the hair follicle bulge [95]. Epithelial stem cells have been further characterised by labelling specific stem cell populations in transgenic mice (Tg), and by lineage analysis during embryonic development, normal homeostasis or healing response (reviewed in [117]). Lineage analysis of stem cells revealed that interfollicular epidermis stem cells are responsible for maintenance of skin as well as re-epithelialization of wound, while hair follicle stem cells are mainly involved in homeostasis of the hair follicle itself, and only migrate to the interfollicular epidermis during wound response (reviewed in [256]).
Although the epidermis has been the focus of stem cell research in skin, stem cell characteristics such as multi-potency and expression of stem cell markers have also been identified in the dermal compartment of skin and hair follicles (reviewed in [117]).

1.5.2 **Interfollicular stem cells**

Histological studies of mice more than 40 years ago showed that the epidermis is organised in columns of cells with the 10 basal cells arranged in a hexagon configuration. This structure was hypothesised to be the structure of the epidermal proliferative unit (EPU), with one central stem cell at the base of the unit [257, 258].

The existence of EPUs was tested in lineage-tracing analyses by transfecting cultured keratinocytes with Lac-Z virus followed by transplantation into nude mice, and by direct infection with Lac-Z virus [259-261]. In these experiments labelled individual cells formed discrete columns of blue cells which could be maintained for extended periods of time, suggesting that EPU exist in the basal layer of skin epithelium (reviewed in [15]). Further experiments support the proliferative heterogeneity in human epithelial cell culture, which showed some cells generated rapidly expanding colonies with undifferentiated morphology, while others generate small clones [262].

The epithelial stem cells at the base of EPU were identified based on early label retaining studies (discussed in 1.5.1). Existence of label retaining cells in the basal layer of epidermis [252] supports the EPU hypothesis and defined it as slow cycling stem cell in each EPU which generates the pool of transient amplifying cells with restricted proliferation potential (reviewed in [263]).

The long-term lineage tracing experiments in mice done by Clayton *et al.* in 2007 [264] show more complex proliferative heterogeneity in the epidermis. Lineage tracing experiments, of up to one year, revealed that the shape and size of EPUs are different and some clones expand laterally [264]. These authors conducted the first quantitative clonal analysis of the interfollicular epidermis and showed that progenitor cells at the basal layer can become two mitotic cells, two post-mitotic cells or undergo asymmetrical cell division.
and produce one cell of each type, with a 10:10:80 fixed probability in healthy tissue [264] (reviewed in [117]).

According to this study, epidermal homeostasis is maintained by a process named ‘Neutral competition’, in which reduction in the size and number of clones is compensated for by expansion of neighbouring clones (reviewed in [117]). This proliferative heterogeneity and neutral competition among the stem cell progeny have also been shown to occur in other epithelial tissues such as in the oesophagus and intestine (reviewed in [117]).

Mascre et al. [254] further characterised interfollicular epithelial stem cells in mice by labelling with two different promoters; one targeting committed progenitors and the other targeting a more quiescent label-retaining population. They showed different regenerative and homeostatic properties in these two populations of cells. Mascre et al. demonstrated a more quiescent label-retaining cell population located in the basal layer in rodent epidermis, which expresses higher levels of stem cell markers such as α6-integrin and β1-integrin, while committed progenitor cells with higher proliferation rates express lower levels of stem cell markers [254]. They showed that although the progeny of committed progenitor clones transiently contributes to the initial stages of wound healing, the clones derived from the more quiescent population last longer in the repaired epidermis [254].

The location of stem cells in human skin is under debate (reviewed in [265] and [117]). Currently there is no assay to test if a specific marker identifies epithelial stem cells. Furthermore, currently there is no in vivo experimental technique to identify slow-cycling cells in human [265]. Another difficulty is that hairy dorsal skin, which is the source of keratinocytes in most of murine experiments, is very different from the usual source of cells in experiments with human keratinocytes which is the neonatal foreskin [265]. Therefore, murine keratinocyte experiments may not be applicable to the human case [265].

The structure of human skin is different from mice skin. In human, the epidermis is thicker than in mice and the undulation of the basal layer of epidermis forms deep epidermal ridges (rete ridges) that help anchor it to the dermis. The structure of monkey palmar
epidermis however, is very similar to that of the human palm [141]. Based on cell morphology and the location of label-retaining cells in monkey palm epidermis, Lavker et al. showed that, interfollicular slow-cycling cells are located at the base of rete ridges [141]. This location for stem cells would provide more protection than any other site within the interfollicular epidermis [141] (reviewed in [15]).

Recently histological examination of arrector pili muscles in mice by Torkamani et al. [137] in our department showed that the keratinocytes in distal arrector pili muscle attachment site also express epithelial stem cell markers including keratin15and α6 integrin. In humans, the distal APM attachment is mostly located at the base of the rete ridges. These results support the finding of Lavker et al. that showed the epidermal stem cells are located at the base of rete ridges [141].

To identify the location of stem cells in human skin, the expression of stem cell markers has been studied in epidermis. Cells at the base of the ridge in human adult skin, express higher levels of keratin 15 (K15) protein, which is considered a stem cell marker [266], and display a higher clonogenic potential than the more proliferative upper rete ridge basal cells [267-269] (reviewed in [117]).

On the other hand, there is also strong evidence from young human epidermis histology studies, which shows, that cells at the top of the rete ridges express stem cell markers such as β1 integrin, and LRIG1 [270-272]. These contradictory results are probably due to the differing age of the epidermis studied in these experiments, and new approaches are required to identify and characterise the keratinocyte stem cells and the mechanism of skin homeostasis and repair (reviewed in [117]).
1.5.3 **Hair follicle epithelial stem cells**

A heterogeneous population of epithelial stem cells have been characterised within the hair follicle; in the bulge, secondary hair germ, and upper bulge area, in both humans and mice (reviewed in [273, 274] and Table 1-3). Figure 1-7 shows characterised epithelial stem cell markers and their spatial organisation within the mouse telogen follicle (reviewed in [274]).

<table>
<thead>
<tr>
<th>Table 1-3</th>
<th><strong>Common stem cell markers in mice and in human hair follicles [273]</strong></th>
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<tbody>
<tr>
<td><strong>Species</strong></td>
<td><strong>Marker</strong></td>
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<tr>
<td><strong>Mice</strong></td>
<td></td>
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<tr>
<td></td>
<td>CD34</td>
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<tr>
<td></td>
<td>TCF3</td>
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<td></td>
<td>NFATC1</td>
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<td>NESTIN</td>
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<td>Label retaining cells</td>
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<tr>
<td></td>
<td>KRT15</td>
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<td>LGR5</td>
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<td></td>
<td>LHX2</td>
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<td>SOX9</td>
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<td></td>
<td>MTS24</td>
</tr>
<tr>
<td></td>
<td>ITGA6LowCD34–Sca-1–</td>
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<tr>
<td></td>
<td>LRIG1</td>
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<td></td>
<td>LGR6</td>
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<td>BLIMP1</td>
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<td>SOX2</td>
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<td></td>
<td>VCAN</td>
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<tr>
<td></td>
<td>ALPL</td>
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<tr>
<td></td>
<td>NEXIN1</td>
</tr>
<tr>
<td></td>
<td>CD133</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td><strong>Mesenchymal stem cell markers</strong></td>
</tr>
<tr>
<td><strong>KRT15</strong></td>
<td>Bulge/isthmus</td>
</tr>
<tr>
<td><strong>CD200</strong></td>
<td>Bulge/isthmus</td>
</tr>
<tr>
<td><strong>KRT19</strong></td>
<td>Bulge/isthmus</td>
</tr>
</tbody>
</table>
Bulge stem cells

The first study to discover a stem cell population in hair follicle was published in 1990 by Cotsarelis et al, [95]. They identified the slow-cycling cells in the bulge area, in the mid portion of the follicle at the attachment of the arrector pili muscle. The bulge area can be identified as a prominent structure in rodents and human foetal hair follicle. In this study, tritiated [3H] thymidine was given to mice for a long period of time (two weeks). All dividing cells incorporated the thymidine isotope into newly synthesised DNA. Subsequently, the labelled cells were chased for an additional four weeks. Using autoradiography of 3µm plastic embedded tissue sections, only those cells that divided very rarely retained their label. In this experiment on the examination after four weeks, 95% of label-retaining cells (LRC) in mouse skin were found in the bulge area (named for the swelling evident at this site in rodents). Later, the high clonogenic and multi-potency capacity of bulge cells from micro-dissected rat vibrissae was shown in vitro and in vivo transplant experiments [275, 276]. In adult humans there is no prominent structure at the bulge [118] area; however, laser-capture micro-dissection and microarray-based analysis of human anagen hair follicles has identified distinctive human bulge cells with clonogenic potential at the APM attachment site [277].

Various sub-populations of cells have been described in the bulge area based on the expression of stem cell markers, their migration during the hair cycle and wound healing...
One of the first cell markers observed in the bulge area is keratin 15, a type I cytokeratin, which is expressed in the bulge area in mice and humans [277, 279]. The involvement of K15+ cells in wound re-epithelialisation was shown in mice by Ito et al; however, K15-positive cells reside only temporarily in the healing epithelium [136]. CD34 is another molecular characteristic of mouse bulge cells [280], which is not expressed in humans [277].

Other populations of stem cells in the bulge have been identified by different levels of expression of α6-integrin, (α6-integrin\textsuperscript{high} and α6-integrin\textsuperscript{low}) in epithelial cells. As discussed in 1.5.2 α6-integrin is a stem cell marker expressed in the basal layer of the interfollicular epidermis. In the bulge, cells with high levels of α6-integrin are more proliferative in anagen and located closer to the basement membrane than are cells with lower levels of α6-integrin [281].

In addition to distance from the basement membrane, cells in the bulge are characterised according to their vertical axis position [117]. The lower region of the bulge is characterised by a higher level of proliferation and higher expression of LGR5 (Leu-rich repeat-containing G protein-coupled receptor 5), than is the upper bulge region [282-285]. The LGR5+ cells in the lower bulge region also express Hedgehog-activated transcription factor, GLI1 (GLI-Kruppel family member). GLI1 is not expressed in the middle region of the bulge; however, it is expressed in the upper part [224]. These upper-bulge GLI-expressing, but CD34-negative and K15-negative cells, migrate to the interfollicular epidermis and contribute to a long term repopulating in regenerated epidermis after wounding [224], while Keratin 15+, CD34+ bulge cells are temporary residents in healing epithelium [136].

LGR5+ GLI1+ cells are also found in the secondary hair germ during catagen [284, 286]. Secondary hair germ also shares other stem cell characteristics of bulge cells, such as the presence of CD34 and keratin 15. However, during anagen, the LGR5+ GLI1+ cells are found in the lower part of the outer root sheath and lose other bulge stem cell markers such as CD34) [284]. These changes during the hair cycle suggest cell trafficking from bulge to the SHG, occurs during catagen [284, 287]. In addition, the ability of SHG cells to
repopulate the bulge when depleted of its stem cells [287] supports the concept of cell trafficking between the bulge and the secondary hair germ.

Other stem cell markers expressed in the bulge area of rodent and human are: CD34, SOX9, S100A4, transcription factor 3 (TCF3) [288], LIM homeobox 2 (LHX2) [289], nuclear factor of activated T cells calcineurin-dependent 1 (NFATc1) [290] and Nestin [291].

**The Secondary hair germ**

The secondary hair germ is an additional set of epithelial stem cells, which appears in hair follicles at the end of catagen, between the club hair and the dermal papilla (reviewed in [86, 102], Figure 1-8). While bulge stem cells give rise to the outer root sheath, the hair germ develops into the hair shaft and also the matrix at the base of the mature follicle (reviewed in [102]).

![Figure 1-8 Hair follicle anatomy in anagen and telogen.](image)

At the beginning of anagen, the first proliferative activity in response to dermal papilla signals occurs within the hair germ [98]. As discussed above and shown in Figure 1-7 and Table 1-3 the hair germ displays some stem cell markers similar to bulge cells, such as keratin 15, SOX9, and LGR5 [98], which suggests possible cell trafficking between these two anatomical regions. BrdU pulse-chase studies also support this hypothesis and the supposition that the bulge can be considered the precursor of the HG [98].
The location of the secondary hair germ in direct contact with the two stem cell populations of dermal papilla and the bulge during the telogen (shown in Figure 1-8), suggests it may have an important role in communication and cell trafficking involved in the follicle cycle [287]. This role was confirmed by advanced lineage-tracing techniques. Using histone H2B (H2B)–GFP label-retaining assays, Hsu et al. have shown that hair germ cells originated from lower bulge cells. These cells in turn contribute to the cycling portion of follicles during anagen, which can subsequently return to the stem cell niche in the bulge and be stored for the next hair follicle cycle [278].

**The upper bulge area**

**The junctional zone and isthmus**

In contrast to stem cells in the bulge, CD34 and K15 are not expressed in the isthmus, the area that is located above the bulge (Figure 1-7). In this area, cells are characterised by expression of LGR6 and MTS24 [292-294]. These cells are mainly involved in homeostasis of the junctional zone and sebaceous glands [292]. However, they are able to migrate to wound areas and become the permanent residents of healing epithelium [292].

**The infundibulum**

Higher in the infundibulum, cells express the stem cell marker LRIG1⁺ (Leucine-rich repeats and immunoglobulin-like domains protein) [295]. These cells contribute to normal homeostasis of the infundibulum and sebaceous glands; however, when LRIG1⁺ cells are transplanted to a wound area they are found to participate in the entire hair follicle as well as the interfollicular epidermis [295]. Another stem cell marker expressed in the upper portion of the infundibulum is Sca-1, which can give rise to long-term stable IFE (long term resident), but not the hair follicle [294].

**The sebaceous gland**

While stem cells from the isthmus (LRIG1⁺ cells), infundibulum (LGR6⁺ cells) and bulge contribute to maintenance of the sebaceous glands, there is a population of stem cells
expressing Blim1 (B lymphocyte-induced maturation protein 1), which originate in the bulge. Blim1-positive cells seem to be restricted to sebaceous gland differentiation [296].

1.5.4 Hair follicle epidermal cells in wound healing

The contribution of hair follicle cells to wound re-epithelialization was noticed may decades ago. Bishop et al. (1945) reported that superficial wounds, where portions of hair follicles remained intact, healed more quickly [297]. They observed, in deep wounds where the hair follicles were destroyed, re-epithelialisation began from the margins of the wound, whereas when hair follicles remained relatively intact, wounds re-epithelialized evenly across its entire surface, and healed more quickly [297].

During the last two decades, participation of hair follicle cells in wound re-epithelialisation has been studied in several lineage analysis animal experiments. In these studies labelled hair follicle cells were tracked in the epidermis at different time points during wound healing. The first marker used to identify the hair follicle stem cells was long-term retention of a DNA label, such as 5-bromodeoxyuridine (BrdU) or tritiated thymidine [95]. Taylor et al. (2000) found that during wound healing, the slow-cycling hair follicle cells labelled with BrdU moved upwards and participated in wound epithelialization [298].

![Figure 1-9](image)

**Figure 1-9** Follicular epithelial stem cells involved in wound re-epithelialization in mice.
Heterogeneous populations of cells have been characterised by lineage-tracing of hair follicle-derived cells during wound healing (Figure 1-9). In 2005 Ito et al. [136] showed that labelled cells under K15 promoter migrated to the healing epithelium and contributed to wound repair. In skin, Keratin 15 is mainly expressed in hair follicle bulge cells and only 1% of basal cells in the interfollicular epidermis are K15⁺; however, K15⁺ cells contributed to 30% of epithelium healing after wounding [136].

In this study, K15⁺ cells were labelled by crossing the transgenic (Tg) mice (Krt1-15-Cre/PGR)22Cot mice with R26R reporter mice that express lacZ under the control of a ubiquitous promoter after Cre-mediated removal of an inactivating sequence. CrePGR is a fusion protein consisting of Cre recombinase and a truncated progesterone receptor that binds the progesterone antagonist, RU486. In Tg (Krt1-15-cre/PGR)22Cot mice, CrePR1 remains inactive in the cytoplasm of the K15⁺ cells except during treatment with RU486, which permits CrePR1 to enter the nucleus and catalyse recombination.

After treatment with RU486, K15⁺ cells were mainly detected in the hair follicle bulge and only 1% of epidermal basal cells expressed K15 protein. However, during wound healing K15⁺ cells appeared in the epidermis of the wound area and in a linear manner toward the centre of the wound, and by day eight after wounding lacZ positive cells were found to contribute up to 30% of cells in the healing epithelium. Labelled cells in this experiment were temporarily resident in the healing epithelium and their contribution to it had diminished to 2.1 ± 1.3% by day fifty after wounding [136]. Therefore, the hair follicle K15 positive cells only temporarily participated in wound epithelium. These findings suggested that either the K15⁺ cells were progenitor cells with limited regenerative capacity, or they migrated back to their bulge niche after the new epithelium had been repaired.

The above study by Ito et al. [136] could be criticised, as the K15⁺ labelled cells in the healing epithelium could have originated from the small population of K15⁺ cells in the interfollicular epidermis rather than the hair follicle itself. However, the participation of hair follicle epithelial cells in wound healing was subsequently confirmed by another study by Levy et al. (2007) [299], which used more specific labelling of hair follicle cells. In this
In vivo lineage tracing, using inducible and constitutive Cre recombinase expressed from the Sonic hedgehog locus (Shh), Levy et al. confirmed that follicular cells participated in the initial resurfacing of the wound. They also showed that their progeny persisted in wound epidermis for months after the wound was healed [299].

Gli1 is a transcription factor activated by sonic hedgehog signalling in the upper bulge area [224] and, as shown by Brownell et al. Sonic hedgehog is produced by sensory neurons, which activate the Hedgehog pathway in hair epithelium. They found that the Gli1+, K15− cells from bulge migrate to wound epidermis and unlike K15+ bulge cells, they make a permanent contribution to wound re-epithelialization [224].

By lineage tracing and labelling hair follicle cells with different stem cell markers, heterogeneous populations of stem cells that originated from bulge or upper bulge area were found to participate in wound re-epithelialization, (as shown in Figure 1-9 and Table 1-4) [300].

<table>
<thead>
<tr>
<th>Stem cell markers</th>
<th>Location in Hair follicle</th>
<th>Wound Re-epithelialization</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>K15+</td>
<td>Bulge</td>
<td>Transiently</td>
<td>[136]</td>
</tr>
<tr>
<td>GLI1+</td>
<td>Upper Bulge &amp; secondary hair germ</td>
<td>Long lasting contribution</td>
<td>[224, 299]</td>
</tr>
<tr>
<td>LGR5+</td>
<td>Lower bulge</td>
<td>Long lasting contribution</td>
<td>[284]</td>
</tr>
<tr>
<td>SOX9+</td>
<td>Bulge</td>
<td>Long lasting contribution</td>
<td>[301, 302]</td>
</tr>
<tr>
<td>LGR6+</td>
<td>Upper bulge, Isthmus</td>
<td>Long lasting contribution</td>
<td>[292]</td>
</tr>
<tr>
<td>Lrig1+</td>
<td>Junctional zone</td>
<td>Long lasting contribution</td>
<td>[295]</td>
</tr>
</tbody>
</table>

Lrig1 (Leucine-rich repeats and immunoglobulin-like domains 1) positive cells are found in the junctional zone and are able contribute to the entire follicular, sebaceous and interfollicular lineage [295]. LGR5 (Leucine-rich repeat-containing G protein-coupled receptor 5) positive cells are located in the lower bulge and in contrast to cells of the upper bulge cells, they are not slow cycling [284].

Unlike K15+ bulge cells, which are the temporary contributors of wound epithelium, Lrig1, SOX9 and LGR5-expressing cells permanently contribute to new epithelium [284, 302], (Table 1-4).
1.5.5 **Hair follicle dermal stem cells**

The epithelial component of hair follicle has been the main focus of hair follicle stem cell research and dermal components of the hair follicle are mainly recognised as supporting structures for epithelial progenitor and stem cells. The dermal components of the hair follicle are comprised of the dermal sheath and dermal papilla that contribute to the epithelial stem cell ‘niche’ in the bulge and bulb area respectively (section 1.5.3 and see below).

In addition to the contribution to epithelial stem cell niche, hair follicle dermal cells also have mesenchymal stem cell characteristics. However, their exact role in homeostasis and repair is not yet understood.

**Stem cell niche**

The concept of a “niche” as a specialized micro-environment, was first introduced by Schofield almost 30 years ago [303]. He hypothesized that the stem cells are seen associated with other cells, which determine their stemness behaviour. Once stem cells are removed from their niche, unless they can occupy a similar microenvironment, they differentiate, age and lose their stem cell characteristics [303].

The ultrastructure of a stem-cell niche is complex and comprised of cells, paracrine and endocrine signals, extracellular matrix, neural input and close contact with microvasculature (reviewed in [304]). The stem cell niche is critical for regulating stem-cell function [304], and should be considered an essential component of hair follicle epithelial and mesenchymal stem cells.

The arrector pili muscles and the nerve fibres around the hair follicle are in close contact with bulge stem cells and considered as important components of this stem cell niche [102]. Nerve fibres around the hair follicle are involved in Hedgehog signalling and maintain the expression of Gli1 in upper bulge stem cells (Table 1-4) [224]. This Gli1+ population migrates to interfollicular epidermis and contributes to the wound re-epithelialisation [102, 224]. Recently in our department, Torkamani et al. [137] showed that keratin15 and α6 integrin positive basal keratinocytes in the interfollicular epidermis
in mice, are located in the distal arrector pili muscle attachment site located at the base of the rete ridges. These findings suggest a close relationship between the APM and the epithelial stem cell niche in the bulge and the interfollicular epidermis.

There is supporting evidence that subcutaneous fat deep in the dermis is involved in regulation of hair follicle stem cells. Subcutaneous adipocytes proliferate during anagen. Mature adipocytes express BMP2 and keep the hair follicle niche in quiescence during the resting phase (telogen), while immature adipocytes induce hair growth [192, 195] (reviewed in [102]).

Hair follicle dermal cells have been considered as a supporting structure for epithelial stem cells in the bulge and bulb area (reviewed in [116]). Dermal cells are involved in producing the signalling pathways required for differentiation of epithelial cells during the induction and organogenesis of the hair follicle and hair cycling (reviewed in [102]). Therefore, hair follicle dermal papilla and dermal sheath cells are considered as crucial elements of the epithelial stem cell ‘niche’ in the bulb and bulge area.

Improvement in isolation and culturing techniques of hair follicle dermal cells has led to further understanding of the interaction between the epithelial and mesenchymal components of hair follicles during development and cycling. These studies have led, to the discovery of the hair induction capacity of the dermal papilla [113] and dermal sheath cells [104], and the development of hair reconstitution assays [104, 114] (reviewed in [116]).

The inductive property of dermal cells was shown by Oliver in 1967 when he transposed dermal papillae beneath the upper half of amputated vibrissa hair follicles [113]. He transplanted these dermal papillae into a follicular skin and induced hair growth [305]. Cultured dermal papilla and dermal sheath cells were also able to form a new functioning dermal papilla when transplanted into the skin and induce hair follicle [100, 104, 112, 114, 120] (reviewed in [116]).

Using an in vivo microscopy imaging system, Rompolas et al. (2013) [306] have shown that at the beginning of the anagen phase, epithelial cell division occurs in the activated
hair germ compartment that is in contact with the mesenchymal DP niche. They also showed that the dermal papilla is required to initiate the hair cycle. Laser ablation of dermal papilla just before the hair regeneration phase kept the hair follicle in the resting phase and prevented initiation of anagen [306].

The signalling molecules involved in hair follicle induction, morphogenesis and hair cycling include secreted molecules of the Wnt/wingless family, the hedgehog family, and members of the TGF-β/BMP (transforming growth factor-β/bone morphogenetic protein), FGF (fibroblast growth factor) and TNF (tumour necrosis factor) families, and transcription factor NFIB (reviewed in [102], discussed in 1.2.2 and 1.3.5). In the process of morphogenesis and hair cycle, dermal cells are considered as inducers and the epithelial cells as responders. However, the signals between these two cell types are two-sided and complicated (reviewed in [116]).

The dermal papilla not only induced hair formation and hair growth, but also regulated hair regression during catagen. A recent study by Mesa et al. [307] showed that ablation of dermal papilla reduced the cell death in the hair follicle epithelium during the regression phase [307].

**Hair follicle Mesenchymal stem cells**

In addition to their hair induction capacity and contribution to the epithelial stem cell niche, dermal cells have also been found to have similar characteristics to bone marrow mesenchymal stem cells.

Lako et al. [101] have shown that hair follicle dermal cells can produce haematopoietic colonies *in vitro*, reconstitute bone marrow, and contribute to all blood lineages when transplanted in lethally irradiated recipient mice [101]. It has also been shown that hair follicle mesenchymal cells have similar morphology and doubling time in cell culture environment, as bone marrow mesenchymal stem cells. In addition, hair follicle dermal cells express the same cell surface marker (CD44+, CD73+, CD90+ and CD34+) as bone marrow mesenchymal stem cells [308]. Hair follicle dermal papilla and dermal sheath cells are also multi-potential (Table 1-11) [309-311], while interfollicular dermal cells have not
shown this multi-potency [308]. The multipotency of hair follicle dermal cells will be discussed in 1.7.3.

Dermal papilla and dermal sheath cells form fibroblast-like cell cultures when grown in 10% (v/v) foetal calf serum in coated cell culture flasks [101, 309]. In non-adherent culture flasks with serum free media, they grow as floating spheres [106, 312]. Cultured dermal papilla and dermal sheath cells are multipotential and are able to differentiate into various lineages such as neurons and glia [312] bone and adipose tissue [309], skeletal muscle [310] and contractile smooth muscle cells [311]. Consequently, the hair follicle has been considered as a source of mesenchymal adult stem cells suitable for cell therapy and tissue engineering. This will be discussed in 1.6.4, and in chapter 3.

Although cultured hair follicle dermal cells have mesenchymal stem cell characteristics, the exact role of dermal sheath and dermal papilla cells in hair and skin homeostasis and repair is not yet understood [102] (this will be further discussed in the following section).

1.5.6 Hair follicle dermal cells in wound healing

In 2001, Jahoda et al. hypothesized that hair follicle dermal cells, like bulge stem cells, may act as the reservoir of mesenchymal stem cells involved in hair and skin homeostasis and repair. The dermal sheath and dermal papilla are likely to provide key cellular input into granulation tissue formation, and participate in angiogenesis and nerve fibre remodelling during wound healing [313] (reviewed in [314]).

Although the function of the dermal component of the hair follicle is mainly to send signals required for hair induction and hair cycling, it has been hypothesised that hair follicle dermal cells also participate in wound healing.

Hair follicle dermal cells the origin of myofibroblasts and pericytes

There is some evidence to suggest that hair follicle dermal cells may be the precursors of myofibroblast, blood vessels and sub-dermal fat involved in wound healing. First of all, α-smooth muscle actin (α-SMA), an actin protein isoform found in smooth muscle cells around the blood vessels (Pericytes) [315] and myofibroblasts [316], is expressed in
dermal sheath cells within the hair follicle both in vivo and in vitro [317]. Dermal papilla cells do not express α-SMA in vivo; however, they express more α-SMA in culture than interfollicular fibroblasts [317]. These findings suggest that hair follicle dermal papilla and dermal sheath cells are specialized fibroblasts that may be the origin of myofibroblasts and pericytes in healing wounds [313, 317].

Furthermore, isolated dermal papilla cells and cultured cells originating from the dermal component of hair follicles are able to differentiate into smooth muscle cells and adipocytes in vitro [309, 318-320] and in vivo [321, 322]. The animal experimental model developed by Gharzi et al. showed that transplanted labelled dermal papilla cells induce new hair follicle development (hair induction assay) and then participate in granulation tissue from reconstituted hair follicle during skin-wound healing. In this experiment, labelled dermal papilla cells persist in healing dermis in a similar manner to the normal skin fibroblast [321].

Hair follicle dermal cells may participate in granulation tissue vascularisation. Amoh et al. (2004) have shown that nestin-positive cells originated from transplanted follicles, form blood vessels, express endothelial cell markers, the CD31 and Von Willebrand factor (VWF), and connect to the host vasculature in skin dermis [323]. Nestin+ cells that are believed to be neural crest in origin [320], are located in bulge and in the dermal component of the hair follicle [256, 312].

“Skin-derived precursors” or SKPs cells are one of the early cultured cells from dermis introduced by Toma et al. in 2001 [320]. These cells are also believed to arise from the neural crest [320]. SKPs have been cultured from skin dermis and dermal papilla. Lavoie et al. (2009) showed that when the SKPs were transplanted at the site of fractured bone, they migrated around the blood vessels. These cells express α-smooth muscle actin (pericyte marker) and not CD31 (endothelial marker), which suggests differentiation to pericyte [324]. In another study by Biernaskie et al. (2009) when SOX2+ Skin-derived precursors cultured cells, derived from dermal papilla and dermal sheath, were transplanted into skin, they differentiated to dermal fibroblast and adipocytes in the hypodermis and contributed
to wound healing [322]. Hair follicle neural crest stem cells and SKPs will be further discussed in 1.5.9.

Although there has been some evidence suggesting possible involvement of isolated hair follicle dermal cells in wound myofibroblasts and vasculature, direct participation of hair follicle cells in granulation tissue myofibroblast has not been shown and this hypothesis was not supported by a recent lineage analysis experiment performed in rodents.

Driskell et al. (2013) characterised two distinct cell populations in upper dermis, including hair follicle dermal cells, and lower dermis. They showed that from E18.5 B-lymphocyte-induced maturation protein 1 (Blimp1) was selectively expressed only in the upper dermal fibroblast, including dermal papilla, dermal sheath and endothelial cells, while fibroblasts in the lower dermis expressed Delta-like homologue 1 (Dlk1). To label these two populations of fibroblasts in skin, Driskell et al. used two different Cre-mediated recombinant transgenic mice. In Dlk1-CreER 3LSL-td Tomato mice, the upper skin fibroblasts, including hair follicle dermal cells, were labelled in red; while, the lower skin fibroblasts in Blimp1-Cre 3LSL- green fluorescent protein (GFP) mice were labelled green in histology examinations.

Using 8 mm excisional wound models, they showed that it is mainly the lower dermal fibroblasts which invade the granulation tissue and participate in dermal repair during wound healing, while upper dermal fibroblasts, including hair follicle dermal cells, are recruited only at a later stage (day 17) and only appear in upper dermis, underneath the fully re-epithelialised epidermis [50].

Based on this result, fibroblasts and myofibroblasts in granulation tissue have not originated from hair follicle dermal cells, at least at the early stage of wound healing. The appearance of Dlk1 driven GFP+ positive cells beneath the newly formed epithelium after 17 days of wounding, which might be originated from hair follicle dermis, suggest that hair follicle dermal cells may have a supportive role for new epithelium at a later stage of healing.
1.5.7 **Cell trafficking in hair follicle stem cell niche**

Dynamic morphological changes during the hair cycle, suggest cell trafficking between various anatomical structures of the hair follicle during the hair cycle. Cell trafficking between epidermal components of hair follicle was confirmed by lineage-tracing techniques [278]. Hsu *et al.* (2011) have shown that hair germ cells have originated from lower bulge cells. During anagen, germ cells contribute to the cycling portion of hair, then return to the stem cell niche in the bulge and are stored for the next hair follicle cycle [278].

Cell trafficking has also been suggested to occur between the two dermal components of the hair follicle, the dermal papilla and the dermal sheath. In hair induction experiments, transplanted dermal papilla or dermal sheath cells were incorporated into the dermal component of the newly formed hair follicle, or into pre-existing hair follicles. This raised the idea of cell trafficking between hair follicle dermal papilla and dermal sheath cells, which was suggested by Oliver in 1991 and Jahoda in 1998 [325, 326]. The dermal sheath has been considered as a cellular reservoir of dermal papilla cells during the hair follicle cycle [103]. The similarity between cell markers of dermal papilla and dermal sheath cells also supports the idea of cell trafficking between these two mesenchymal components during the hair cycle [100, 103, 104, 326] (reviewed in [102]).

In a recent study using an *in vivo* multiphoton microscopy imaging system with genetic lineage tracing, Rompolas *et al.* [306] visualized the genetically labelled cell in the hair follicle over time. They used a transgenic mouse line (K14-H2BGFP) to label hair follicle epithelial and transgenic Lef1-RFP mouse to label mesenchymal hair follicle populations. The results showed that dermal sheath cells were capable of repopulating the dermal papilla after laser ablation [306]. They also demonstrated that following laser ablation of hair follicle bulge or hair germ cells, the niches were able to recover and both bulge and hair germ were able to repopulate each other following laser ablation [306]. These findings support the hypothesis of cell trafficking between the dermal papilla and the dermal sheath.
1.5.8 **Hair follicle melanocyte stem cells**

Melanocytes originate from the neural crest and migrate to eye, skin and hair follicle before birth. These cells produce melanin pigments that function as a protection against radiation and DNA damage. In humans, melanocytes are found in the interfollicular epidermis and hair follicle, while in mice skin melanocytes are only found in hair follicle (reviewed in [327]).

Hair follicle melanocyte stem cells are located in the bulge area [97]. It is believed that the communications of melanocyte with dermal papilla and bulge cells are essential for the survival and behaviour of melanocyte stem cells [117, 328]. The differentiated progeny of activated melanocytes from bulge migrate to the bulb area, where they produce pigment for matrix cells (reviewed in [327]).

Activation of melanocyte stem cells is under the influence of signals secreted by bulge epithelial stem cells during the hair cycle. Examples are the TGFβ signals during the telogen [329] and WNT signals at the beginning of anagen, which activate both the melanocytes and bulge stem cells [330]. The differentiated progeny of activated melanocytes migrate to the bulb area, where they produce pigment for matrix cells (reviewed in [327]).

Experimental observations in human vitiligo patients suggest that melanocytes migrate from the hair follicle to interfollicular epidermis in UV irradiated vitiligo lesions [331, 332]. Recent studies in mice also showed that after wounding or UVB irradiation hair follicle melanocyte stem cells also migrate to the healing epithelium [333]. In adult mice, there are no melanocytes in the interfollicular epidermis of hairy skin [65], where the hair coat covers and protects the epidermis. It is possible that mice have evolved to acquire the ability to regenerate epidermal melanocytes to protect their skin in the event of injury or UVB irradiation [333].
1.5.9 Neural crest stem cells

In addition to melanocytes that are derived from embryonic neural crest, it has long been known that the mesenchymal tissue of the face, including dermis, derived from the neural crest [59, 109].

Migration of neural crest cells in the mouse embryo was further investigated by Yoshida et al. [48] (2008). Using the Wnt1Cre R26R mice, they detected migration of neural crest cells to craniofacial mesenchyme with a boundary just posterior to the eye. Jinno et al. (2010) confirmed the diversity in the developmental origin of dermis, by lineage tracing in several Cre-reporter mouse lines [71]. Recently using a Wnt1Cre transgenic mouse, which permanently labels early migratory neural crest populations Rinkevich et al. (2015) [49], detected Wnt1 lineage–positive fibroblasts within the oral dermis.

Diversity in the embryonic origin of skin dermis also applies to the dermal component of the hair follicle as the hair follicle dermal papilla and dermal sheath are derived from skin dermis. This has been recently confirmed by comprehensive lineage analysis [50].

Before the characterisation of neural crest cells in embryonic skin, several cultured cells with neural characteristics had been produced. However, it was not clear if they had originated from the neural crest. Using the Wnt1Cre combined with the Rosa26 reporter transgenic mouse, which permanently labels early migratory neural crest cells, Fernandes et al. in 2004 [106] found neural-crest derived cells in vibrissae hair follicle dermal papillae.

“Skin-derived precursors” cells are one of the early cultured cells from dermis which are believed to have originated from the neural crest as introduced by Toma et al. in 2001 [320]. They identified a group of multipotent cultured cells from rodent skin that were able to proliferate and differentiate in culture, which they called “Skin-derived progenitor” (SKP). SKPs are floating spheres produced from skin dermal cells, cultured in a non-adherent, uncoated flask in a special neural culture medium [320].

There was a question on the origin of SKP cells at that time, as SKPs do not express two common neural crest cell markers, p75 neurotrophin receptor and PSA-NCAM [320].
However, SKP cells express nestin, which is a neural crest stem cell marker [106]. Nestin is also expressed in the dermal papilla and bulge hair follicle [323, 334]. In addition to nestin, SKPs express other neural-specific proteins such as fibronectin and Sox2 [106, 322].

To prove that SKPs are neural crest in origin, Fernandes et al. [106] injected the labelled SKP cells from back skin of neonatal mice, to the chick neural crest migratory stream in vivo. They showed that half of the transplanted cells migrated to the peripheral neural crest and few migrated into the neural tube and some migrated to the skin dermis (after three days). They concluded that cultured dermal cells were neural crest related.

In addition to vibrissae dermal papillae, which are neural crest in origin, dorsal and ventral trunk skin were also used to produce cultured SKPs in the experiments done by both Toma’s [320] and Fernandes’ group [106]. However, cells that originated from ventral and dorsal skin are not expected to have neural crest characteristics, as they are mesoderm in origin and not neural crest.

The neural crest origin of SKP was questioned by Jinno et al. [71]. They compared the SKPs from dermal hair follicle cells in the face, which are neural crest derived with the dorsal trunk, which are not neural crest in origin and derived from somite. They showed that both of these two cell populations were able to differentiate to neural lineage and have similar gene expression in microarray analysis. They argued that despite the different developmental origin of SKPs from these two locations, cells were functionally similar and exhibited neural crest characteristics. Jinno et al. concluded that cells could exhibit characteristics of neural crest cell derivatives, even though they have a non-neural crest origin [71].

SKPs are multi-potential and have been differentiated into neurons, glia, smooth muscle cells, adipocytes and pericytes [106, 320]. SKPs were also integrated into the periosteum when transplanted in to the fracture region [324] (Table 1-11). Nestin-expressing cells originating from the hair follicle also form blood vessels in the dermis. Amoh et al. (2004) showed that after transplantation of hair follicles from nestin GFP transgenic mice into
nude mice, GFP-expressing cells originated from the transplanted hair follicles, formed blood vessels and developed connection with the surrounding recipient vasculature [323] (Table 1-11).

Sox2 is another embryonic and neural stem cell marker expressed in the dermal papilla and lower part of the dermal sheath. Using Sox2 GFP transgenic mice, Bienaskie et al. (2009) have shown that isolated Sox 2-expressing cells are able to differentiate into myofibroblasts in the dermis [322]. Sox2 positive cells also formed SKP spheres in vitro [322]. The SKP spheres induced hair follicle formation when transplanted into severe combined immunodeficiency (SCID) mice. They also formed chimeric hair follicles which express Sox2 in the dermal papilla and the lower part of the dermal sheath. Lineage tracing of transplanted chimeric follicles also shows the labelled Sox2-positive cells (originated from the dermal component of the transplanted hair follicle) are able to migrate and participate in the granulation tissue after wounding. [322].

In addition to SKP cells that are floating sphere cultured cells, there is a type of cultured, neural crest-originated cell in the form of an adherent cell, called epithelial neural crest stem cells (EPI-NCSC). These cells were developed by Sieber-Blum et al. by culturing micro-dissected bulge cells in a special serum-free medium [335] which forms adherent culture cells. Similar to SKPs, EPI-NCSC also showed activation of Wnt signalling [335], which suggests their neural crest origin [312, 335]. EPI-NCSCs have differentiated to neurons, Schwann cells and cartilage in vitro [335] and when grafted to injured spinal cord, differentiate into neurons and oligodendrocytes, improving touch perception [336]. Interestingly, despite the fact that transplantation took place only on one side of the spinal cord, a bilateral improvement was observed, suggesting possible neurotrophic or, angiogenic factors, secreted from the transplanted EPI-NCSCs [336, 337].

Hair follicles are an accessible source of multipotent epithelial, mesenchymal and neural crest stem cells. Hair follicles are considered an ideal source of stem cells, not only for hair and skin regeneration, but also for cell therapy and tissue engineering in other organs [273]. Application of hair follicle stem cells in regenerative medicine will be discussed in 1.6.4.
1.6  **Wound healing**

Skin is the largest organ in the body, which provides protection against dehydration, physical and biological injuries. The wound healing response is an essential and vital biological process [1].

Primitive animals such as small amphibians and reptiles [4] are capable of complete wound healing; however, in larger animals regeneration is no longer possible. In humans and other mammals, excepting the case of injury to foetal skin [5], skin repair is inevitably associated with scar formation, which probably provides a survival advantage by preventing infection and mechanical deformation in larger tissues [2]. One of the leading hypotheses is that the immune system is involved in scar formation and scarless healing in the human fetus is related to the immature immune system [327].

The complex process of wound repair has evolved to rapidly prevent bleeding and replace the skin defect by re-epithelialization and formation of a new dermal matrix. This section contains a review firstly of the mechanism of acute skin repair, and secondly of the physiopathology of chronic wounds. The role of the hair follicle in wound healing will be discussed in more detail in Chapter 6.

1.6.1  **Acute wound healing**

Using laboratory animal experiments the sequence of events following skin damage, and the various cells and molecules involved in these processes have been characterised. These studies have resulted in the definition of different sequential and overlapping stages in acute wound healing. These stages in both human and animal studies include: 1) Haemostasis, 2) Inflammation, 3) Proliferation and migration and 4) Maturation and remodelling [338-341].
As shown in Figure 1-10, various stages of healing have also been characterised by migration of specific immune cell populations. Tissue damage initiates a burst of coagulation and inflammation and repair response [342]. Studies in zebra fish, Xenopus, and Drosophila have shown that tissue injury results in damage response signals within minutes, including hydrogen peroxide, which is essential for recruitment of immune cells to the wound [343, 344]. Epidermal calcium concentration produced upon injury, stimulates actin polymerization and wound contraction [344]. Other known biological factors considered as early response signals are prostaglandins PGH2 and PGE2, which stimulate progenitor cell proliferation [345].

**Haemostasis**

Both the coagulation and inflammation phases begin immediately after an acute skin injury and they share many mediators and cells which initiate the wound healing response.

Different clotting cascades are initiated either by factors released by damaged skin (extrinsic factors) or activation of platelets by exposed collagen (intrinsic factors), which results in aggregation and degranulation of platelets and formation of a blood clot [346]. The clot provides a source of cytokines and growth factors needed for the subsequent
healing response, and furnishes a scaffold structure which migrating cells fill to repair the
defect [346].

Damage to blood vessels is followed by vasoconstriction lasting five to ten minutes which
is triggered by the platelets [346]. Formation of a blood clot and initial vasoconstriction
around the wound prevent bleeding and also provide temporary sealing of the wound. The
initial vasoconstriction is followed by vasodilatation of arterioles which generate redness
and swelling around the wound [346].

Table 1-5  Regulation of wound repair by growth factors and cytokines [347]

<table>
<thead>
<tr>
<th>Responses</th>
<th>EGF</th>
<th>FGF</th>
<th>GMCSF</th>
<th>IL-1</th>
<th>PDGF</th>
<th>TGF-β1</th>
<th>VEGF</th>
<th>CTGF</th>
<th>IGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast proliferation</td>
<td>++</td>
<td>+</td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Keratinocyte proliferation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrix formation</td>
<td>+</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory cell migration/chemotaxis</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

EGF = epidermal growth factor; FGF = fibroblast growth factor; GMCSF = granulocyte-macrophage colony-stimulating factor; HGF = hepatocyte growth Factor; IL-1 = interleukin-1; IGF-1 = insulin growth factor-1; PDGF = platelet-derived growth factor; TGF-β1 = transforming growth factor-β1, VEGF = vascular endothelial growth factor, CTGF = connective tissue growth factor.

The chemotactic factors released by platelets, attract other platelets, and also leukocytes
and fibroblasts. The inflammatory process is activated by cytokines and growth factors
released by platelets and leukocytes. Leukocytes and platelets also provide factors to
stimulate matrix protein synthesis, angiogenesis, re-epithelialisation and proliferation of
keratinocytes and fibroblasts (Table 1-5) [347, 348].

Table 1-5, is a simplified description showing cytokines and growth factors involved in
wound healing. In this table only the main effects of cytokines and growth factors are
noted, while the actions of growth factors are often context-specific and vary according to
the biological situation [347].
**Inflammation**

The inflammatory phase of wound healing begins during coagulation. Several inflammatory cells have been characterised in each stage of wound healing. In early studies, anti-sera were used to deplete specific cell populations and to study their function in wound healing (reviewed in [349]), and advanced transgenic technologies were subsequently employed to investigate the cells and cytokines involved in wound healing in different knock-out mice (reviewed in [349]). Table 1-6 shows the role of individual cytokines released by various inflammatory cells during wound healing.

<table>
<thead>
<tr>
<th>Table 1-6</th>
<th>Inflammatory cells and their functions in wound repair [349]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell type</strong></td>
<td><strong>Functions</strong></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Phagocytosis of infectious agents</td>
</tr>
<tr>
<td></td>
<td>Macrophage activation through phagocytosis</td>
</tr>
<tr>
<td></td>
<td>Amplify inflammatory response</td>
</tr>
<tr>
<td></td>
<td>Stimulate repair response</td>
</tr>
<tr>
<td>Macrophage</td>
<td>Phagocytosis of neutrophils and fragments of tissue degradation</td>
</tr>
<tr>
<td></td>
<td>Amplify inflammatory response</td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory function</td>
</tr>
<tr>
<td></td>
<td>Stimulate repair response: angiogenesis, fibroplasia</td>
</tr>
<tr>
<td></td>
<td>Fibrolysis</td>
</tr>
<tr>
<td>Mast cell</td>
<td>Control vascular permeability</td>
</tr>
<tr>
<td></td>
<td>Control influx of PMN</td>
</tr>
<tr>
<td>T cell: Th1/Th2</td>
<td>Regulate tissue remodeling</td>
</tr>
<tr>
<td>γδ T cells</td>
<td>Keratinocyte proliferation and differentiation</td>
</tr>
<tr>
<td></td>
<td>Hyaluronic acid synthesis in Keratinocytes αβ</td>
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<td></td>
<td></td>
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</tbody>
</table>

As shown in Figure 1-10, the time line of migration of immune cell populations correlates with the various phases of wound healing [338]. The inflammatory phase can roughly be divided into an early phase with neutrophil recruitment and a late phase with the appearance and transformation of monocytes (reviewed in [346]).
**Neutrophils**

Neutrophils are the first inflammatory cells to appear in a wound. Within a few hours after injury, neutrophils cross capillary walls (extravasation), and are recruited to a wound [349].

Factors involved in extravasation and recruitment of neutrophils are summarised in Table 1-7. The expression of adhesion molecules on endothelial cells and their interaction with cell membrane integrins presented by neutrophils are essential for extravasation of neutrophils. In addition, chemokines and their receptors are crucial mediators for the expression of endothelial adhesion molecules. Another important factor involved in extravasation and recruitment of neutrophils are bacterial products, such as formyl-methionyl peptides and lipopolysaccharides, which accumulate in the infected wound [349].

<table>
<thead>
<tr>
<th>Table 1-7</th>
<th>Factors involved in extravasation and recruitment of neutrophils [349]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines involved in expression of endothelial cells’ adhesion molecules</strong></td>
<td>IL-1β, IL-1α, TNF-α, IFN-γ</td>
</tr>
<tr>
<td><strong>Endothelial cells’ Adhesion molecules</strong></td>
<td>Endothelial P- and E-selectins, ICAM-1, ICAM-2.</td>
</tr>
<tr>
<td><strong>Integrins on neutrophils</strong></td>
<td>CD11a/CD18, CD11b/CD18, CD11c/CD18, CD11d/CD18</td>
</tr>
<tr>
<td><strong>Cytokines involved in Neutrophil recruitment</strong></td>
<td>IL-8, growth related onco gene-α, and monocyte chemoattractant protein-1 (MCP-1)</td>
</tr>
<tr>
<td><strong>Bacterial products</strong></td>
<td>Lipopolysaccharides, formyl-methionyl peptides</td>
</tr>
</tbody>
</table>

Debridement of necrotic tissue and killing of bacteria by phagocytosis, antimicrobial substances and proteinase secretion, are the crucial functions of neutrophils during the first few days after injury [346]. Neutrophils secrete antimicrobials such as cationic peptides, eicosanoids, and proteinases such as elastase, cathepsin G, proteinase 3 and a urokinase-type plasminogen activator [349]. Neutrophils also act as chemoattractants for other cells [349]. *In vitro* experiments have also shown that neutrophils modulate the cytokine profile expression and the phenotype of macrophages [350].
Although neutrophils and macrophages are crucial in defence against bacteria [349], an experiment on guinea pigs by Simpson et al. (1972) showed depleted neutrophils with antiserum had no significant effect on tissue repair in incisional wound under sterile conditions [351]. Recent studies have confirmed that depletion of neutrophils does not impair dermal repair, but surprisingly, lack of neutrophils in wounds significantly accelerates re-epithelialization [352]. The role of inflammation in chronic wounds will be discussed in 1.6.2.

**Monocyte and macrophages**

In the absence of bacterial infection, neutrophil infiltration ceases after a few days [349]. The second stage of the inflammatory phase is characterised by the presence of monocytes in the wound area. Monocytes appear around day 2 after injury and in addition to resident macrophages, the majority of macrophages infiltrate through blood vessels to the wound site [349]. Interaction of the a4b1 integrin and endothelial vascular cell adhesion molecule-1 [353, 354] as well as chemotactic factors, including growth factors, pro-inflammatory cytokines, and chemokines macrophage inflammatory protein 1a, MCP-1 RANTES [348, 355] are involved in the process of monocyte recruitment. These chemokines are secreted by fibroblasts, wound edge keratinocytes, and leukocytes. [349].

Monocytes differentiate to mature macrophages as they extravasate. These transformations involve major alterations in gene expression and numerous changes in cell surface receptors. These process are influenced by mediators in the wound and are considered an adaptation to the specific wound microenvironment [356] (reviewed in [349]).

The important role of macrophages in wound healing was shown more than 30 years ago by Leibovich et al. (1975) [357]. They demonstrated that depletion of macrophages by antisera leads to a significant delay in wound healing. These results have been confirmed in recent studies. Mirza et al. [358] used transgenic mice expressing diphtheria toxin (DT) receptor under the control of the CD11b promoter to specifically ablate macrophages during wound healing. These studies showed that macrophage depletion had a profound effect on wound healing and resulted in delayed re-epithelialization, impaired
angiogenesis, reduced collagen deposition, and decreased cell proliferation in the healing wounds.

Further experiments using glycosylates double P- and E-selectins [359] and ICAM-1-deficient mice [360] showed the importance of adhesion molecules in infiltration of macrophages, and confirmed that lack of macrophages at the wound site delay wound healing.

**T cells**

In the absence of infection during the remodelling stage of wound healing, T cells are the dominant leukocyte at wound sites [361]. Like macrophages, T cells differentiate to different subtypes and each subtype, such as T helper or γδ T cells, is characterised by a specific cell marker. As shown in Table 1-6 each subtype of T cells secretes a specific cytokine profile (460). For example the Th1 cells are a primary source of IFN-γ while Th2 cells are the source of IL-4 and IL-13 [341].

As mentioned previously, these cytokines have a major effect on the macrophage function and angiogenesis. Depletion of γδ T cells in skin showed a delayed healing response following skin injury in mice (reviewed in [349]). Recent studies have also shown that T helpers influence the remodelling of wounds and might be involved in regulation of collagen deposition or wound breaking strength [362].

**Proliferation and migration**

When haemostasis has been achieved and the inflammatory response has settled, the proliferation stage can start to replace the tissue defect [363]. This phase of wound healing is associated with formation of new blood vessels and re-epithelialisation that occurs by migration and proliferation of keratinocytes. Dermal defects are replaced by migration and proliferation of fibroblasts, deposition of the extracellular matrix and formation of granulation tissue [364].
Angiogenesis

Initially the wound is hypoxic due to the loss of vascular perfusion. With the newly formed capillaries vascular perfusion is restored to provide oxygen and nutrient for the heightened demands of regenerative tissue [365]. It is presumed that angiogenesis has a critical role in the wound healing process; however, this has not been investigated in the context of repair (reviewed in [366]).

Immediately after injury and during the healing process, several pro-angiogenic cytokines and growth factors are released from platelets, keratinocytes and macrophages, including IL6, IL8, TNF, vascular endothelial growth factor (VEGF), PDGF, FGFs and TGF-β (Table 1-5) [1, 341] Hypoxia regulates VEGF expression by activation of hypoxia-inducible factor-1α in keratinocytes (reviewed in [367]). VEGF also mobilizes bone marrow, the endothelial progenitor cells to wound, through CXCR4 ligand and SDF-1 (stromal cell-derived factor 1) [368] (reviewed in [1]).

Pericytes are believed to play a role in regulating vascular formation, stabilization and remodelling; however, so far their precise functional relevance is largely unknown [341].

New blood vessels appear in the granulation tissue around day 2 [1]. New vasculature forms via a complex process of sprouting of capillaries from the pre-existing blood vessels (angiogenesis) and spontaneous formation of blood vessels and generation of blood vessel islands from circulating endothelial precursor cells (reviewed in [369]). Although formation of new vasculature in granulation tissue is mainly through angiogenesis, there is an increasing body of evidence that circulatory endothelial progenitor cells also participate in vascularization of granulation tissue (reviewed in [341]).

Using a haematopoietic chimera murine experiment, Crosby et al. [370] showed that four months after irradiation and haematopoietic recovery 0.2% to 1.4% of epithelial cells in normal dermis vasculature derived from circulating haematopoietic progenitors, while 8.3% to 11.2% of cells in newly formed blood vessels in sponge-induced granulation tissue derived from circulating haematopoietic progenitors. The contribution of circulating
endothelial cells varies in different tissues and also depends on the gradient of hypoxia and ischemia within the injured tissue (reviewed in [371]).

Angiogenesis is associated with proteolytic degradation of the basement membrane around endothelial cells by matrix metalloproteinases (MMPs,) [369]. In addition, expression of αvβ3 integrin in surrounding ECM is associated with endothelial cell proliferation and migration [1, 369]. Formation of blood vessels and migration of endothelial cells are guided by the gradient growth factors such as VEGF, placental-derived growth factor, and fibroblast growth factor-2 (FGF-2) (reviewed in [369]).

**Re-epithelialization**

Re-epithelialization starts about 24 hours after skin injury (reviewed in [1]). Keratinocyte stem cells and transient amplifying cells from surrounding hair follicles (Discussed in 1.5.3 and 1.5.4) and the interfollicular epidermis (discussed in 1.5.2) contribute to re-epithelialization [372].

In an early study of wound re-epithelialization, Matolsty *et al.* (1970) used 3H-thymidine to label and lineage to trace basal keratinocytes. They found labelled cells appeared at supra basal and stratum corneum at day 1 and 3 day post-application of incisional wound respectively. They also showed that re-epithelialization is achieved by a combination of migration and proliferation of keratinocytes [373].

It is believed that re-epithelialization in humans mainly occurs through cell migration in smaller incisional wounds (reviewed in [374]) and may take only 24-38 hours, (reviewed in [375]). In larger wounds, migration of cells alone is insufficient to close the defect and re-epithelialization depends on proliferation of basal keratinocytes as well as migration. In the latter case the process may take several weeks [376]. Proliferation of keratinocytes commences in the basal layer, within 300 – 400 cell diameters from the wound edge within a few hours after wounding. It peaks in 1-2 days post wounding and lasts several weeks (reviewed in [1]).
Keratinocytes at the wound edge undergo significant changes to be able to proliferate, migrate, and replace the epidermal defect [377] (reviewed in [341]). This transformation of keratinocytes is known as epithelial-mesenchymal transition (EMT), which is an embryological process leading to proliferation and migration of keratinocytes [378]. EMT is associated with changes in cell membrane adhesion molecules and intra cellular filaments. Several signaling and interactions between epithelial cells, fibroblasts, endothelial cells as well as growth factors and matrix proteins are involved in this process [379] (reviewed in [347]).

**Mechanism of re-epithelialization**

Migrating keratinocytes elongate and flatten to facilitate movement [380] (reviewed in [1]). Re-organisation of intracellular monofilaments such as actin and keratin intermediate filament (K) in keratinocyte, are believed to provide the plasticity and flexibility necessary for migration while still maintaining resilience of the intracellular scaffold [341, 377]. For example, K1 and K10 expressed in supra basal cells decrease and flexible keratin fibres such as K6 and K16 increase (reviewed in [1]). Keratin 6 and 16 are also expressed in proliferating keratinocytes in places such as the hair follicle’s outer root sheath and these are considered proliferation markers for keratinocytes [381, 382].

Mechanical stimulation during skin injury may provide the starting signal for re-epithelialization (reviewed in [383]). Keratinocyte injury alters the ratio of magnesium and calcium ions. Calcium ions are involved in the modulation of desmosomal adhesion (cell adhesion molecules are discussed in (1.2.1)). Calcium ions may initiate the complex process of keratinocyte migration [384, 385]. Several molecules such as GFs, keratinocyte cell membrane protein such as integrins and matrix metalloproteases (MMPs) have been found to be involved in the process of keratinocyte proliferation and migration (reviewed in [385]).

It has long been noted that growth factors and cytokines such as epidermal growth factors (EGF), keratinocyte growth factors (KGF), IGF-1 and NGF are released at a wound site and influence keratinocyte migration and proliferation [348, 376] (Table 1-5).
Enzymatic loosening of intercellular desmosomes and hemi-desmosomes facilitate the migration of keratinocytes through provisional structural matrix components (reviewed in [346, 347]). Several enzymes including matrix metalloproteinases (MMPs) have been found to increase the migration of keratinocytes including MMP-1 (Collagenase 1), MMP-2 (Gelatinase A), MMP-10 (Stromelysin 2), MMP-7 (Matrilysin-1) and MMP-28 (Epilysin) [385].

Integrins are another important class of molecules involved in migration of keratinocytes [386]. The integrins are transmembrane receptors, which modulate the cell-cell and cell-matrix interactions involved in cell adhesion, signal transduction, gene expression and other fundamental biological processes (reviewed in [341]). Integrins are also a component of hemidesmosomes, which bind the extracellular matrix (ECM) to cytoskeletal structures (reviewed in detail in 6.1.3 skin anatomy).

Integrins consist of at least 24 αβ heterodimers (18 α, and 8 β subunits) [385]. Integrins have dynamic expression patterns during wound healing. Changes in integrin expression are found to be involved in keratinocyte migration. For example, α3β1 integrin promotes lamellipodia (the cytoskeletal actin protein projection on the leading edge of the motile cells) formation in keratinocytes [387].

Re-arrangement of actin fibres as well as the dynamic integrin profile in keratinocytes is believed to be the cytoskeletal mechanism of keratinocyte migration. Polymerization of actin fibres in the excrescence and formation of new adhesions to the ECM in the wound bed is mediated by integrins, which lead to lamellipodial crawling keratinocyte migration along the preformed fibrin blood clot in the higher layers of granulation tissue (reviewed in [346, 385]).

**Basement membrane synthesis**

During re-epithelialisation, the basal lamina components such as collagen IV, laminin and heparin sulfate are synthesised by both fibroblasts and keratinocytes. The basement membrane appears in the wound around day 7 to 9 [54]; however, maturation of anchoring fibrils may take up to 3 years (reviewed in [1]).
Matrix synthesis

The extra cellular matrix within the granulation tissue, not only provides a scaffold for the healing dermis, but also regulates cell functions such as cell adhesion, migration and proliferation (reviewed in [341]). The ECM interacts with cell membrane proteins such as integrin. It also binds to growth factors such as TGF-β, to act as a reservoir for growth factors (reviewed in [35]).

The components of the extracellular matrix change continuously as healing progresses. In the earlier stage of wound healing, a blood clot consisting of fibrin and fibronectin provides a substrate for migration of inflammatory cells and fibroblasts within the granulation tissue (reviewed in [341]). During the proliferation phase of healing, the appearance of provisional matrix components, such as vitronectin and tenascin, facilitate cell adhesion, migration and proliferation (reviewed in [1]).

A greater number of cross-links between collagen fibres results in a more insoluble collagenous matrix (reviewed in [1]). As the healing progresses the extra cellular matrix also consists of collagen type III and IV and at a later stage of healing type I collagen bundles appear in the dermis, which increases the wound’s tensile strength (reviewed in [341]).

Hyaluronic acid is another abundant component of the provisional matrix. It creates a highly hydrated structure and provides less resistance to cell migration (reviewed in [347]). TGF-β1 is the main growth factor found to be involved in up-regulation of hyaluronic acid in granulation tissue [388]. Interestingly, the hyaluronic acid-rich environment in embryo dermis is thought to be one of the factors responsible for the “scarless” wound healing in human embryos [389]. Elastic fibres can be detected at the end of the second trimester using electron microscopy, and it is at this time that fetus skin shifts from non-scarring to scarring wound repair [43].

At later stages of wound healing, during remodelling, hyaluronic acid is degraded by hyaluronidase and replaced by sulfated proteoglycans, which provide a more rigid structure in the scar tissue [347].
Other important components involved in the healing response of the extra cellular matrix are matrix-degrading metalloproteinases (MMP). MMPs are pro-enzymes that are produced either as soluble molecule in matrix or cell membrane-anchored proteinases [390]. In addition to the critical roles of MMPs in keratinocyte migration, it is believed that they also regulate several healing processes in the dermis (reviewed in [385, 391]).

Most MMPs are secreted as pro-enzymes and are subsequently activated in the extracellular space either by other MMPs or by an oxidation reaction through reactive oxygen species such as nitric oxide [390], produced by leukocytes (reviewed in [392]).

So far, 23 human MMPs have been identified. MMPs degrade all the components of the extra cellular matrix in dermis and basement membrane and are involved in tissue remodelling. MMPs also release bioactive fragments from the ECM and basement membrane macromolecules. They also regulate leukocyte influx and angiogenesis at the appropriate time during wound healing (reviewed in [393, 394]). For example, MMP-2 and MMP-9 expose a cryptic epitope within collagen IV that promotes angiogenesis (reviewed in [393]). In addition, MMPs enzymes free the growth factors and cytokines stored within the ECM (reviewed in [394]). Examples are vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGFβ), which are mobilized and activated by MMPs (reviewed in [347, 393]).

**Fibroblast, myofibroblast and mesenchymal stem cells**

Fibroblasts proliferate and migrate during wound healing. In a manner similar to migrating keratinocytes, dynamic expression of integrin and fibronectin receptors and down-regulation of receptors for collagen are also seen in fibroblasts surrounding the injury site [395, 396]. In addition, several paracrine and autocrine factors are found to be involved in fibroblast migration and proliferation including: TGF-β family, IGF-1, PDGF, CTGF and hypoxia-induced heat shock protein 90 (reviewed in [1]).

Fibroblasts within the granulation tissue differentiate into myofibroblasts. Myofibroblasts are morphologically and functionally intermediate between fibroblasts and smooth muscle cells [1] and express a high level of intra cellular α-smooth muscle actin (α-SMA) [316].
They are believed to participate in contraction of wounds (reviewed in [397]). Myofibroblasts do not express smooth muscle markers such as smooth muscle myosin heavy chain, h-caldesmon, smoothelin (reviewed in [397]). Desmin has also been identified as a negative marker of myofibroblasts; however, there are a few studies that have reported desmin in myofibroblasts (reviewed in [397]).

Pericytes express similar proteins to myofibroblasts. Therefore, it is difficult to distinguish the myofibroblasts from the pericytes based on the expression of specific proteins. The pericytes are mainly characterised by their specific anatomical location around the blood vessels (reviewed in [397]).

Myofibroblasts were first described in healing skin wounds by Gabbiani et al. (1971) and are characterised by their prominent microfilament within the cytoplasm, which differentiate them from normal fibroblasts [398]. In a later study, using immunohistochemistry, fully differentiated myofibroblasts were characterised by high levels of expression of intra cellular α-smooth muscle actin (α-SMA) [316]. Myofibroblasts are further characterised by expression of cell adhesion protein, osteoblast (OB) cadherin [399] and the presence of a splice variant form of fibronectin (ED-A fibronectin) in the microenvironment which is not seen in α-smooth muscle actin negative fibroblasts [400].

Both mechanical and biological factors are found to be involved in the differentiation of myofibroblasts (reviewed in [341, 401]). Examples of biological factors are the growth factors such as TGF-β1 and IL-6, and the ECM proteins such as the fibronectin splice variant ED-A FN (reviewed in [341, 401]). As the healing process progresses, myofibroblasts undergo either apoptosis or reverse differentiation, and abnormal persistence of myofibroblasts results in hypertrophic scar and keloid scar (reviewed in [341]).

**Origin of myofibroblasts and mesenchymal stem cells**

There have been several hypotheses about the origin of myofibroblasts in wound granulation tissue [397]. Suggested sources for wound myofibroblasts are local dermal and
hair follicle fibroblasts, perivascular fibroblasts or pericytes, and circulatory fibrocytes that have originated from bone marrow.

**Local fibroblasts and hair follicle dermal cells**

Local fibroblasts next to the wound have been considered the main origin of wound myofibroblasts (reviewed in [397, 402]). However, as discussed in 6.1.3, heterogeneous populations of fibroblasts have been characterised within the normal dermis. The fibroblasts are heterogeneous in their embryonic origin, and in function [397]. There are at least three populations of fibroblasts recognised in the dermis, the superficial or papillary fibroblasts, reticular fibroblasts in the deep dermis and hair follicle fibroblasts, including the dermal sheath and dermal papilla [397].

As discussed in 1.5.6, it has been hypothesised that the dermal component of hair follicle is the origin of myofibroblasts. Although it has been shown that transplanted hair follicle dermal cells differentiate to wound myofibroblasts (discussed in 1.5.6), this hypothesis was not confirmed in lineage tracing experiments by Driskell et al. [50].

Using comprehensive lineage analysis in rodents Driskell et al. (2013) characterised two distinct cell populations in the upper dermis (including hair follicle dermal cells) and lower dermis. From E18.5 B-lymphocyte-induced maturation protein 1 (Blimp1) was selectively expressed in the upper dermal fibroblast and CD31+ endothelial cells, while from E18.5 the Delta-like homologue 1 (Dlk1) was expressed in the lower dermis [50].

Driskell et al. labelled two different populations of upper and lower skin fibroblasts using Cre-mediated recombination (Dlk1-CreER 3LSL-tdTomato mice and Blimp1-Cre 3 LSL-GFP mice respectively). They showed that it was mainly the lower dermal fibroblasts, which invaded the granulation tissue. The upper dermal fibroblasts (including hair follicle dermal cells) were recruited only at a later stage (day 17 in 8 mm excisional wounds in mice) and only appeared in the upper dermis, beneath the fully re-epithelialised epidermis [50].
**Pericytes**

Another suggested source for myofibroblasts is the perivascular smooth muscle cells or pericytes (reviewed in [397]). In addition to mesenchymal stem cell characteristics (reviewed in [403]), pericytes have certain markers in common with dermal fibroblasts and myofibroblasts, which raised the idea that during the healing process pericytes may lose their phenotypic features such as desmin, differentiate to myofibroblasts, and participate in wound granulation tissue ([404, 405] and reviewed in [397]). In skeletal muscles, pericytes can differentiate into muscle fibres; however, currently there is no *in vivo* evidence that myofibroblasts originate from pericytes (reviewed in [403]).

**Bone marrow mesenchymal stem cells and circulating fibroblast**

Haematopoietic and mesenchymal cells are the two main cellular lineages in bone marrow. Haematopoietic cells are generated from haematopoietic stem cells (HSCs), and mesenchymal cells are thought to be derived from mesenchymal stem cells (MSCs) in the bone marrow; however, the definitive nature of MSCs is still under investigation [406, 407].

Blood-borne fibroblast-like cells (fibrocytes) in the circulation or bone marrow mesenchymal stem cells are another suggested source for wound myofibroblasts. It has been hypothesised that circulating fibrocytes, like inflammatory cells, enter the injured skin and contribute to granulation tissue (reviewed in [397]).

Bucala *et al.* (1994) described circulating fibrocytes as a subpopulation of bone marrow mononuclear cells, which adhere to the plastic flask when cultured and show fibroblast morphology [408]. Similar to tissue fibroblasts, these cultured cells expressed CD34, Collagen I⁺ and Vimentin⁺. Bucala *et al.* also identified similar cell populations, in wound exudate in mouse experiments, and concluded that these cells originated from circulating fibroblasts or fibrocytes [408]. However, collected exudate fluid from wound chambers in sex-mismatched bone marrow chimeric mice did not confirm that the CD34 collagen I positive cells in wound originated from bone marrow [408]. They argued that the
circulatory fibroblasts arose from radio-resistant bone marrow progenitor cells or other tissue sources [408].

The idea of ‘bone marrow-derived circulatory cells’ contributing to wound myofibroblasts was supported by several experiments using intravenous injection of labelled bone marrow mesenchymal stem cells (BM MSC), which were found in the wound granulation tissue [409-411]. There is also some evidence that bone marrow derived cells differentiate into endothelial cells and pericytes [411, 412] (reviewed in [369]), and even trans-differentiate into keratinocytes [413] and participate in wound re-epithelialisation [411, 412, 414] (reviewed in [415]). However, the results of some bone marrow transplant and lineage tracing experiments in mice did not support this hypothesis [50, 402, 416, 417] (reviewed in [403]).

Using a comprehensive lineage analysis and transplantation assay in rodents, Driskell et al. (2013) ruled out the participation of bone marrow derived cells in dermal fibroblasts [50]. They first showed that platelet-derived growth factor receptor-α (Pdgfr-α) is a pan-fibroblast marker and expressed in both the upper and lower dermis at all stages of development [50]. Using Bone marrow (BM) reconstitution experiments in sublethally irradiated wild-type mice reconstituted with bone marrow of mice expressing GFP protein in all cells (eGFP under the control of β-actin CMV promoter (ACP–eGFP)), GFP+ cells appeared in wound dermis. However, in irradiated wild-type mice reconstituted with bone marrow coming from Pdgfr-α-H2B–eGFP mice, there were no GFP+ cells in the wound dermis [50]. These results suggested that although bone marrow derived cells, such as immune cells, participated in wound granulation tissue, bone marrow was not the origin of fibroblast or myofibroblasts in newly formed dermis [50]. These controversial results may be due to the degree and nature of the wound (excisional wounds vs. skin graft [413]) or variation in the irradiation protocols used in bone marrow transplant experiments and the resistance of bone marrow cells to radiation [408].

In 2010, Wagner et al. showed that allogeneic bone marrow transplantation in humans can partially improve skin blistering in patients with Recessive Dystrophic Epidermolysis Bullosa (RDEB) [418]. In RDEB a mutation in collagen VII, which is the main collagen in
the basement membrane [12], leads to fragility at the dermal-epidermal junction and thence to blistering disease. A recent study also showed that circulating PDGFRα+ cells restore type VII collagen in RDEB mouse skin graft [419]. Participation of transplanted bone marrow in restoring basement membrane proteins supports the theory of participation of bone marrow derived circulatory fibroblasts and mesenchymal stem cells in wound granulation tissue.

This result and the presence of transplanted donor cells in skin were also confirmed in animal experimental studies. In an animal experimental model of RDEB, bone marrow derived stem cells differentiated to epithelial cells by molecules (High mobility group box 1 HMGB1) released by grafted skin [413]. Other chemokines and their receptors also found to be involved in recruiting bone marrow mesenchymal stem cells to injured tissues are the C-X-C type chemokine ligand 12 (CXCL12), known as stromal cell-derived factor 1α (SDF-1α), and its receptor, CXCR4 (reviewed in [419]).

**Remodelling**

After re-epithelialisation and vascularization of the wound and when the scar tissue is formed, the wound enters the remodelling phase [341]. Wound maturation and remodelling is associated with degrading the extra cellular matrix proteins such as fibrin, fibronectin, laminin and collagen III with Matrix Metalloproteinases (MMPs) and synthesis of collagen I and elastin fibres [366]. The balance between MMPs and their inhibitors in the granulation tissue plays a critical role in wound remodelling [391].

In normal adults, type I collagen accounts for 80% and type III for 10% of total collagen in dermis, while in early stages of healing, around day 2-7 after wounding, type III Collagen is the predominant collagen in granulation tissue. Over a period of one year collagen III is gradually replaced with collagen I bundles with more cross links which increase the wound’s tensile strength [420] (reviewed in [421]). The tensile strength of scar tissue gradually increases to 40% of strength before the injury at 1 month and by 1 year, reaches up to 70% of its pre-injury strength [420] (reviewed in [421]).
Remodelling is also associated with apoptosis of myofibroblasts [422], regression of vascular structure and normalization of vascular permeability. In addition, apoptosis and efflux of inflammatory cells from scar tissue lead to resolution of the inflammatory response [341].

1.6.2 Chronic wound

A chronic wound is defined as a wound which fails to progress through a normal orderly and timely process of healing [423]. The healing process is slow in chronic wounds and the sequence of overlapping stages in normal wound healing is lost [347]. The recurrence of chronic wounds in patients suggests an intrinsic healing impairment [423].

Despite several treatment options being available, the treatment of chronic wounds is still far from satisfactory and curing them remains a challenge in a clinical setting, which leads to a considerable socioeconomic burden on patients and the community [6].

In recent years, there has been a focus on stem cell therapy and tissue engineering as an attractive alternative treatment for chronic wounds, with the hope that not only may the physical defect be repaired, but cytokines and growth factors may be provided, so that the wound may be replaced with tissue possessing a normal injury response.

Chronic wounds are usually associated with systemic diseases and classified based on their underlying cause [424]. Vascular insufficiency, diabetes mellitus, and local-pressure are the major causes of non-healing wounds. Advanced age, chronic mechanical stress, compromised nutritional or immunological status are also associated with chronic wounds [424].

The exact biological profile and mechanism of a chronic wound is not clear. Several molecular and cellular abnormal findings have been discovered in patients with chronic wounds; however, the major limitation in wound research is the unavailability of animal models [347].

There is increasing evidence showing the abnormal behaviour of cells in a non-healing wound microenvironment. Acute wound fluid stimulates fibroblast and endothelial cell
growth [425] (reviewed in [1]), while it has been shown that chronic wound fluid decreases proliferation and migration of fibroblasts, endothelial cells and keratinocytes [426-428]. Several molecules, including ROS and the mitogen-activated protein kinase pathway, were found to be involved in this process [429, 430].

In addition to the effect of the wound microenvironment, cells from chronic wounds behave differently to cells from acute wounds when cultured in similar environments and show altered response to cytokines and growth factors. *In vitro* experiments have shown that chronic wound fibroblasts do not proliferate as much as normal wound fibroblasts [431]. Also, chronic wound fibroblasts are less responsive to cytokines and growth factors such as transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF), than acute wound fibroblasts [432-434]. In addition, a decrease in expression of growth factor receptors is found in fibroblasts from chronic wounds [432, 433].

**Physiopathology of chronic wounds**

**Persistent inflammation**

During acute wound healing, the inflammatory response ceases as healing progresses. However, a chronic persistent inflammation is one of the characteristics of most chronic wounds [435] (reviewed in [366]). Despite persistent inflammation, some functions of immune cells, such as bactericidal and phagocytic activities are impaired [436]. It is believed that a large density of immune cells in chronic wounds is likely to inhibit many repair processes [366]. The microbial flora might have an important role in non-healing wounds, either by producing the inflammatory response or directly by their actions on keratinocytes or wound fibroblasts [366]. However, it is difficult to differentiate whether exposure to microbes in an open wound is the cause of the chronic inflammation, or vice versa, or both [366].
Old age and Senescence of cells

There is a strong association between the chronic wounds and old age [6]. The risk factors for chronic wounds such as vascular diseases and diabetes are more common in old age. This would explain why non-healing wounds are more common in the elderly.

Old age may also have a direct effect on healing. Telomere shortening is the main known mechanism of aging: it is related to instability of chromosomes, cancer and wound healing (reviewed in [437]). Telomerase is an enzyme that prevents telomere shortening; telomerase deficient mice have short telomeres [437]. Rudolph et al. [437] showed delay in re-epithelialization in excisional 3 mm punch biopsy wounds in aged telomerase-deficient mice compared to controls. In this experiment, there was no difference in BrdU incorporation, suggesting a delay in re-epithelialization may not be directly associated with the proliferative index [437].

Impaired mobilization of stem cells is one of the mechanisms suggested for impaired wound healing in older adults [438]. Flores et al. [439] showed that in telomerase-deficient mice there is less reduction in label-retaining cells following treatment with 12-Otetradecanoylphorbol 13-acetate (TPA), a potent tumour promoter [28] that activates LRCs to give numerous progeny. Flores et al. concluded that stem cell mobilization is impaired in telomerase-deficient mice. This result was also supported by other studies in which enhancing telomerase activity increased the rate of wound closure and mobilization of epithelial cells in rabbit and mouse experiments [440, 441].

An impaired healing response has also been observed in mice with increased activation of transcription factor P 53, which express an early aging phenotype or telomere shortening [442]. It has been suggested that healing impairment in old age may be related to poor stem cell pools in skin [443-446].

Venous ulcers

Most chronic wounds occur in the lower limbs and venous insufficiency is the most common leg ulcer [424]. Vein insufficiency results from incompetence of the valves in the
lower limb venous system, or from hypertension associated with blockage or damage of
the veins. The exact mechanism by which venous hypertension leads to ulceration and the
intense fibrosis seen around venous ulcers (lipodermatosclerotic) is not clear [424, 447]
and several hypotheses have been proposed (reviewed in [347]). Normal oxygenation in
limbs with venous ulcers rules out hypoxia as a mechanism in venous leg ulcers (reviewed
in [448]).

Presence of macromolecules in wound exudate might be related to healing impairment in
venous ulcers. It is hypothesised that venous hypertension leads to leakage of large
molecules, especially fibrinogen, into interstitial fluid and deposition of fibrin around the
capillary (reviewed in [347] and [447]). There is evidence that growth factors bind to the
macromolecules leaking into the dermis in chronic venous ulcers [449, 450]. It has been
hypothesised that these large molecules can trap growth factors and make them unavailable
for healing process [450-452] (reviewed in [347]).

Another hypothesis focuses on Matrix metalloproteinases (MMPs). Excessive amounts of
metalloproteinases accumulate in venous ulcers and other types of chronic wound which
may be able to break down the extracellular matrix, cytokines and growth factors and
interfere with healing [347, 453, 454].

**Hypoxia**

During the initial stage of wound healing, wound sites are often hypoxic. This is due to
disruption of vasculature surrounding the wound, and the high metabolic demands of cells
at a wound site [455]. Hypoxia contributes to initiation of the healing process. A hypoxic
environment promotes accumulation of inflammatory cells and re-epithelialisation [456],
and enhances activity and proliferation of dermal fibroblasts (reviewed in [455]).

However, the wound microenvironment ultimately requires normoxic condition for
successful healing. Hypoxia in chronic wounds damages fibroblast production of ECM,
collagen synthesis, differentiation of fibroblasts into myofibroblasts and ultimately wound
closure (reviewed in [455]).
**Arterial ulcers and wound vascularity**

Peripheral arterial disease (PAD) is a common manifestation of atherosclerosis. Smoking, diabetes mellitus, hypertension, dyslipidemia and hyperhomocysteinemia (a medical condition characterised by an abnormally high level of homocysteine) are the risk factors for atherosclerosis. [457]. Peripheral arterial disease is diagnosed in 25% of patients with chronic leg ulcer, of which most have combined peripheral arterial disease and venous insufficiency [458]. Atherosclerotic disease results in reduced arterial blood supply, tissue hypoxia and tissue damage. This is considered the pathophysiology of arterial ulcers [457].

In the clinic, it is assumed that the angiogenesis occurring during wound repair plays a crucial role in healing; however, this has not been extensively researched in the context of wound healing [366].

**Neuropathy and wound innervation**

Adult wounds become hyper-innervated by sensory nerves within days of wounding, and remain so throughout the healing process [459-461]. Impaired skin wound healing is often observed in patients suffering from peripheral nerve lesions following trauma, spinal cord lesions or diabetic neuropathy [462, 463]. It is believed that cutaneous innervation is important for wound healing; however, this has not been extensively explored in the context of wound healing [366, 424]).

Studies of embryonic development and regeneration in lower species; however, have shown that nerves have a very important role in limb regeneration in species such as amphibians. Limb denervation at the level of the brachial plexus inhibits limb regeneration in salamanders (reviewed in [464]). The initial signalling from ensheathing Schwann cells, such as newt anterior gradient protein (nAG), is found to induce an epithelial expression of nAG and is involved in limb regeneration in salamanders. Without this initial signal the wound stump heals, but no limb grows back (reviewed in [464]). Studies in the chick embryo also suggest a positive association between nerves and wound repair [465].
Several neuropeptides have been shown to stimulate synthesis of ECM components and proliferation of fibroblasts and keratinocytes. The concentration of substance P (SP) and the density of SP-immunoreactive nerve fibres in dermis increases during wound healing [459]. Both Calcitonin gene-related peptide (CGRP) and SP have been shown to stimulate fibroblasts and keratinocyte renewal and trigger neurogenic inflammation [466-469]. Neuropeptide vasoactive intestinal peptide (VIP) has been shown to stimulate the proliferation and migration of keratinocytes [470, 471].

In an in vitro experiment using injured human skin explant incubated with rat primary sensory neurons from dorsal root ganglia (DRG) or different neuropeptide, Cheret et al. [472] showed that sensory neurons and their derived-neuropeptides are able to promote skin wound healing. In this experiment neuromodulators increased fibroblast and keratinocyte proliferation. Furthermore, the enzymatic activities of matrix metalloproteinases (MMP-2 and MMP-9) were increased in the first days of the wound healing process [472]. Incubation with neuromediators also promotes the adhesion of human dermal fibroblasts and their differentiation into myofibroblasts [472].

**Diabetes**

Diabetes mellitus is the world’s most common metabolic disease [1]. Patients with diabetes mellitus have a 25% risk of developing chronic wounds in the feet [473, 474] and 15% of diabetic patients with foot ulcers will eventually undergo a lower extremity amputation (reviewed in [457]).

It is not clear to what extent impaired healing is due to the direct effects of insulin deficiency or its sequelae, including hyperglycaemia, hyperlipidaemia, peripheral neuropathy, or obesity. Diabetic neuropathy is considered to be the major cause of foot ulcers in diabetic patients [457, 475]. Patients with diabetes have reduced nerve numbers in their skin compared to control subjects [476-478]. Diabetic foot ulcers often result from unnoticed injury on the plantar surface of the foot (particularly over bony prominences), and the senses of light touch, vibration, and position are usually lost in the affected foot due to neuropathy (reviewed in [1]). It may also be associated with paraesthesia,
anaesthesia and pain [1]. Autonomic neuropathy also plays a role; it can lead to abnormal vascular auto regulation and decreased cutaneous perfusion and oxygen saturation [479], as well as loss of sweating, which makes the skin dry and susceptible to infection [475] (reviewed in [1]).

Peripheral vascular disease and microvasculature disease are also considered important etiologic factors in diabetic ulcer [1, 457]. Diabetic ulcers are also prone to infection (reviewed in [1]) and impaired neutrophil activities have been reported in diabetic patients [480].

Over 100 known physiologic factors are found to contribute to wound healing deficiencies in diabetic patients [481] including abnormalities in matrix metalloproteinase and their inhibitors [482], angiogenic response [374, 483], macrophage function, keratinocyte, fibroblast and endothelial cell migration and proliferation [484]. Alteration in growth factors and cytokines in the wound microenvironment [484], as well as changes in cellular response to cytokine stimulations [485] have also been identified in the pathophysiology of impaired wound healing in diabetic patients.

**microRNAs**

microRNAs (miRNAs) are small non-coding RNA molecules that can be found within the intron of coding or non-coding genes [486]. They are formed of only 19-24 nucleotides and have a function different to that of messenger RNAs. Messenger RNAs transmit the information between DNA and proteins, while miRNAs bind to the three untranslated regions of the target messenger RNA and regulate the gene expression [486]. Several miRNAs have been found to regulate skin development, homeostasis and other diseases [486, 487].

The expression levels of specific miRNAs have been found to be altered during wound healing and regulate the healing process. An example is miR-201, a miRNA that influences keratinocyte proliferation [488]. Another example is miR31, which is up-regulated in wound-edge keratinocytes [489]. Over-expression of miR-31 is found to promote cell proliferation and migration, while inhibition of miR-31 had the reverse effect [489]. Thus,
it has been hypothesized that chronic wounds may occur as the result of misregulation of miRNAs (reviewed in [490, 491]).

1.6.3 Chronic wound treatment

Chronic wounds are the complex result of abnormal healing responses in the skin as well as ischemia, infection and pressure. Healing is highly dependent on targeting these factors.

Standard wound treatments include antiseptics and antibiotics to treat and control wound infection, debridement of necrotic tissue, and wound dressing. Specific treatments depend on the pathophysiology of chronic wounds. Treatment of underlying systemic disease such as diabetes or heart failure improves healing. For example, arterial ulcers can benefit from restoration of peripheral arterial blood flow, either by surgical reconstruction (e.g. femoral popliteal bypass) or by less invasive endovascular interventions (e.g. femoral angioplasty) (reviewed in [458]). Clinical trials have shown that venous ulcers benefit from compression bandages (reviewed in [458]). In the following section, the classical treatment of wounds will be discussed, and thereafter, the application of stem cell therapy and tissue engineering in the field will be reviewed.

Dressing

The main function of dressing is to protect against trauma, bacterial infection, and to provide an optimal environment for healing, which may vary according to the precise stage of the process.

Dressing should absorb excessive fluids while maintaining a moist environment. The studies of Winter and Scales in 1963 showed that air-dried wounds developed thicker scabs and re-epithelialized at a slower rate, resulting in deeper scars [492]. Maintaining a moist environment helps healing and results in better cosmetic outcomes [341]. Live epithelial cells survive and are able to migrate to cover the wound in a moist environment (reviewed in [341]). In addition, many endogenous factors are involved in healing such as growth factors and cytokines found in fluid (reviewed in [341]). It has also been suggested that a moist wound environment is able to maintain the electrical gradient between the wound...
and normal skin, which is believed to promote epidermal cell migration from surrounding skin [493] (reviewed in [341]).

**Topical negative pressure therapy**

Topical negative pressure therapy stimulates wound healing by applying sub-atmospheric pressure (100–125 mm Hg) to the wound surface, and is used mainly for venous diabetic ulcers (reviewed in [457]). It applies negative pressure using an open pore foam conduit which is cut to the wound size and sealed with a semi-occlusive drape and attached to a vacuum source (reviewed in [457]). It has been shown that topical negative pressure therapy alters molecules involved in chronic wounds such as MMP’s, increases perfusion, removes the exudate, reduces the oedema and enhances the formation of granulation tissue (reviewed in [494]).

**Growth factors**

As mentioned above, growth factors play a crucial role in wound healing (1.6.1), and applying growth factors to wounds has therefore been one of the targeted approaches to treating chronic wounds. Experimental studies have shown that the topical application of certain growth factors such as PDGF-BB, endothelial growth factor (EGF), FGF-2, and granulocyte-macrophage colony-stimulating factor (GM-CSF) accelerate wound repair (reviewed in [348] (reviewed in [495]).

Topical PDGF-BB (Becaplermin) is the only FDA-approved growth factor for treatment of diabetic ulcers and has been shown to improve healing in clinical trials and to increase the incidence of wound closure up to 48% compared to 33% for control subjects [496]. Topical FGF-2 is also approved for use in China and Japan (reviewed in [495]).

The use of cytokines and growth factors in humans is limited by loss of drug activity in the protease rich environment of chronic wounds [495], and they are effective only when combined with optimal wound bed preparation and elimination of other factors that impair healing [347].
**Skin graft**

In non-healing wounds or extensive skin defects such as large burns, skin graft improves healing. Skin graft procedures can be divided into two classes: split-thickness skin graft (STSG) which includes the epidermis and only part of the dermis, and full thickness skin graft (FTSG), consisting of the epidermis and full thickness dermis (reviewed in [495]).

STSG can be meshed and expanded over a wide area of the recipient site, and has better survival rate in wounds with less vascularity compared to FTSGs. However, it has the disadvantage of forming skin contracture in the recipient site during healing. Autologous Split-thickness skin graft is a routine treatment for non-healing wound and burn patients. However sometimes, for example, in an extensive burn, there is inadequate non-burned autologous donor tissue (reviewed in [495]).

Xenograft and allograft dermal substitutes can be used to treat large areas of a skin defect in extensive burns and are considered a temporary wound cover and biological dressing which stimulates the healing process by providing growth factors, cytokines and extracellular matrix [497]. However, Xenograft and allograft dermal substitute ultimately undergo immune rejection in 3 to 4 weeks [498]. DNA analysis of wounds after the application of non-autologous skin substitutes shows complete disappearance of grafted cells after 2 months [499].

Overall, skin auto grafts are not always available in sufficient quantity and skin allograft has the disadvantage of rejection by the host immune system. The limitations of living skin grafts have led to development of bioengineered skin substitutes and cell therapy (reviewed in [495]).

**Stem cell therapy and tissue engineering**

The emergence of cell therapy and tissue engineering leads to the development of novel strategies to replace the damaged tissue and provide the signals, cytokines and growth factors needed for tissue regeneration and ultimately restoring function. Although technological advances have been made towards developing bio-compatible materials, the source of multi-potential stem cells remains a challenge for future research. The candidate
sources of stem cells would be embryonic stem cells, adult stem cells and induced pluripotent stem (IPS) cells, which are adult cells reprogrammed to a multi-potent state.

Adult stem cells have the advantage over embryonic stem cells of being autologous and unlikely to be rejected by the patient, and as opposed to embryonic stem cells, no moral concerns are raised at their use. In addition, in contrast to embryonic stem cells and IPS cells, adult stem cells are not tumourigenic.

For these reasons adult stem cells are a suitable source of stem cells in regenerative medicine. In fact, several adult stem cells have been tried in a number of phase I/II and III clinical trials and used successfully in a clinical setting to treat various diseases including chronic wounds as well as renal failure, liver failure, heart failure and myocardial infarction (reviewed in [500]). Hair follicles, too, have been considered as a source of epithelial and mesenchymal adult stem cells in regenerative medicine [273]. Application of hair follicle cells in regenerative medicine and skin repair will be discussed in 1.7 and 1.7.4.

Both epithelial and mesenchymal stem cells have been tried in tissue engineering and cell therapy to treat chronic wounds.

**Bioengineered skin equivalents**

Advances in tissue culture technique allowing the production of large quantities of keratinocytes and fibroblasts, have led to generation of the bio-engineered skin substitute. Bioengineered skin is only helpful in ulcers that are of long duration and have not responded to conventional therapy (reviewed in [347]). These bioengineered skins can be classified into epidermal substitute, dermal substitute and a combination of dermis and epidermis.

The autologous epidermal substitutes are used in patients with partial and full thickness burns. In this technique, to expand the keratinocytes, a skin biopsy is first obtained from the patient and then keratinocytes are cultured *in vitro* with irradiated murine fibroblasts that serve as a feeder layer. Cultured keratinocytes form a cell sheath with a thickness of 2-
8 cells, which can be transplanted over the skin defect [497]. Cultured keratinocytes also can be sprayed as individual cells onto the wound surface [501]. The primary disadvantages of autologous epidermal substitutes are their short shelf-life and their expense. Epidermal grafts can also only be used in clean wounds, and it may take three weeks to culture and expand keratinocytes [497]. In addition, cultured keratinocytes by themself are not suitable for deep wounds and need to be combined with a dermal substitute in full thickness wounds [497].

Allograft of dermal or epidermal cells has been approved for use in clinical trials, examples are neonatal foreskin fibroblasts incorporated in an absorbable suture material [502], or bilayer engineered skin formed by fibroblasts and keratinocytes from neonatal foreskin [503]. In these allografts there is no prolonged engraftments of cells [504], as they are eventually rejected by the host immune system and in clinical trials they have been applied repeatedly to the wound to stimulate healing (reviewed in [347]).

Acellular dermis (allogeneic or xenogeneic) or synthetic dermal substitutes are easier to manufacture than cell-containing substitutes. In addition, bioengineered skin equivalents are able to incorporate into wounds without rejection. However, survival of skin graft is completely dependent on revascularization. The maximum thickness of skin substitute that can easily become vascularised is only 0.4 mm [505] and failure of vascularization leads to loss of the graft [506]. Although freeze-dried human donor dermis (ALLODERM) [507] has been used successfully in other reconstructive surgeries, dermal substitutes (with or without incorporation of keratinocytes or fibroblasts) do not re-vascularize well in skin, and their engraftment has not been successful as a dermal replacement [495].

**Mesenchymal stem cell therapy**

As mentioned above, autologous epithelial cells and stem cells have been used in chronic wounds to replace the epidermis. In addition to epithelial stem cells, mesenchymal stem cells including bone marrow and adipose-derived stem cells have been tried in experimental (Table 1-8) and clinical trials (Table 1-9), and shown to improve healing (reviewed in [508, 509]).
Bone marrow and adipose tissue has been considered the main autologous source of mesenchymal stem cells for wound healing. These cells can proliferate in vitro and cultured cells have shown chemotaxis toward a variety of wound healing cytokines in vitro [523, 524] and in vivo animal studies [511, 525].

It is believed that mesenchymal stem cells improve healing mainly through paracrine factors (reviewed in [508]). Early studies showed that transplanted MSC and ASCs reduce inflammation [511], recruit endothelial cells [510], promote angiogenesis [512, 516], and produce new connective tissue and reduce fibrosis [409, 526]. However, limited engraftment and survival at the wound site are still major concerns and the clinical translation of these cells are still in the early stages (reviewed in [508]).

Other candidates for wound cell therapy are IPS cells and embryonic stem cells. As discussed above, in addition to the ethical issues surrounding the use of embryonic stem
cells, it is currently still difficult to induce differentiation to suitable cells while avoiding transplant rejection, and concerns about the tumourigenicity of IPS cells restrict their use in clinical studies [527].

**Table 1-9  Registered clinical trials of mesenchymal stem cell treatment in skin wounds [508]**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Design</th>
<th>Wound type</th>
<th>Time frame</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allogeneic BM-MSCs</td>
<td>Injection: test of four different doses</td>
<td>Second-degree burns (&lt;20% TBSA)</td>
<td>No less than 52 weeks</td>
<td>Not yet recruiting</td>
<td>NCT02104713a</td>
</tr>
<tr>
<td>Autologous BM-MSCs</td>
<td>Forty injections of CD34+ cells</td>
<td>Chronic critical limb ischemia, diabetic foot</td>
<td>≈17 weeks</td>
<td>79% limb salvage</td>
<td>NCT01232673a, [528]</td>
</tr>
<tr>
<td>Autologous BM-MSCs</td>
<td>BM-MSCs in fibrin spray</td>
<td>Non-healing</td>
<td>24 weeks</td>
<td>Ongoing</td>
<td>NCT01751282a</td>
</tr>
<tr>
<td>hASCs</td>
<td>Multiple injections within and periphery of the wound</td>
<td>Diabetic foot venous/pressure ulcer</td>
<td>≈10 weeks</td>
<td>Recruiting</td>
<td>NCT02092870aASCs</td>
</tr>
<tr>
<td>hASCs</td>
<td>Injection (3–8×10^6 cells) + PRP matrix</td>
<td>Burns</td>
<td>Not defined</td>
<td>Ongoing</td>
<td>2012-001596-36b</td>
</tr>
<tr>
<td>hASCs</td>
<td>Injection and spraying (4.5×10^6 cells)</td>
<td>Burns</td>
<td>Not defined</td>
<td>Ongoing</td>
<td>2009-016365-29c</td>
</tr>
<tr>
<td>SVF</td>
<td>Injection</td>
<td>Venous ulcers</td>
<td>Not defined</td>
<td>Not yet recruiting</td>
<td>d</td>
</tr>
<tr>
<td>Autologous lipoaspiration</td>
<td>Injection</td>
<td>Diabetic/venous stasis wounds</td>
<td>52 weeks</td>
<td>Unknown</td>
<td>NCT00815217a</td>
</tr>
</tbody>
</table>

*a: Identifier on US clinical trials; b: Identifier on EU clinical trials; c: Identifier on EU clinical trials; d: Australian Clinical trials. BM-MSCs, bone marrow mesenchymal stem cells; TBSA, total body surface area; hASCs, human adipose stem cells; PRP, platelet-rich plasma; SVF, stromal vascular fraction.*

The hair follicle has been considered an accessible source of stem cells in cell therapy and tissue engineering, particularly for skin regeneration, which is in any case the biological function of hair follicle cells. Isolated cells from hair follicle have been used for skin cell therapy and tissue engineering, which is discussed in detail in 1.7.4.
1.6.4 Rodent wound healing

Wound healing has been studied in many species of animals. Although swine and primates have similar wound healing mechanisms to humans, their use is limited in experimental studies because they are expensive and not accessible.

Rodents are an attractive candidate for wound healing studies because of low cost and ease of handling. In addition, genetic manipulation of rodents provides an extremely helpful tool to study new gene pathways and the molecules involved in wound healing. However, human and rodents show major differences in their skin structure and wound healing mechanisms. The differences between humans and mice in these respects are summarized in Table 1-10 (reviewed in [529]).

| Major differences between human and mice skin. (table modified from [529]) |
|-----------------|-----------------|-----------------|
| **Hair cycle**  | **Mouse**       | **Human**       |
|                 | Approximately 3 weeks | Highly variable, region-dependent |
| **Epithelial architecture** | **No rete ridges** | **Rete ridges present** |
| **Apocrine sweat glands** | **Not present in skin, extensive in mammary glands** | **Present in axilla, inguinal, and perianal skin regions** |
| **Biomechanical properties** | **Thin, compliant, loose** | **Thick, relatively stiff, adherent to underlying tissues** |
| **Hypodermal thickness** | **Hair cycle-dependent** | **Less variable** |
| **Subcutaneous muscle layer** | **Present throughout as panniculus carnosus** | **Present only in neck region as platysma** |
| **Major method of wound healing** | **Contraction** | **Granulation tissue formation and re-epithelialization** |

Mouse skin has a thin muscle layer under the skin (called panniculus carnosus) which produces wound contraction and plays an important role in rodent skin wound repair, whereas re-epithelialization and granulation tissue formation are the major mechanisms involved in human wound healing (reviewed in [529]). Lack of a suitable experimental model for chronic wounds is one of the important obstacles to chronic wound research [529].
Nude mice are a long-standing host of choice for transplantation studies and are commonly used in wound healing experiments. The lack of immune response in nude mice allows allograft and xenograft experiments, and prevents the immune reaction to GFP protein [530] in grafted hair follicle, which could potentially alter the healing experiment.

The immune deficiency and abnormal skin and hair follicle phenotype in nude mice may potentially alter wound healing experiments; however, recent studies have shown that nude mice have almost normal healing with less scar formation after wounding than normal mice [531].
1.7 Hair follicle stem cells and regenerative medicine

Primitive species such as amphibians have the capacity to regenerate certain body parts, including limbs, tail, jaw, and retina [532, 533], and this ability exists to a limited extent in the human foetus [5]. However, in adult humans, this ability is lost and healing occurs with scar formation and loss of function. Examples include heart failure after myocardial infarction or skin contracture and scar formation after burns. This limited regenerative response is further curtailed by vascular or innervation impairment [534-536] and in systemic metabolic diseases such as diabetes where tissues lose their normal repair response.

Stem cell research provides prospects for treatment and healing. These cells are not only a potential source for the structural materials of damaged organs, but they also have the possibility of replacing damaged tissue with cells that possess a normal injury response. In addition stem cells provide a suitable microenvironment for healing by secreting cytokines and growth factors (GFs), and promoting innervation and vascularity [2].

The candidate sources of stem cells are embryonic stem cells (ESCs), adult stem cells (ASCs) and induced pluripotent stem (IPS) cells, which are adult somatic cells reprogrammed to a multi-potent state. Each of these three groups of stem cells has their own potentials and limitations.

ESCs are isolated from the inner mass of the blastocyst. Collection of ESCs usually results in destruction of the entire embryo and destruction of human embryos is morally and ethically unacceptable to many people [527]. Besides embryonic stem cells are allogenic and evoke an immune response in recipient host tissue. Therapeutic cloning using somatic cell nuclear transfer (transferring an adult cell nucleus into an enucleated egg) [537] is a technique that has been used to overcome the allogeneic potential of embryonic stem cells; however, the ethical concerns remain [527]. The third major challenge relating to the use of ESCs in tissue engineering is managing the risk of uncontrolled differentiation after transplantation, including formation of teratoma and teratocarcinoma.
IPS cells can be produced with high efficiency from patients’ own somatic cells. IPS cells are a source of multi-potential autologous stem cells for regenerative medicine. IPS cells are non-immunogenic and overcome the ethical issues associated with ESCs, as harvesting of IPS cells does not involve injury to anyone other than the beneficiary. Unfortunately, like ESCs, IPS cells also carry the risk of uncontrolled differentiation after transplantation, including formation of teratoma and teratocarcinoma. ASCs are found in various tissues. ASCs express early developmental markers and are involved in homeostasis and repair of adult tissue. Initially ASCs were thought to be able to differentiate into a limited number of cell lineages within their respective germ layer. However, further characterisation of these cells now shows that adult stem cells have the potential to trans-differentiate into cell types of differing germ layers, making these stem cells promising candidates for regenerative medicine and disease management.

Adult stem cells have the advantage over embryonic stem cells of being autologous and not liable to rejection by the patient; also, there are no moral concerns raised with their use. Furthermore, in contrast to embryonic stem cells and IPS cells, adult stem cells are not tumourigenic. For these reasons, ASCs are currently the most suitable source of stem cells in regenerative medicine.

The best-characterised ASCs are the bone marrow derived stem cells. Bone marrow derived stem cells include both haematopoietic stem cells and non-haematopoietic stem cells. The non-haematopoietic stem cells are also known as ‘stromal stem cells’ or ‘mesenchymal stem cells’ (reviewed in [500]).

Bone marrow mesenchymal stem cells are able to differentiate into both mesenchymal and non-mesenchymal cell lineages. Although bone marrow is considered a rich source of mesenchymal stem cells, these pluripotent stem cells are rare and only represent between 0.01 and 0.001% of the total nucleated cells within bone marrow [538, 539]. This small number has led many researchers to search for alternative sources of adult stem cells, and this remains a challenge in regenerative medicine.
Hair follicles (HFs) have exceptional regenerative capacity in the adult human. Hair grows rapidly [540] and HFs undergo cycles of degeneration and regeneration throughout life [541]. The hair follicles are considered to be a reservoir of stem cells in skin or “the bone marrow” of skin [81].

Various populations of epithelial and mesenchymal stem cells are recognised within HFs. The epithelial stem cells are not only responsible for continuous cyclic transformation of hair follicles, but are also involved in re-epithelialisation of skin during wound healing. In addition to the stem cell population in the epithelial compartment of the hair follicle, multipotent mesenchymal stem cells also reside in the dermal compartment of the hair follicle; i.e. the dermal papilla and dermal sheath. These cells are multi-potential and are able to differentiate into fat, bone, cartilage, skeletal muscle, and smooth muscle cells [309-311]. The abundance of HFs and their accessibility makes them a potential alternative source of pluripotent stem cells for regenerative medicine.

1.7.1 Methods for isolating hair follicle and hair follicle cells

There have been recent advances in isolation and culture of dermal and epidermal hair follicle cells, which leads to further characterisation of cells and the exploration of their capacities in various areas of regenerative medicine.

Hair follicle epithelial cells can be obtained and cultured from plucked hair either by enzymatic digestion [542] or, alternatively, epithelial cells can simply grow out of explants [543, 544]. However, plucked hair only contains transit-amplifying epithelial cells with short-term culture potential, and hair follicle micro-dissection is needed in order to isolate epidermal stem cells from the bulge area or and mesenchymal stem cells from the dermal papilla or dermal sheath [545].

Micro-dissection is another technique used to isolate anatomically defined tissues from hair follicle, such as the dermal papilla or dermal sheath [112, 309, 312, 546], or bulge area [277, 547]. In this laborious technique, fine forceps (No 5) are used to handle the tissue under a dissecting microscope, and a fine needle (21 or 25 Gage) is generally used as a small knife to dissect a specific area from the hair follicle such as the dermal papilla,
dermal sheath or bulge. When culturing dermal papilla cells, further enzyme digestion (usually with collagenase or dispase) \[312\] or mechanical cutting \[548\] is needed to break basal lamina and facilitate the migration of dermal papilla cells from the explants. Isolated cells can be cultured under specialized culture conditions to propagate specific cell populations or to differentiate stem cells toward a specific lineage.

1.7.2 Hair induction and restoring hair growth

Engineering hair follicles has an important application in hair restoration in alopecia as the result of burns or common clinical diseases such as androgenic alopecia, which is a miniaturization of hair and shortening of the anagen phase due to the effect of testosterone and dihydrotestosterone on androgen receptors in the dermal papilla \[549, 550\]. Currently the main treatment for hair loss is hair follicle transplantation \[551\]. In this technique, follicles from a hair follicle rich area are dissected and transplanted into the bald skin. Although hair follicle transplantation is the gold standard for hair restoration, sufficient donor hair follicles are not always available; and for a long time engineering a functional hair follicle has therefore been the focus of the scientific efforts to treat hair loss.

During the embryonic period, signals from mesenchyme can generate hair follicle (discussed in 1.3.2). This characteristic of hair follicle dermal papilla is not restricted to the embryonic period, and various experiments in both animals and humans have shown that dermal components of adult hair follicles also are able to induce complete hair follicle formation when transplanted into dermis \[99, 100, 113, 114\]. Similar results have been observed even in transgender \[99\] and trans-species \[552\] tissue recombination assays.

Culturing dermal papilla cells may prove a successful approach to expanding cell numbers. Although cultured cells initially retain their induction capacity \[114, 553\], this function is lost after several passages \[122, 554, 555\]. Various strategies are used to preserve hair induction ability in cultured dermal papilla cells, including optimising the culture medium \[556-558\] and activation of signalling pathways involved in hair induction such as Wnt or Bmp \[122, 124\].
Silva-Vargas et al. have shown that, similar to the two-way signals passed between epidermis and mesenchyme during hair follicle morphogenesis (discussed in 1.3.2), the epidermis also induces dermal papilla formation in adult skin by Wnt pathway activation [559]. These studies suggest hair follicle dermal cells have great potential for hair follicle engineering and the treatment of hair loss.

1.7.3 Multi-potential stem cells

Multi-potential neural crest and mesenchymal stem cells have been characterised in the dermal sheath and dermal papilla. Hair follicle dermal sheath and dermal papilla cells have even been differentiated to haematopoietic lineages and contributed to all blood lineages when transplanted in lethally irradiated recipient mice [101]. Dermal sheath and dermal papilla cells are also able to differentiate into adipocytes, osteocytes, chondrocytes [309], smooth muscle [311, 560], and skeletal muscle [310] in vitro (Table 1-11).

Isolated hair follicle bulge stem cells have been differentiated to keratinocytes, also incorporated into the epidermis when transplanted in mouse skin [281, 561]. Hair follicle bulge cells can also differentiate to smooth muscle cells [319]. The bulge is also considered a niche for neural crest stem cells (discussed in 1.5.9). Nestin positive K15-stem cells originating from the bulge have been differentiated to astrocytes, oligodendrocytes, keratinocytes, smooth muscle, and melanocytes [291].

Several cell populations with neural crest stem cell characteristics have been isolated and cultured from the bulge and the dermal component of the hair follicle. Examples are SKP cells and EPI-NCSCs (discussed in 1.5.9). Hair follicle neural crest stem cells have been differentiated to neural lineages such as Schwann cells and neurons as well as adipocytes [320], chondrocytes and osteocytes [324]. Table 1-11 shows differentiation of hair follicle dermal and epidermal cells in to different lineages in vivo and in vitro experiments.

As shown in Table 1-11, the hair follicle is an accessible source of multipotent cells, which makes it an ideal source of stem cells, not only for hair and skin regeneration, which is the biological function of these cells, but also for cell therapy and tissue engineering in other organs [261].
<table>
<thead>
<tr>
<th>Stem cell population</th>
<th>Differentiated</th>
<th>Selection and characterisation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP &amp; DS</td>
<td>Adipocyte</td>
<td>Oil Red O staining</td>
<td>[309, 318]</td>
</tr>
<tr>
<td>DP &amp; DS</td>
<td>Osteocyte</td>
<td>von Kossa staining</td>
<td>[309, 318]</td>
</tr>
<tr>
<td>DP &amp; DS</td>
<td>Chondrocyte</td>
<td>Alcian Blue stain</td>
<td>[309]</td>
</tr>
<tr>
<td>Mixed Hair follicle</td>
<td>Chondrocyte</td>
<td>Alcian Blue stain</td>
<td>[562]</td>
</tr>
<tr>
<td>dermal cells (DP+DS)</td>
<td>Osteocyte</td>
<td>Von Kossa stain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adipocyte</td>
<td>Oil Red O staining</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Smooth muscle</td>
<td>α-smooth muscle, Calponin &amp;</td>
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</tr>
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<td></td>
<td></td>
<td>myosin heavy chain IHC</td>
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<tr>
<td>DP &amp; DS, micro-dissection</td>
<td>Neural</td>
<td>βIII-tubulin &amp; NF200,</td>
<td>[318]</td>
</tr>
<tr>
<td>Dermal papilla &amp;</td>
<td>Haematopoietic cells</td>
<td>Repopulation of the mouse</td>
<td>[101]</td>
</tr>
<tr>
<td>dermal sheath micro-</td>
<td></td>
<td>haematopoietic system</td>
<td></td>
</tr>
<tr>
<td>dissection</td>
<td>Dermal sheath (micro-dissection)</td>
<td>Histology study: Incorporation in</td>
<td>[321]</td>
</tr>
<tr>
<td></td>
<td>Dermal fibroblast</td>
<td>to the epidermis when</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>transplanted in wound</td>
<td></td>
</tr>
<tr>
<td>DP (micro-dissection)</td>
<td>Skeletal muscle</td>
<td>PCR analysis of MyoD &amp;</td>
<td>[310]</td>
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<td></td>
<td>myogenin, myotube formation</td>
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<tr>
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<td>Smooth muscle cell</td>
<td>α-SMA promoter activity, α-SMA,</td>
<td>[311, 560]</td>
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<tr>
<td></td>
<td></td>
<td>Calponin, Actinin, Contractility assay</td>
<td></td>
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<tr>
<td>Bulge cells</td>
<td>Smooth muscle cell</td>
<td>α-SMA, Contractility assay</td>
<td>[319]</td>
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<tr>
<td>Bulge cells</td>
<td>Keratinocyte</td>
<td>Incorporation in to the epidermis when transplanted in mouse skin</td>
<td>[281]</td>
</tr>
<tr>
<td>Bulge Nestin positive K15-</td>
<td>Neuron</td>
<td>bIII-tubulin, In vitro &amp; in vivo</td>
<td>[291]</td>
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<tr>
<td>hair follicle stem</td>
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<td>Neural differentiation (transplant under ski),</td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>Astrocyte</td>
<td>GFAP</td>
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<tr>
<td></td>
<td>Oligodendrocyte</td>
<td>CNPase</td>
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</tr>
<tr>
<td></td>
<td>Keratinocyte</td>
<td>K5 K8</td>
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<tr>
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<td>Smooth muscle</td>
<td>α-SMA</td>
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</tr>
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<td>ND-GFP containing melanin</td>
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<td>Schwann cell</td>
<td>Transplanted SKP cells myelinate</td>
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<td></td>
<td></td>
<td>CNS axons of the dysmyelinated brain of neonatal shiverer mice &amp; enhance nerve repair in peripheral nerve injury</td>
<td></td>
</tr>
<tr>
<td>SKP (DP)</td>
<td>Neuron &amp; glia</td>
<td>βIII-tubulin, p75, MAP2ab Or S100, p75NGFR, GFAP CNPase</td>
<td>[312]</td>
</tr>
<tr>
<td>Stem cell population</td>
<td>Differentiated</td>
<td>Selection and characterisation</td>
<td>Ref.</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------------</td>
<td>---------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>SKP (DP &amp; Bulge)</td>
<td>Neuron &amp; glia</td>
<td>In vitro Diff &amp; enhance repair in spinal cord injury in vivo</td>
<td>[334]</td>
</tr>
<tr>
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<td>Nestin, βIII-tubulin, neurofilament-M</td>
<td>[320]</td>
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<td>GFAP, CNPase</td>
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</tr>
<tr>
<td></td>
<td>Smooth muscle</td>
<td>a-SMA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adipocyte</td>
<td>Oil Red O</td>
<td></td>
</tr>
<tr>
<td>SKP</td>
<td>Osteocyte</td>
<td>integration into the periosteum when transplanted in to the fracture region, Alizarin Red,</td>
<td>[324]</td>
</tr>
<tr>
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<tr>
<td></td>
<td>Schwann cell</td>
<td>P75 - S100β,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pericytes</td>
<td>a-SMA</td>
<td></td>
</tr>
<tr>
<td>SKP (Dermal)</td>
<td>Neural crest precursor</td>
<td>Fate mapping &amp; Lineage analysis when Injecting in embryo</td>
<td>[106]</td>
</tr>
<tr>
<td>Hair follicle nestin expressing cells (DP &amp; Bulge)</td>
<td>Blood vessels</td>
<td>Nestin expressing blood vessel originated from transplanted hair follicle</td>
<td>[323]</td>
</tr>
<tr>
<td>Dermal papilla &amp; dermal sheath, sox2 positive cells from sox2 EGFE transgenic mice</td>
<td>Dermal fibroblast</td>
<td>Histology study: Incorporation in to the dermis when transplanted in skin</td>
<td>[322]</td>
</tr>
<tr>
<td>Dermal papilla &amp; dermal sheath, sox2 positive cells from sox2 EGFE transgenic mice</td>
<td>Adipocyte</td>
<td>Histology study: Incorporation in to the adipocyte rich hypodermis when transplanted in skin</td>
<td>[322]</td>
</tr>
<tr>
<td>EPI-NCSCs</td>
<td>Chondrocytes</td>
<td>Collagen type II</td>
<td>[335]</td>
</tr>
<tr>
<td></td>
<td>Schwann Cell</td>
<td>Glial fibrillary acidic protein (GFAP) expression</td>
<td></td>
</tr>
<tr>
<td>EPI-NCSCs</td>
<td>Neuron</td>
<td>Form neurons &amp; improves sense of touch when transplanted in spinal injury (B-III tubulin, GAD67),</td>
<td>[564]</td>
</tr>
</tbody>
</table>

### 1.7.4 Skin regeneration and wound healing

The body of evidence showing the participation of hair follicle cells in wound healing (discussed in 1.5.4 and 1.5.6), has raised the idea of using hair follicle dermal and epidermal cells to enhance wound healing and generate engineered skin. Emerging techniques for isolation and culturing of hair follicle cells has led to the generation of engineered skin-tissue equivalents from hair follicle dermal and epidermal cells.
Hair follicle keratinocytes have been cultured and expanded simply from plucked scalp hair, to produce an epithelial cell sheet or skin substitute to treat chronic leg ulcers [565-568]. Although epidermal cells obtained from plucked hair are transit-amplifying epithelial cells with limited proliferation capacity [545], they have improved healing in clinical trials (shown in Table 1-12).

Another advantage of using hair follicle epidermal cells is that, in contrast to interfollicular epidermis, the proliferation ability of outer root sheath keratinocytes is not limited by the age of the donor [565], and hair follicle keratinocytes from patients can be expanded in culture irrespective of their age, which is very important, as chronic wounds are more common in old age.

<table>
<thead>
<tr>
<th>Hair follicle derived cells used in engineered skin tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorporation of cultured keratinocytes from human plucked hair (combined with dermal equivalent, Human dermal fibroblast + collagen) forms tissue engineered skin.</td>
<td>Hoeller, et al.2001 [566]</td>
</tr>
<tr>
<td>Hair follicles transplanted into the tissue engineered skin (Integra). (Case report)</td>
<td>Navsaria, et al.2004 [569]</td>
</tr>
<tr>
<td>Cultured HF-derived keratinocytes and melanocytes used in chitosan–gelatin membrane developed a pigmented skin in nude mice.</td>
<td>Liu, et al.2011 [570]</td>
</tr>
<tr>
<td>Dermal Papilla Cells and keratinocytes from human hair follicle incorporated in tissue-engineered skin improve the wound healing Process and Generate Hair Bud-Like Structures in nude mice.</td>
<td>Leiros et al, 2014 [571]</td>
</tr>
<tr>
<td>FAC sorted Lgr6 positive cells in a hydrogel vehicle increased epithelization, hair growth and angiogenesis of wounds in nude mice.</td>
<td>Lough et al.2014 [572]</td>
</tr>
</tbody>
</table>

In addition to keratinocytes, the dermal component of the hair follicle has also been used in skin tissue engineering. Recently Leiros et al. showed that incorporation of human hair follicle dermal papilla cells into engineered mouse skin promotes wound vascularization and improves healing in nude mice [571]. Adding hair follicle derived melanocytes also formed a pigmented skin substitute [570]. Current strategies for the use of hair follicle cells in skin tissue engineering are summarised in Table 1-12.
1.7.5 Nerve regeneration and repair

In addition to the neurotrophic properties of the hair follicle (discussed in 1.4.3), various populations of neural crest stem cells have been characterised in the dermal papilla, dermal sheath and bulge area. Nestin$^+$ and Sox2$^+$ cells, which are neural crest cell markers, are expressed in these areas. Various types of cultured cells, such as epithelial neural crest stem cells (eNCSC) and SKP cultured cells, are also believed to be originated from the neural crest and have the potential to differentiate into neural crest lineages (Table 1-11). Hair follicle neural crest stem cells have been discussed in detail in 1.5.9.

eNCSC can be differentiated to Schwann cells in vitro [335]. The hair follicle nestin-expressing cells and SKP cells derived from them are able to differentiate into neuron and glia, both in vitro [291, 320], and in vivo [334]. In addition, SKP cells are able to enhance tissue repair when transplanted to the injured spinal cord [334]. In addition, SKPs produce myelin proteins and improve repair when transplanted in mice with peripheral nerve injury [563].

1.7.6 Smooth muscle differentiation and vascular tissue engineering

Hair follicle dermal cells may be potential candidates for vascular tissue engineering [311, 573]. As discussed earlier in this review (1.3.6) the hair cycle and the active growth (anagen) phase of the hair cycle is associated with an increase in vascularity [10, 200], implying a pro-angiogenic effect of the hair follicle. In addition, the hair follicle dermal component expresses $\alpha$-smooth muscle actin ($\alpha$-SMA) (discussed in 1.5.5) suggesting that these cells might be the origin of myofibroblast and pericytes in skin wound.

Blood vessels contain both endothelial cells and the smooth muscle cells or pericytes. Endothelial cells form the inner layer of vessels where their selective permeability allows the transferral of substances between the blood, interstitial space and cells. Endothelial cells are surrounded by smooth muscle cells that control the blood flow by dilatation and constriction of vessel walls in response to vasoactive agonists.
As mentioned in 1.5.5, in animal experimental models, hair follicle cells participate in both the endothelial and pericyte component of vessels. Nestin+ cells originated from transplanted hair follicle form blood vessels and express endothelial cell markers (CD31 and VWF) [323]. In another study, when cultured SKP cells were transplanted at the site of fractured bone, injected SKP cells appeared around the blood vessels, and expressed α-SMA (pericyte marker) and not CD31 (endothelial marker), which indicates differentiation to pericyte [324] and not to endothelial cells.

Liu et al. [311] have shown the presence of smooth muscle progenitor cells in the hair follicle. In their experiment, α-SMA-expressing cells were isolated from hair follicle explants culture by transfection with retrovirus encoding for enhanced green fluorescent protein under the α-SMA promoter. Using fluorescence-activated cell sorting, they showed that selected cells exhibited high proliferation capacity, expressed several SMC markers and were contractile. Liu et al. called this cell population “smooth muscle progenitor cells” [311]. By culturing with a decellularized natural biomaterial (decellularized small intestinal sub-mucosa), the same group also showed that smooth muscle progenitor cells could migrate to the matrix and secrete collagen and elastin (the two main components of the vascular wall) [573]. Liu et al. introduced “hair follicle smooth muscle progenitor cells” as a suitable and accessible source of cells for cardio-vascular tissue engineering, including heart valves, vascular grafts, and even cardiac patches for life threatening cardiac disease [311].

1.7.7 Reprograming hair follicle cells

Induced pluripotent stem cells (iPSCs) are adult cells that are reprogrammed to an undifferentiated state. Similar to embryonic and adult stem cells, they have the potential to differentiate into different lineages. In 2006, Takayshi and Shinya Yamanaka showed that by introducing different embryonic transcription factor genes (Oct4, SOX2, cMyc, and Klf4) with a retroviral vector, mice fibroblasts can be made to reprogram and form embryonic-like stem cell colonies in cell culture [574]. Reprogrammed cells were able to differentiate into cells from all germ layers both in vitro and in vivo, and form teratomas.
when injected in to the nude mice. Later, human iPSCs were produced from human fibroblasts [575] and even from human renal epithelial cells presented in urine [576].

Hair follicle cells have been attractive candidates for reprogramming. In addition to their accessibility, follicular keratinocytes could be reprogrammed with higher efficiency than normal fibroblasts [577]. In addition, dermal papillae cells endogenously express three of the reprogramming factors SOX2, c-Myc, and Klf4, and are reprogrammed more rapidly with only Oct4 reprogramming factor [578].

Although advances in reprogramming technology have come from the use of different vectors such as plasmids, and even small molecules that mimic the effect of transcription factors [579], the oncogenicity of IPS cells remains a challenge for clinical application of IPS cells. Another concern in regard to the clinical use of iPSCs is the immunogenicity of autologous iPSCs due to genetic instabilities [580].

1.8 Normal physiologic response of hair follicle to skin wounds

The influence of the hair follicles on the healing properties of skin has long been known. Zawacki (1967) showed that the thickness and vascularity of rodent skin are constantly undergoing cyclic changes related to hair follicles, which results in significant difference in healing of experimental burns [581]. He showed that the anagen phase of the hair cycle was associated with an increase in thickness and vascularity in skin. Therefore, otherwise similar burn injuries may induce a partial thickness skin loss in thick anagen skin, but whole-thickness skin loss in thin skin during telogen [581]. However, it is only recently that the complex relationship of the hair cycle and skin wound has been explored.

In an experimental study Ansell et al. (2011) showed that full thickness wounds in mouse skin surrounded with anagen hair follicles (induced by hair pluck) heal more quickly than similarly sized wounds surrounded by telogen hair follicle in other mice of the same age [78]. On the other hand, skin wound also affects the hair cycle: around small cutaneous wounds in the mouse with synchronised telogen hair, hair fibres grew longer, which suggests a shift from telogen to anagen [582] as follows:
Several changes associated with the anagen phase of the hair follicle may explain the mechanism by which the hair cycle influences wound healing. As discussed in 1.3.6, active hair growth (anagen) is associated with angiogenesis [10] and innervation in skin [11]. As both vascularization [534, 535] and skin innervation [536] play critical roles in wound healing (discussed in 1.6.2), by adding epithelialization, angiogenesis and innervation as a possible mechanism by which a shift in the hair cycle may influence wound healing, we can complete the above algorithm to:

\[ \text{Wound} \rightarrow \text{Anagen} \rightarrow \text{Wound healing} \]

The connection between the hair cycle and the innervation, vascularity and healing of skin, has not been reported in human, as the hair cycle is asynchronous [184, 188, 189] (discussed in 1.3.6) and the influence of hair cycling in skin is less prominent [76].

If these findings are confirmed in human studies, clinical applications may follow, and skin innervation, vascularity and wound healing may perhaps be accelerated by manipulating hair folicle cycling [214]. For example, plucking hair, which induces anagen, may be found to speed wound healing in chronic wounds and surgical wounds [214].
1.9 **Hypothesis**

Despite several treatment options being available, the treatment of chronic wounds remains a challenge in a clinical setting, which leads to a considerable socioeconomic burden on patients and the community [6].

The hair follicle has exceptional regenerative characteristics. Hair follicles are able to recover from a continuous cycle of regression and regeneration throughout life [76, 81], and are even capable of regenerating themselves following partial amputation [99, 113, 552].

The hair follicle has been considered a reservoir of stem cells or “the bone marrow” of skin [81]. Various populations of epithelial and mesenchymal stem cells have been identified in the hair follicle, which participate in hair and skin regeneration as part of their normal physiologic response.

In addition to being the stem cell reservoir for skin repair, hair follicles also respond to injury by shifting to rapid hair growth [582] and increasing in vascularity [10] and innervation [11] within the skin, which may influence the healing process. In rodent experiments in which the hair cycle is synchronised, active hair growth is associated with better wound healing [78].

In the present thesis the hypothesis was that transplanted hair follicles around a skin wound would improve wound healing.

In this study, follicular transplantation was used to deliver hair follicle stem cells to an injury site. The structure and function of the hair follicle was preserved by isolating whole hair follicles and then transplanting them to the wound site, instead of isolating a specific hair follicle cell population. Follicular transplantation not only provides a population of epithelial and mesenchymal stem cells, but may also improve healing by promoting angiogenesis and innervation.
1.10 **Aims, Scope and Methodology**

This thesis explores the potential of hair follicle transplantation in skin wound healing. An animal model of hair follicle transplantation, similar to human hair restoration surgery, was established. Follicles from green fluorescent protein transgenic mice were transplanted into the nude immune-deficient mice. The effects of hair follicle transplantation on wound healing were investigated including the migration and participation of the fluorescent-labelled cells originating from the hair follicle.

The investigations of this aim were conducted in the following manner:

1. Establishment of a hair follicle transplantation technique in nude mice.

2. Establishment of the excisional wounds with and without hair transplant in nude mice, to investigate the role of transplanted hair follicles in wound healing, particularly the participation of hair follicle dermal and epidermal cells in the wound area.

3. Establishment of incisional wounds with intact or transected hair follicles, to assess the role of damage to hair follicles in releasing the follicular cells and participation in dermal and epidermal healing.
Chapter 2

General Materials and Methods

2.1 Animals

All animal experiments were done with the approval of St Vincent’s Health Animal Ethics Committee (AEC) according to the Code of Practice for the Care and Use of Animals for Scientific Purposes developed by the Australian National Health and Medical Research Council (NH&MRC, 7th Edition, 2004).

Animals were maintained in a PC2 level animal house room with a 12 hours light/ dark cycle, and feed and water was available ad libitum. Information about animals used in the following chapters has been summarized in Table 2-1 and detailed information has been provided in relevant chapters.

Table 2-1 List of animals used in vivo and in vitro experiments.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Animal source</th>
<th>Protocol number</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57/BL6</td>
<td>Animal Resource Centre, Western Australia.</td>
<td>ACE 050/08</td>
<td>Establish the mouse vibrissae micro-dissection</td>
</tr>
<tr>
<td>Nude mice (BALB/c Foxn1nu /Arc)</td>
<td>Animal Resource Centre, Western Australia.</td>
<td>AEC 045/10</td>
<td>Recipient for hair follicle transplantation and wound healing (Chapter4, 5 and 6)</td>
</tr>
<tr>
<td>GFP mice C57BL6/Tg14 (act-EGFP) OsbY01</td>
<td>Dr Maree Faux Ludwig Institute for Cancer Research Originated from: Genome Research Centre, Osaka, University, Japan</td>
<td>ACE 045/10 Institutional Biosafety Committee (IBC) protocol No.192</td>
<td>Donor hair follicle in hair follicle transplantation and wound healing (Chapter4, 5 and 6)</td>
</tr>
</tbody>
</table>

2.1.1 GFP mice

4-6-week-old male GFP transgenic mice express GFP under the control of a chicken beta-actin promoter and cytomegalovirus enhancer on a pure C57Blk6 background (Mice—C57BL6/Tg14 (act-EGFP) OsbY01 mice, Genome Research Centre, Osaka University,
Osaka) [583]. Figure 2-1 shows the GFP construct in the transgenic mice used. All tissues from the transgenic mice appeared green under excitation light [583, 584]. GFP transgenic mice were kindly provided by Dr Maree Faux (Ludwig Institute for Cancer Research, Royal Melbourne Hospital), with permission from Dr Masaru Okabe (Genome Information Research Centre Osaka University). Vibrissae from transgenic mice (act-EGFP) were used as donor hair follicles for hair transplantation into nude mice.

![GFP construct diagram]

Figure 2-1  The green fluorescent protein construct in GFP⁺ transgenic mice used by Okabe, M. et al (1995). All tissues from the transgenic mice appeared green under excitation light [583].

2.2 Aseptic technique and anaesthesia methods

To avoid contamination, all surgical instruments were sterilised at 125°C for 15 min. For hair follicle transplantation, incisional and excisional wounding and wound imaging animals were anaesthetised by inhalation of 2% (v/v) Isoflurane (Baxter Healthcare Ltd. Qld Toongabbie, NSW, Australia). Depth of anaesthesia as well as respiration rate was monitored throughout the surgical procedures. All surgical procedures were carried out in a sterile environment and the animal was secured on the operating table by taping down all limbs.

2.3 Bone marrow harvesting

Bone marrow was collected from the animals’ femurs and studies were conducted using fluorescence-activated cell sorting (FACS) to identify and quantify the GFP⁺ cells.
After cervical dislocation and harvesting of the wounds, the hind limb muscles were dissected away to expose the bone. The femurs from both hind limbs were collected from just above the knee joint to just below the hip joint. PBS with antibiotics (100 Units/ml penicillin G and 100 mg/ml streptomycin sulfate) was used to first wash the bones and then rinse the bone marrow. The bone marrow was collected in 10 ml tubes by flushing each bone with approximately 10 ml of PBS (+antibiotic), and then passing each sample through a 70 µm nylon mesh (cell strainer, BD biosciences). The cell suspension was then centrifuged (at 1500 rpm for 6 minutes) and resuspended in 1ml lysis buffer for 5 minutes at room temperature. Harvested bone marrow cells of each animal were washed twice in PBS 2% FBS (v/v). For fluorescence-activated cell sorting the final cell pellet was resuspended in 1ml of 2% (v/v) FBS. Cells were sorted using a BD FACS Aria cell sorter (BD Biosciences).

2.4 Fluorescence-activated cell sorting

Harvested bone marrow specimens (2.3) from nude mice with hair transplantation were examined by Fluorescence-activated cell sorting (FACS), on a BD FACS Aria (BD Biosciences). To identify and quantify migrating GFP+ cells, originated from transplanted follicle, in bone marrow, cells were sorted with excitation 488 nm and 530/30-emission filter for GFP. For each preparation, collected bone marrow from a C57BL/6 mouse was used to determine the GFP negative gate. A total of 50,000 cells were studied by Flow Cytometry in each animal. Results were analysed with FlowJo software Version 8.8.6.

2.5 Histology tissue preparation

Immunohistochemistry (IHC) was performed on paraffin embedded or frozen sectioned tissue and also on cells cultured in chamber slides.

2.5.1 Paraffin embedded tissue

Harvested tissues were fixed in PFA for 24 hours and then were stored in PBS at 4°C prior to processing. During processing, tissues were dehydrated through a graded alcohol series and infiltrated with paraffin in an automated tissue processor (Shandon Hypercentre XP).
Immediately following processing, the tissues were embedded in paraffin blocks. Paraffin embedded tissues were cross-sectioned (Biocut 1130 microtome) at 5μm thickness and mounted on poly-lysine coated glass slides (Thermo scientific), dried at 37°C overnight then used for histology staining.

2.5.2 Frozen tissue preparation

Formaldehyde-fixed frozen skin tissue samples were used to visualise GFP+ cells originating from GFP transgenic mice, as described before [585]. In brief, harvested skin tissues were placed in 10% (w/v) paraformaldehyde (PFA) for six hours, followed by 15% (w/v) sucrose for 24 hours at 4°C. Specimens were then immersed in optimal cutting temperature compound (OCT, Sakura Finetek CA USA) and frozen with liquid nitrogen. Frozen tissues were cut to 18μm thick sections (Reichert-Jung Cryocut 1800), dried at room temperature overnight and stained the next day or stored in -80°C freezers.

Cells containing GFP can be easily visualized in frozen sections [585]. To detect GFP positive (GFP+) cells, slides with frozen sections simply washed in distilled water and counterstained with diamidino-2-phenylindole (DAPI) (1μg/mL) for 1 min, then washed in PBS and mounted in a water-based mounting medium, and covered with a coverslip. Cell nuclei were stained with DAPI to visualize the tissue structure and the presence of the GFP+ cells originating from the transplanted hair follicles was studied in wounds.

2.6 Histochemical staining

Haematoxylin and Eosin staining

Paraffin embedded sections were stained with Haematoxylin and Eosin (H&E) to show general morphology of the tissue as described in Table 2-2.
### Table 2-2  Detailed protocol for Haematoxylin and eosin staining

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reagent</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-Wax</td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td>Hydrate</td>
<td>100% Ethanol 1</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol 2</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>70% Ethanol</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>3 washes with dH2O (Distilled water) 10 dips per wash</td>
<td></td>
</tr>
<tr>
<td>Nuclear staining</td>
<td>Mayer’s Haematoxylin</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>3 washes with dH2O 10 dips per wash</td>
<td></td>
</tr>
<tr>
<td>Bluing agent</td>
<td>Ammonia water</td>
<td>2min</td>
</tr>
<tr>
<td></td>
<td>3 washes with dH2O 10 dips per wash</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm stain</td>
<td>Eosin</td>
<td>2 min</td>
</tr>
<tr>
<td>Dehydrate</td>
<td>70% Ethanol</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol</td>
<td>1min</td>
</tr>
<tr>
<td>Clear</td>
<td>Histolene</td>
<td>2 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>2 min</td>
</tr>
<tr>
<td>Mount coverslips</td>
<td>DPX mounting medium</td>
<td>Allow slides to dry over night</td>
</tr>
</tbody>
</table>

**Masson’s trichrome staining**

Masson’s trichrome staining was performed on the paraffin embedded wound sections as described in Table 2-3. In Masson’s trichrome staining, mature collagen in undamaged epidermis appears intense blue and can be discriminated from the light and diffuse blue color of the granulation tissue with poor collagen bundles.
### Table 2-3: Detailed protocol for Masson’s trichrome staining

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reagent</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-Wax</td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td>Hydrate</td>
<td>100% Ethanol 1</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol 2</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>70% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>Wash in 3 changes of tap water</td>
<td></td>
</tr>
<tr>
<td>Enhancement of staining intensity</td>
<td>Bouin’s solution</td>
<td>60 min at 60 °</td>
</tr>
<tr>
<td></td>
<td>Washed with tap water until the yellow colour disappeared</td>
<td></td>
</tr>
<tr>
<td>Nuclear staining Black/ Blue</td>
<td>Weigert’s haematoxylin</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Wash in 2 changes of tap water</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm and muscle fibres stain red</td>
<td>Acid Fuchsin</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>Wash in 2 changes of tap water</td>
<td></td>
</tr>
<tr>
<td>Remove red from collagen</td>
<td>Phosphotungstic acid</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Drain slides only</td>
<td></td>
</tr>
<tr>
<td>Collagen dye light blue</td>
<td>Aniline blue</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>Wash in 2 changes of tap water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1% Acetic acid</td>
<td>2 min</td>
</tr>
<tr>
<td></td>
<td>No Wash slides directly into the next solution</td>
<td></td>
</tr>
<tr>
<td>Dehydrate</td>
<td>70% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>Clear</td>
<td>Histolene</td>
<td>2 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>2 min</td>
</tr>
<tr>
<td>Mount coverslips</td>
<td>DPX mounting medium</td>
<td>Allow slides to dry over night</td>
</tr>
</tbody>
</table>

### 2.7 Immunohistochemistry

Table 2-4 and Table 2-5 shows the primary and secondary antibodies used in histology studies. Detailed protocol for each immunohistochemistry and immunofluorescence staining is described below.
## List of primary antibodies used for IHC and Immunofluorescence

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host</th>
<th>Species raised against</th>
<th>Clonality</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Supplier</th>
<th>CAT-No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD31</td>
<td>Rat</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>IgG</td>
<td>1/150</td>
<td>Proteinase K (Dako)</td>
<td>BD Bioscience</td>
<td>#553370</td>
</tr>
<tr>
<td>Anti-F4/80</td>
<td>Rat</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>IgG2b</td>
<td>1/100</td>
<td>Proteinase K (Dako)</td>
<td>Abcam</td>
<td>AB6640</td>
</tr>
<tr>
<td>Anti-PGP9.5</td>
<td>Rabbit</td>
<td>Mouse</td>
<td>Polyclonal</td>
<td>IgG</td>
<td>1/1500</td>
<td>-</td>
<td>Abcam</td>
<td>Ab10404</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>Rabbit</td>
<td>Jellyfish</td>
<td>Polyclonal</td>
<td>IgG</td>
<td>1/1500</td>
<td>-</td>
<td>Abcam</td>
<td>AB 290</td>
</tr>
<tr>
<td>Anti-Cytokeratin</td>
<td>Rabbit</td>
<td>Cow</td>
<td>Polyclonal</td>
<td>Unfractionated Anti-serum</td>
<td>1/1000</td>
<td>Proteinase K (Dako)</td>
<td>Dako</td>
<td>Z0622</td>
</tr>
</tbody>
</table>

## List of secondary antibodies used for IHC and immunofluorescence

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Host</th>
<th>Species raised against</th>
<th>Clonality</th>
<th>Dilution</th>
<th>Supplier</th>
<th>CAT-No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated rabbit anti rat Ig-Biotin</td>
<td>Rabbit</td>
<td>Rat</td>
<td>Polyclonal</td>
<td>1/200</td>
<td>DAKO</td>
<td>E0468</td>
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<tr>
<td>Goat anti rabbit IgG AF 488</td>
<td>Goat</td>
<td>Rabbit IgG</td>
<td>Monoclonal</td>
<td>1/200</td>
<td>Jackson ImmunoResearch</td>
<td>111-545-144</td>
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<tr>
<td>Cy3 Anti rabbit IgG</td>
<td>Goat</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1/200</td>
<td>Jackson ImmunoResearch</td>
<td>111-165-003</td>
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</tbody>
</table>
Table 2-6 Detailed protocol for IHC using CD31 antibody (Auto strainer or manual staining)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reagent</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-Wax</td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td>Hydrate</td>
<td>100% Ethanol 1</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol 2</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>70% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>3 washes with dH(_2)O</td>
<td>10 dips per wash</td>
</tr>
<tr>
<td></td>
<td>Wash sections in buffer (TBS)</td>
<td>3× 5'/wash</td>
</tr>
<tr>
<td>Peroxidase Block</td>
<td>3% H(_2)O(_2) in 50% methanol / dH(_2)O</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Wash sections in buffer (TBS)</td>
<td>3× 5'/wash</td>
</tr>
<tr>
<td>Antigen retrieval</td>
<td>DAKO proteinase K</td>
<td>8 min</td>
</tr>
<tr>
<td></td>
<td>Wash sections in buffer (TBS)</td>
<td>3× 5'/wash</td>
</tr>
<tr>
<td>Blocking serum</td>
<td>DAKO blocking reagent</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>Wash sections in buffer (TBS)</td>
<td>3× 5'/wash</td>
</tr>
<tr>
<td>Primary antibody</td>
<td>Rat anti mouse CD31 Bioscience</td>
<td>1h</td>
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<tr>
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<td>Wash sections in buffer (TBS)</td>
<td>3× 5'/wash</td>
</tr>
<tr>
<td>2(^{nd}) antibody</td>
<td>Rabbit anti rat IG –Biotin 1/300</td>
<td>30 min</td>
</tr>
<tr>
<td>ABC Detection system</td>
<td>Vector ABC kit</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>Substrate–chromogen</td>
<td>4 min</td>
</tr>
<tr>
<td></td>
<td>1 drop of DAB + 1ml of buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 washes with dH(_2)O</td>
<td>10 dips per wash</td>
</tr>
<tr>
<td>Counterstaining</td>
<td>Haematoxylin</td>
<td>2 min</td>
</tr>
<tr>
<td>Dehydrate</td>
<td>As per H&amp;E protocol</td>
<td></td>
</tr>
<tr>
<td>Mount + coverslips</td>
<td>DPX mounting medium</td>
<td>Allow slides to dry over night</td>
</tr>
<tr>
<td>Reaction</td>
<td>Reagent</td>
<td>Time</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------------------------------------</td>
<td>---------------</td>
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<tr>
<td>De-Wax</td>
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<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td>Hydrate</td>
<td>100% Ethanol 1</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol 2</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>70% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>3 washes with dH2O 10 dips per wash</td>
<td></td>
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<td>Wash sections in buffer (TBS)</td>
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<td>EDTA-Na2 0.01 M, pH8 in 95°C water bath</td>
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<td>3% H2O2 in 50% methanol / dH2O</td>
<td>5 min</td>
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<tr>
<td></td>
<td>Wash sections in buffer (TBS)</td>
<td>3× 5′/wash</td>
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<td>Wash sections in buffer (TBS)</td>
<td>3× 5′/wash</td>
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<tr>
<td></td>
<td>Blocking serum</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>Wash sections in buffer (TBS)</td>
<td>3× 5′/wash</td>
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</tr>
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<td>Wash sections in buffer (TBS)</td>
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<td>Substrate–chromogen</td>
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<tr>
<td></td>
<td>1 drop of DAB + 1ml of buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 washes with dH2O 10 dips per wash</td>
<td>5 min</td>
</tr>
<tr>
<td>Counterstaining</td>
<td>Haematoxylin</td>
<td>2 min</td>
</tr>
<tr>
<td>Dehydrate</td>
<td>As per H&amp;E protocol</td>
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</tr>
<tr>
<td>Mount coverslips</td>
<td>DPX mounting medium</td>
<td>Allow slides to dry over night</td>
</tr>
<tr>
<td>Reaction</td>
<td>Reagent</td>
<td>Time</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------------------</td>
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<td>De-Wax</td>
<td>Histolene Histolene</td>
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<tr>
<td>Hydrate</td>
<td>100% Ethanol 1 100% Ethanol 2 70% Ethanol</td>
<td>1 min 1 min 1 min</td>
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<td>3 washes with dH2O 10 dips per wash</td>
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<td>Wash sections in buffer (TBS)</td>
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<td>Peroxidase Block</td>
<td>3% H2O2 in 50% methanol / dH2O</td>
<td>5 min</td>
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<td>2 min</td>
</tr>
<tr>
<td>Dehydrate</td>
<td>As per H&amp;E protocol</td>
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<td>DPX mounting medium</td>
<td>Allow slides to dry over night</td>
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<tr>
<td>Reaction</td>
<td>Reagent</td>
<td>Time</td>
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<td>-------------------------</td>
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<td>5 min</td>
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<tr>
<td>Hydrate</td>
<td>100% Ethanol 1</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol 2</td>
<td>1 min</td>
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<tr>
<td></td>
<td>70% Ethanol</td>
<td>1 min</td>
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<td>3 washes with dH2O 10 dips per wash</td>
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<td>Wash sections in buffer (TBS)</td>
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<td>Peroxidase Block</td>
<td>3% H2O2 in 50% methanol / dH2O</td>
<td>5 min</td>
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<tr>
<td>Blocking serum</td>
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<tr>
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<td>Substrate–chromogen</td>
<td>4 min</td>
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<tr>
<td></td>
<td>1 drop of DAB + 1ml of buffer</td>
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<td>3 washes with dH2O 10 dips per wash</td>
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<td>Counterstaining</td>
<td>Haematoxylin</td>
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<td>Dehydrate</td>
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<td>2 min</td>
</tr>
<tr>
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<td>100% Ethanol</td>
<td>2 min</td>
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<tr>
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<td>100% Ethanol</td>
<td>2 min</td>
</tr>
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<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td>Mount+ coverslips</td>
<td>DPX mounting medium</td>
<td>Allow slides to dry over night</td>
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<tr>
<td>Reaction</td>
<td>Reagent</td>
<td>Time</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------------------------------------------</td>
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<td><strong>De-Wax</strong></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td><strong>Hydrate</strong></td>
<td>100% Ethanol 1</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol 2</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>70% Ethanol</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>3 washes with dH2O</td>
<td>10 dips per wash</td>
</tr>
<tr>
<td></td>
<td>Wash sections in buffer (TBS)</td>
<td>3× 5′/wash</td>
</tr>
<tr>
<td><strong>Peroxidase Block</strong></td>
<td>3% H2O2 in 50% methanol / dH2O</td>
<td>5 min</td>
</tr>
<tr>
<td><strong>Blocking serum</strong></td>
<td>DAKO blocking reagent</td>
<td>30 min</td>
</tr>
<tr>
<td><strong>Primary antibody</strong></td>
<td>Rabbit Anti GFPAb (Ab260), 1/1500</td>
<td>1h</td>
</tr>
<tr>
<td></td>
<td>Wash sections in buffer (TBS)</td>
<td>3× 5′/wash</td>
</tr>
<tr>
<td><strong>EnVision+ Detection</strong></td>
<td>Peroxidase labelled polymer</td>
<td>30 min</td>
</tr>
<tr>
<td>system</td>
<td>(DAKO EnVasion K 4011)</td>
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<tr>
<td></td>
<td>Substrate–chromogen</td>
<td>4 min</td>
</tr>
<tr>
<td></td>
<td>1 drop of DAB + 1ml of buffer</td>
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</tr>
<tr>
<td></td>
<td>3 washes with dH2O</td>
<td>10 dips per wash</td>
</tr>
<tr>
<td><strong>Counterstaining</strong></td>
<td>Haematoxylin</td>
<td>2 min</td>
</tr>
<tr>
<td><strong>Dehydrate</strong></td>
<td>70% Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td></td>
<td>100% Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td><strong>Clear</strong></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Histolene</td>
<td>5 min</td>
</tr>
<tr>
<td><strong>Mount + coverslips</strong></td>
<td>DPX mounting medium</td>
<td>Allow slides to dry over night</td>
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### Table 2-11  Detailed protocol for anti CD31 and anti GFP immunofluorescence double staining

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<td>De-wax sections</td>
<td>As per H&amp;E staining</td>
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</tr>
<tr>
<td>Wash sections in buffer (TBS) *5’/wash</td>
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<td></td>
</tr>
<tr>
<td>Antigen retrieval</td>
<td>Proteinase K</td>
<td>6 min</td>
</tr>
<tr>
<td>Wash sections in buffer TBS</td>
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<td></td>
</tr>
<tr>
<td>Incubate in blocking reagent</td>
<td>DAKO blocking reagent</td>
<td>30 min</td>
</tr>
<tr>
<td>Incubate in primary antibody</td>
<td>Rat anti mouse CD31 1:150</td>
<td>1 h</td>
</tr>
<tr>
<td>Wash sections in buffer (TBS)</td>
<td></td>
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</tr>
<tr>
<td>Incubate in secondary antibody</td>
<td>Biotinylated Rabbit anti Rat Ab (E0468)</td>
<td>1 h</td>
</tr>
<tr>
<td>Wash sections in buffer (TBS)</td>
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<tr>
<td>Incubate in detection systems:</td>
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</tr>
<tr>
<td>Apply colour detection system</td>
<td>Streptavidin –AP 1/500</td>
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<td>Wash sections in buffer TBS</td>
<td></td>
<td>3×5'/wash</td>
</tr>
<tr>
<td>Apply colour detection system</td>
<td>Fast red (ref)</td>
<td>2 min</td>
</tr>
<tr>
<td>Wash sections in d H2o</td>
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<td></td>
</tr>
<tr>
<td>Wash sections in buffer (TBS)</td>
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<td>3×5'/wash</td>
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<tr>
<td>Incubate in blocking reagent</td>
<td>DAKO blocking reagent</td>
<td>20 min</td>
</tr>
<tr>
<td>Incubate in primary antibody</td>
<td>Rabbit anti GFP (AB 290) 1/1500</td>
<td>1 h</td>
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<tr>
<td>Wash sections in buffer (TBS)</td>
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<td>3×5'/wash</td>
</tr>
<tr>
<td>Incubate in secondary antibody</td>
<td>Goat anti rabbit IgG Alexa Fluor 488</td>
<td>1 h</td>
</tr>
<tr>
<td>Wash sections in buffer (TBS)</td>
<td></td>
<td>3×5'/wash</td>
</tr>
<tr>
<td>Counterstaining with DAPI</td>
<td>1/1000</td>
<td>1 min</td>
</tr>
<tr>
<td>Wash sections in buffer (TBS)</td>
<td></td>
<td>3×5'/wash</td>
</tr>
<tr>
<td>Mount + coverslips</td>
<td>Fluorescence mounting medium</td>
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</table>
Table 2-12  Detailed protocol for anti pan-cytokeratin immunofluorescence staining

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<td>10 dips per wash</td>
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<tr>
<td>Wash sections in buffer (TBS)</td>
<td></td>
<td>3× 5′/wash</td>
</tr>
<tr>
<td>Antigen retrieval</td>
<td>Proteinase K</td>
<td>6 min</td>
</tr>
<tr>
<td>Blocking serum</td>
<td>DAKO blocking reagent</td>
<td>30 min</td>
</tr>
<tr>
<td>Primary antibody</td>
<td>Rabbit Anti cytokeratin Ab.1/1000</td>
<td>1h</td>
</tr>
<tr>
<td>Wash sections in buffer (TBS)</td>
<td></td>
<td>3× 5′/wash</td>
</tr>
<tr>
<td>3 washes with dH2O</td>
<td></td>
<td>10 dips per wash</td>
</tr>
<tr>
<td>2nd Ab</td>
<td>Goat anti Rabbit Ab, Cy™3, 1/2000</td>
<td>1h</td>
</tr>
<tr>
<td>Counterstaining</td>
<td>DAPI</td>
<td>30 sec</td>
</tr>
<tr>
<td>Mount + coverslips</td>
<td>Fluorescence mounting medium</td>
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</tr>
</tbody>
</table>
Chapter 3

Hair Follicle Transplantation in Nude Mice

3.1 Introduction

Hair follicle cells contribute to wound healing, skin circulation and skin diseases including non-melanoma skin cancers such as basal cell skin cancer. The techniques of hair follicle transplantation have the potential to be used in hair biology research to investigate the role of hair follicle cells in normal homeostasis of skin, in wound healing and in skin disease. Cells originating from hair follicles can be distinguished from interfollicular epidermis by transplanting follicles from genetically labelled donors. The effects of manipulating gene expression specifically in follicles can be investigated by transplanting follicles from transgenic mice.

Follicular unit hair transplantation is an established procedure for hair restoration in humans. The method involves harvesting hairy skin from the occipital scalp, micro-dissection into individual follicular units and implantation of these follicular units into bald scalp in naturally occurring units of 1 to 4 hairs [586, 587]. Donor skin is harvested, either by 1 mm punch biopsies or by removing a strip of occipital scalp skin, and then micro-dissected into follicular units with the aid of a stereo-microscope. Each follicular unit is inserted into the bald scalp using either a micro-blade or an 18-21-gauge needle. Minimal damage of the recipient site together with high graft survival rates are advantages of this technique.

Since the initial descriptions of human hair transplantation by Okeda in 1939 [588] and Oreitrich in 1959 [589], the techniques have been constantly refined to improve cosmetic outcomes, graft survival and minimize damage to recipient skin. However, there is no detailed description of an animal hair transplantation protocol in the dermatology literature that incorporates these refinements or has been optimised for animal recipients.
Previously published hair induction studies in animals have employed recombination and implantation of dermal or epidermal parts of hair follicles [113, 114, 120, 590] or cultured cells [114, 291, 591, 592]. In these experiments, there was damage to the transplanted tissue, with either the hair follicle separated into different parts or dissociated cells being cultured prior to implantation. Damage to the transplanted follicles could potentially alter the behavior of the follicles. In another published study, isolated vibrissae were transplanted into an incisional wound and sutured [323]. This procedure is time-consuming, and damage to the recipient skin induces a wound healing response that could alter experimental outcomes.

Amoh et al. [323] transplanted the intact hair follicle vibrissae following micro-dissection from transgenic mice. In this study, isolated vibrissae were transplanted in nude mice either under the kidney capsule or subcutaneously, in an incisional wound where the hair follicle was kept in place with nylon sutures [323]. In this model, the hair was transplanted under the skin or kidney capsule and not in its normal anatomical position, which can potentially alter normal hair physiology. Besides, this technique involves an incisional wound and suturing which is time consuming and can potentially alter the transplanted hair follicle.

Sato et al. [593] micro-dissected pelage hair follicles from GFP\(^+\) transgenic mice and transplanted them into nude mice. In this experiment, the GFP\(^+\) positive interfollicular epidermis was also transplanted into the nude mice, as the size of the pelage follicle made it difficult to exclude surrounding interfollicular epidermis from the hair follicles during micro-dissection.

Transplantation of precisely micro-dissected vibrissae follicles from transgenic labelled mouse in nude mice would provide an opportunity to study the participation and migration of hair follicle cells in wound healing. Migrating hair follicle cells can be recognised from other dermal and epidermal cells in skin when transplanted follicles do not contain any surrounding donor interfollicular epidermis.
In this experiment I established a hair transplant technique in nude mice similar to follicular unit transplantation in humans. This model retains the normal anatomical relationship of hair follicle and surrounding tissue. In addition, minimal damage to the transplanted hair follicle and recipient skin make the model resemble normal physiologic function of hair follicle and skin. This technique is also simpler and quicker than other animal models of hair transplantation; with minimal damage to skin and does not require any suturing.

I selected vibrissae rather than pelage donor follicles, as they are easier to harvest and micro-dissect from surrounding donor interfollicular epidermis. Exclusion of the surrounding donor interfollicular epidermis makes this a suitable experiment to study cell migration and involvement of hair follicle cells during re-epithelialization. In addition, measurement of hair growth and hair cycling is also simpler in transplanted vibrissae rather than pelage follicles.

3.1.1  **Nude mice**

Nude mice were first described as a hairless phenotype raised in an albino strain in 1966 [594]. Nude mice are an immune-deficient strain due to spontaneous mutation in Forkhead box protein N1 (FOXN1) gene (reviewed in [595]), which regulates T-cell maturation as well as keratin gene expression [596].

Nude athymic mice are not truly hairless [597, 598] and in fact exhibit the same number of hair bulbs as normal mice [598]. The relevant genetic mutation does not directly interfere with the cyclic activity of the hair follicles [594, 599], although hair growth and differentiation is deeply affected [598]. The hair shaft is twisted and deformed, the hair canal becomes dilated and most hair shafts do not penetrate the epidermis, those that do so, being weak and fragile [598, 600].

In immune-deficient nude mice, T-cell precursors exist; however, their development is blocked due to dysgenesis of the thymus [601, 602]. This leads to an immunodeficiency that prevents rejection of allograft (grafts from the same species) and even Xenograft
(grafts of tissue from other species), and makes nude mice a valuable host for tissue and organ transplantation.

The hair follicle abnormality in nude mice is independent of thymus function since thymus restoration does not induce hair growth [599].

3.1.2 Hair follicle transplantation in nude mice

Nude mice are a long-standing host in transplantation studies. In addition, the hairless phenotype of nude mice makes them a suitable model to optimise the hair transplantation technique, as it is easy to secure a transplanted hair follicle by dressing, easy to recognise and follow up the transplanted hair follicle under skin and to detect the hair fibre and its growth rate.

In this chapter, an animal model of hair follicle transplantation was established using the vibrissae hair follicles from GFP transgenic mice to the back skin of nude mice. This animal model was then used in Chapter 4 and 5 to study the role of transplanted hair follicles during wound healing and to trace the migration of GFP+ cells originating from transplanted hair follicles in full thickness and incisional wounds.

3.1.3 Study aims

This chapter presents an optimised protocol for vibrissae transplantation in nude mice.

Study aims:

- Establishment of vibrissae hair follicle transplantation in nude mice
- Characterisation of any dispersal of donor cells from hair follicles after transplantation.
3.2  Materials and methods

3.2.1  Experimental design

To establish the hair follicle transplant technique in nude mice a series of experiments was conducted in order to keep the hair follicle in place during the first week after transplantation.

Experiment 1: no dressing was applied after follicular transplantation.

Experiment 2: an adhesive bandage applied for the first five days after transplantation

Experiment 3: the hair fibres were cut short and transplanted follicles covered with Vaseline gauze, a non-adherent absorbent dressing, and then secured with an elastic adhesive bandage for five and three days.

Transplanted follicles were checked every day during the first week following transplantation (Experiment 1) and when the dressing was removed (Experiments 2 and 3). The success rate of each protocol was assessed by the number of visible vibrissae hair shafts on each mouse at six weeks, and expressed as a percentage of the number of originally transplanted follicles.

3.2.2  Animals

Whisker pads of C57/BL6 mice were used to establish the mouse vibrissae micro-dissection technique. For hair transplantation experiments, vibrissae hair follicles were isolated from 4-6-week-old male GFP transgenic mice and transplanted into 4-6-week-old male athymic nude mice (BALB/c Foxn1nu/Arc).

All animal experiments were done with the approval of St Vincent’s Health Animal Ethics Committee (AEC number 045/10) and Institutional Biosafety Committee (IBC 192). One donor animal (GFP mouse) was used for each recipient (nude mouse) and all animals maintained in a PC2 animal housing room with a 12-hour light/ dark cycle. Food and water were available ad libitum.
3.2.3 Aseptic technique and anaesthesia methods

All surgical instruments were sterilised at 125°C for 15 minutes. For hair follicle transplantation procedures recipient nude mice were anaesthetised by inhalation of 2% (v/v) Isoflurane (Baxter Healthcare Ltd. Qld Toongabbie, NSW, Australia) and depth of anaesthesia as well as respiration rate was monitored throughout the procedure. The animal was secured on the operating table by taping down all limbs. All surgical procedures were carried out in a sterile environment.

3.2.4 Mice vibrissae follicle micro-dissection

For the isolation of vibrissae, donor GFP transgenic mice were killed by cervical dislocation at 4 to 6 weeks of age. Hair follicle flip-side micro-dissection was performed as previously described [323]. The upper lip containing the vibrissae pad was excised, and fixed onto sterile gauze on a foam pad using 25G needles, with the dermal surface exposed. Drops of Dulbecco's Modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA, USA) containing 1% (v/v) antibiotic-antimycotic (penicillin, streptomycin and Fungizone, Life Technologies) were added as necessary to prevent the tissue from dehydrating.

![Figure 3-1 Mouse vibrissa hair follicle micro-dissection technique. Follicles were dissected from surrounding tissue with fine forceps (A & B). Hair follicles were plucked from the pad by pulling them gently by the neck with fine forceps (C).](image)

With the aid of a stereo-microscope, the follicles were teased away from surrounding tissue using fine watchmakers’ forceps (Figure 3-1 A, B and C). They were then plucked from the pad by pulling them gently by the neck with fine forceps (Figure 3-1, C). Approximately 25 to 30 hair follicles were micro-dissected from each GFP transgenic
mouse. All follicles were kept in DMEM containing the antibiotics, in a 10-cm petri dish, and kept cold on ice.

3.2.5 Follicular unit hair transplantation

Under a stereo-microscope, 15–40 vibrissae from a GFP transgenic mouse were transplanted into the dorsal skin of each nude mouse (Figure 3-2).

Recipient mice were anaesthetized by inhalation of 2% (v/v) Isoflurane. A hole was made in the recipient skin at an approximate angle of 30° using a 25 gauge needle (Fig 5-3 A). The needle was then removed and the dissected hair follicle was placed in the hole (Figure 3-2, B &C) using No.5 watchmakers’ forceps (No.5 forceps, Inox). The hair follicles were placed into the skin at an angle parallel to the host pelage, at an approximate angle of 30° to the vertical directed dorsally. All needles were fitted on 3 ml syringes for ease of use.

Hair follicles were transplanted 2–3 mm apart. The technique was optimised and the transplanted hair follicles were either left without dressing, or dressing was applied to keep the follicles in place as described in 3.3.1.

In the optimised protocol Vaseline gauze and a non-adherent absorbent dressing (Multigate Medical Products Pty Ltd NSW Australia) were placed over the transplanted follicles and secured with an adhesive bandage (Elastoband light adhesive bandage 75 mm, Beiersdorf Australia Ltd NSW Australia) (Fig 5-3 D).

![Figure 3-2](image)

**Figure 3-2** Follicular unit transplantation technique in nude mouse.

Animals were awake after the procedure and put in individual cages with easily accessible food during the first three days after transplantation. Enrofloxacin (Baytril 25mg/mL from Bayer, Leverkusen, Germany) was added to their drinking water at 200 mg/l for five days
after hair transplantation to prevent infection. The dressings were removed under general anaesthesia after three days.

3.2.6 Measurement of hair growth after transplantation

Six weeks after hair transplantation, hair fibres were plucked using fine forceps (the animals being under general anaesthesia), to induce a new hair cycle. New hair growth was measured fifteen days after plucking by measuring the length of the new hair shaft emerging from the skin. Phototrichograms (Nikon SL-1 Macro Cool-Light and Canon Powershot A520 cameras) were taken under general anaesthesia. The length of hair shafts emerging from the skin was measured in phototrichograms using an image analysis program (ImageJ, NIH, Bethesda MD) and calibrated against an internal scale marker. The result was presented as an average of hair length and standard deviation.

3.2.7 Hair follicle transplantation success rate

Between 24-27 hair follicles were transplanted into each nude mouse. After six weeks visible hair follicles were counted in five animals and success rates were presented as the percentage of visible transplanted thick hairs to the original transplanted hair follicles.

3.2.8 Tissue harvesting and preparation

Transplanted hair follicles were harvested 6 weeks after transplantation. Mice were anesthetised by inhalation of 2% (v/v) Isoflurane and after harvesting the skin tissue, the animals were sacrificed by cervical dislocation.

Skin containing the transplanted follicles was collected with a minimum margin of 5 mm. This was prepared for frozen and paraffin-embedded tissue sectioning as described in 2.4. The tissues were embedded in paraffin or frozen blocks with the section side face-down to allow vertical sectioning of skin.
3.2.9 **Histology**

*Detection GFP positive cells in frozen sections*

Cells containing GFP are easily visualized in formaldehyde-fixed frozen sections [585]. 18 µm frozen sections were prepared as described in 2.4. For each animal, the location of all GFP<sup>+</sup> cells was examined in approximately 50 (47–58) frozen sections. To detect GFP<sup>+</sup> cells, slides with frozen sections were simply washed in distilled water and counterstained with diamidino-2-phenylindole (DAPI) (1 µg/mL) for 1 min, then washed in PBS, mounted and covered with a coverslip. Cell nuclei were stained with DAPI to visualize the tissue structure.

*Anatomical study of transplanted hair follicle*

The anatomy and connection to skin vasculature was assessed in H&E staining (as described in Table 2-2) and innervation of the hair follicle assessed by immunohistochemistry (IHC) in paraffin embedded sections using pan-neural marker antibody (Anti PGP9.5). Detailed protocol of IHC using PGP9.5 antibody is described in Table 2-9.
3.3 Results

3.3.1 Optimising the hair transplantation technique

Animals were put in individual cages after hair transplantation. The nude mice appeared healthy after transplantation and were able to move with the dressing. Food was placed in the cages, so it was easily accessible during the first three days after hair transplantation. Dressings, when applied, were removed after three days. As discussed in 3.2.1 a series of experiments were done in order to keep the hair follicle in place during the first week after transplantation (Table 1-3). When no dressings were applied on transplanted follicles, most of the transplanted follicles separated after one day (60%). We therefore thought it was important to apply a dressing during the first three days after hair transplant, to prevent damage to the follicles consequent upon the animal scratching the transplanted area. The transplanted follicle was covered with adhesive elastic bandage (Experiment 2 in Table 3-1); however, most of the hair fibres (80%) stuck to the adherent bandage and the whole follicles came out when the dressing was removed on day 5 after transplantation.

Table 3-1 Optimising the dressing technique for hair transplantation.

<table>
<thead>
<tr>
<th>Dressing</th>
<th>Dressing changed</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp.1: No dressing</td>
<td>-</td>
<td>Hair follicles came off on day 1 (3 animals)</td>
</tr>
<tr>
<td>Exp.2: Dressing with adhesive bandage alone</td>
<td>Day 5</td>
<td>Hair follicles stuck to the tape (3 animals)</td>
</tr>
<tr>
<td>Exp.3: The hair fibres where cut short and dressed with: Vaseline gauze, a non-adherent absorbent dressing and adhesive bandage.</td>
<td>Day 5 and Day3</td>
<td>Almost 90% success of hair transplantation when dressings were changed on day 3 or day 5 (Total of 6 mice, 3 animals in each group)</td>
</tr>
</tbody>
</table>

In the final optimised protocol (Experiment 3 in Table 3-1), transplanted hair follicles were covered with Vaseline gauze, a non-adherent absorbent dressing, and then secured with an elastic adhesive bandage. The hair fibres were also cut short, so that the transplanted hair
did not stick to the adhesive bandage. The dressings were removed after three days and the transplanted areas left open.

In the case of the alternative protocols (Experiments 1&2 in Table 3-1), most of the transplanted hair follicles (more than 60%) came off and were lost. These animals with few surviving transplanted hair follicles were used to measure hair fibre growth after hair pluck. Mice from the optimised protocol (Experiment 3 in Table 3-1) were used to assess the hair cycle after transplantation, and for histology analysis.

3.3.2 **Spontaneous hair cycle after transplantation**

Shedding of hair fibres was detected on day 3 when the dressing was removed (Experiment 3, Table 3-1), suggesting that transplantation induced hair cycling and entry to telogen and exogen. Even when the hair shaft was shed, the transplanted follicle was still visible as a small lump under the skin. The hair shafts were in place in only 23% (9/40) of transplanted follicles on day 7 and new hair fibres were fine and appeared around day 14 (Figure 3-3) suggesting that the follicles had re-entered anagen, the growth phase of the follicle cycle.

![Figure 3-3](image)

**Figure 3-3** Hair cycle after follicular transplantation. A: Day 0 after follicular transplantation. B: The hair cycle is shifted to catagen after transplantation and by day 7, the hair shafts were in place only in 25% of transplanted follicles. C: New hair fibres emerge after two weeks (white arrow).

3.3.3 **Success rate of hair transplantation**

Most of the transplanted follicles contained a new hair shaft by six weeks. Figure 3-4 shows transplanted follicles in Experiment 3 after removing the dressing on day 3 (A), and after six weeks (B&C).
Transplant success rates were measured in mice from Experiment 3 (Table 3-1), as the number of visible vibrissae hair shafts on each mouse at six weeks, and expressed as a percentage of the number of originally transplanted follicles. In six animals 148 hair follicles were transplanted (20-27 hair follicles each), after six weeks 89±8% (Mean ± SD) of hair follicles were visible and produced hair fibres. Figure 3-4 shows the transplanted follicles after six weeks.

![Figure 3-4 Whisker follicles three days and six weeks after transplantation. A: three days after hair follicle transplant. B&C: Six weeks after hair follicle transplant.](image)

### 3.3.4 Hair growth

Hair lengths were measured in our initial hair transplantation experiments when most of the transplanted follicles were lost (Experiments 1 and 2 in Table 3-1), and each animal had fewer surviving transplanted hair follicles at six weeks, which made it easier to measure hair length in trichogram images. To measure hair shaft growth rates, follicles containing a newly growing hair shaft were plucked six weeks after transplantation. A total of 16 follicles in five mice were plucked (2-5 follicles per mouse). Hair regrew in all transplanted hair follicles (Figure 3-5). The length of the hair shaft emerging from the skin on day fifteen was 6.5 ± 2.0 mm (mean ± SD).
3.3.5 Histology analysis

H&E staining of paraffin embedded tissue sections at six weeks after transplantation showed normal anatomy of transplanted follicles.

Figure 3-5  Hair length measurement in transplanted follicles fifteen days after hair pluck. (N=16)

Figure 3-6  Representative photomicrographs of H&E staining of the transplanted follicle. Transplanted follicle is located above the panniculus carnosus (arrow head) and surrounded by subdermal fat (A). Dermal papilla at the base of the transplanted follicle (B). Cavernous blood vessels of the transplanted vibrissae (C).
Transplanted follicles were readily distinguished in histological examination from host pelage hair (Black arrows, fig 3-6). Transplanted follicles produced hair fibers (double headed arrow, Fig 3-6). The structures of the dermal papillae were readily detected at the base of the transplanted follicles (Figure B, 3-6 and B, 3-7). As shown in Figure 3-6, 3-7 and 3-8, transplanted follicles were extended from the orifice in epidermis deep to the dermis, placed on top of the panniculus carnosus (1.2.1) and surrounded by sub-dermal fat.

![Figure 3-7 Representative photomicrographs of CD31 immunostaining of the transplanted follicle 6 weeks after transplantation. CD31 positive vessels were detected in the HF(A) and DP (B).](image)

The cavernous vessel around the transplanted hair follicles contained blood cells (Figure 3.6, C, black arrow in Figure 3-7, B and white asterisk in Figure 3-8, B&D). Considering that the mean erythrocyte life span in mice is about 6 weeks [603], it is less likely that the donor erythrocytes would still be detected in histology examination 6 weeks after transplantation. Therefore we infer the erythrocytes presented in the cavernus vessels are originated from host. This observation suggests that the transplanted hair follicle was connected to the host’s circulatory system.

Immunohistochemical staining with pgp9.5 (pan neural marker) showed thick nerves were also visible around the transplanted follicles, suggesting that they had been innervated (Figure 3-8, C, D, red arrow).
Fluorescent GFP⁺ cells could readily be detected in cryosections of grafted follicles. Approximately 50 (47–58) frozen sections were evaluated in each animal. Histology examination of the skin with transplanted hair follicles in five animals (from Experiment 3, 3.2.1) showed no GFP⁺ cells in the interfollicular epidermis (Figure 3-9). Histology examination of a total of 21 transplanted follicles present in histology slides also showed that the GFP⁺ dermal and epidermal cells remained confined to the site of implantation and did not expand within the host tissue suggesting that the implanted follicles did not participate in homeostasis of the surrounding skin. As shown in Figure 3-10 GFP⁺ cells were limited to the infundibulum of the transplanted hair follicle (Red arrow B and D).
Figure 3-9 Representative photomicrographs of GFP+ follicles (fluorescence microscopy) six weeks after transplantation (18 μm cryosections) show no GFP positive cells in the interfollicular epidermis (). Green: GFP positive hair follicle. Blue: counterstained with DAPI.

Figure 3-10 Representative photomicrographs of GFP+ follicles six weeks after transplantation. GFP+ cells were confined to the transplanted hair follicle and were not found in the interfollicular epidermis (18 μm cryosections). The dotted line roughly indicates the border between transplanted hair follicle and surrounding tissue and red arrows show the edge of the transplanted follicle in epidermis. White asterisk: transplanted follicle. Scale bar: 100 μm.
3.4 Discussion

In this study, we describe a refined hair follicle transplantation protocol that uses vibrissae from GFP transgenic mice for transplantation into the back skin of recipient nude mice. The graft survival rate was similar to human scalp hair restoration surgery (about 90%). GFP-positive follicles were readily identified in histological sections and integrated normally into GFP-negative skin. Transplanted follicles were innervated and their cavernous blood vessels appeared to be connected to the host circulation system. GFP+ dermal and epidermal cells remained confined to the site of implantation and did not expand within the host tissue. The follicle epidermal cells did not participate in homeostasis of the interfollicular epidermis. This later result was anticipated, as Ito et al. have previously shown that hair follicle cells do not participate in homeostasis of skin and it is only during wound healing that epithelial cells from hair follicle mobilize and participate in epidermal repair [136]. Therefore, no surgical wounding means no migration of follicle stem cells to the interfollicular epidermis, which could otherwise complicate the interpretation of experiments on grafted follicles in Chapter 4 and 5.

Vibrissae follicles enter telogen after transplantation, and then spontaneously re-enter anagen. Similar follicle cycling is seen after human hair transplantation and after transplantation of skin patches in mice [604]. In nude mice, the new hair fibre appears two weeks after hair transplant, suggesting that the transplanted follicle might be in telogen during the first two weeks. This is important for planning experiments that use this model if the timing of the hair cycle affecting the experiment.

Like normal hair follicles, the transplanted vibrissae can be induced to cycle by plucking of their hair shafts. This allows induction of anagen simply by hair plucking, and then observation and measurement of hair growth in experimental studies. The control over the hair cycle timing also makes it possible to investigate the contribution of hair follicle cells at different stages of the hair cycle in this model.

The growth rate of normal vibrissae in mice is about 1mm/day during the anagen phase [605]. After plucking a growing whisker, a delay of 8-11 days is seen before a new hair
shaft appears [605]. Although the lag phase in the follicle producing a hair fibre has not been measured in the transplantation model presented in this chapter, the overall growth of transplanted vibrissae to 6.5±2.0 mm at fifteen days after plucking is consistent with these values.

Vibrissae at different positions in the mystacial pad have different patterns of hair growth [605]. The posterior vibrissae are larger with a longer hair shaft compared to the anterior follicles. The vibrissae appeared to maintain these different characteristics after transplantation, with the larger follicles producing longer and thicker hair shafts. Overall, the grafted vibrissae had the normal anatomy and behaved as normal hair follicles.

3.4.1 Comparing with alternative techniques

The hair transplantation protocol we describe has several advantages over alternative methodologies. The hairless phenotype of the nude mouse hosts is beneficial, making it easy to secure transplanted hair follicles with dressings needed after hair transplant. In addition, it is easy to identify the transplanted follicles under the skin and to detect and measure the growing hair fibres from transplanted follicles.

In a study by Amoh et al. [323] whisker hair follicles were transplanted into the skin of nude mice to study the involvement of hair follicle cells in angiogenesis. In this study, micro-dissected vibrissae follicles were placed inside an incisional wound in the dorsal skin of nude mice and kept in place by suturing the incision. Damage to recipient skin by incisional wounding or suturing can potentially alter the transplanted hair follicles or the skin’s response to wounding. In our technique however, no suturing is required, which makes it simpler and minimises damage to the skin. Vibrissae are easy to isolate and micro-dissect with the flip side technique, and their follicles produce large hair fibres, which are easier to detect and measure than smaller pelage hair follicles [593].

Furthermore, as shown in Figure 3-10 A and B, transplanted vibrissae follicles did not contain any surrounding donor tissue from the interfollicular epidermis. This makes it possible to differentiate migration of hair follicle cells from the interfollicular epidermis in
lineage-tracing experiments using genetically labelled donor follicles. In contrast, it would be very difficult to exclude surrounding interfollicular epidermis from the hair follicles when micro-dissecting pelage hair follicles. This was seen in the transplantation experiments performed by Sato et al. where GFP+ interfollicular epidermis was also transplanted into the nude mice [593].

The disadvantage of this protocol perhaps is that vibrissae follicles are specialized hair follicles. Vibrissae follicles are important proprioceptive sensory organs in mice; with thick capsules containing cavernous blood vessels and no analogous organ in humans has been identified. Previous hair follicle epithelial cell migration in wound epithelium has been studied in the case of pelage hair follicles, and it is not known if vibrissae follicles behave the same way during the skin repair. Although epithelial stem cells have been identified in vibrissae follicles [561], there is a possibility that these cells only contribute to the maintenance of follicle structure during cycling and not the skin repair. This possibility will be investigated in Chapter 4.

Adaptation of this protocol for human scalp follicle transplantation into nude mice would be a suitable model to study human follicle behaviour in wound healing; however, this experiment was not done because of the limited time available during this study.

3.4.2 Application in hair and skin biology studies

Follicle transplantation models have several applications in skin and hair biology research. Grafting follicles from knockout or knock-in mice would enable studies of genetically modified follicles in the context of wild-type skin. This approach allows investigation of signalling pathways and genes of interest, specifically in follicle cells. Investigation of the role of specific genes or pharmacologic agents with a focus on hair follicle may reveal specific regulatory molecules and wider exploitation in hair and skin biology in health and diseases.

The advantage of grafting transgenic follicles over simply looking at the phenotype of the transgenic mouse is that the indirect effects of the gene in other tissues would be removed.
Thus, follicle transplantation models could be used to explore the migration of hair follicle cells and their contribution to homeostasis, wound healing, skin circulation, and diseases such as melanoma and non-melanoma skin cancer. Murine models of human skin disease could be used to investigate the role of follicle cells in these diseases.

Variations to the technique described would be possible using donor follicles from humans, transgenic mice and alternative recipients. Other strains of mice such as SCID mice (also immune deficient) have stronger and thicker skin, which should allow the transplanted hair follicle to be stable in the skin during the first week after transplantation. Considering the size of human scalp hair follicles, which are narrower and longer than mice vibrissae, it should be easy to maintain them in the skin after transplantation, and engraftment might even work better, or faster, given there is no blood sinus or thick capsule.

Transplanting precisely micro-dissected vibrissae follicles makes it possible to study the migration of specific cell populations from hair follicles, by using transgenic mouse donor follicles with cell populations that are fluorescently or luminescently labelled. There are cell populations in hair follicles and skin which are phenotypically similar but which nevertheless may behave differently. For example, several molecular markers such as K15 [136] are common to both stem cells in hair follicles and to interfollicular skin. Transplantation of hair follicles from a genetically labelled donor into a non-labelled host would allow follicular and interfollicular cells to be distinguished and their behaviour to be evaluated in vivo.

### 3.4.3 Application in wound healing research

Overall, the vibrissae follicle transplantation technique established in this chapter was found to be suitable for subsequent experiments that study the involvement of transplanted hair follicles in wound healing (Chapter 4 and 5). In this technique, the GFP⁺ follicles integrated normally into GFP negative skin and the vibrissae follicles grew normally. In addition, the transplanted follicle did not contain the donor interfollicular epidermis and GFP⁺ cells were confined in the transplanted hair follicle and were not found in the
interfollicular epidermis (n=5). This made it possible to distinguish the migrating hair follicle cells in the interfollicular epidermis in wound healing experiments.

A possible limitation of this model is that vibrissae are a special sub-population of hair follicles and in some respects unusual. Vibrissae hair follicles have different patterns of hair cycling compared to pelage follicles. Unlike pelage follicles their hair cycle is not synchronous. In addition, exogen does not occur in vibrissae unless the next hair cycle has started and a new hair follicle is grown [606, 607]. Furthermore, vibrissae follicles have cavernous sinuses and a thick capsule, and this may prevent hair damage during micro-dissection, but may also cause them to behave differently during skin repair. For this reason involvement of transplanted vibrissa follicle in re-epithelialization, dermal repair, innervation and vascularization may not be the same as pelage or human scalp follicles. Nevertheless, rodent vibrissae are a widely used and accepted experimental model for studying many aspects of hair biology.
Chapter 4

Hair Follicle Transplant for Wound Healing

4.1 Introduction

As discussed in Chapter 1 (1.7) multiple populations of keratinocyte stem cells have been recognized in different locations of the skin, including hair follicles. Hair follicles have been considered as an attractive source of stem cells for treatment of chronic wounds (Table 1-12). In addition to being the stem cell reservoir for skin repair, hair follicles also respond to injury by shifting to rapid hair growth and increasing angiogenesis and innervation within the skin, which may influence the healing process.

In this thesis the hypothesis was that hair follicle transplantation around the wound site would not only provide a population of epithelial and mesenchymal stem cells, but also promote healing by angiogenesis and innervation. Using the hair transplantation technique established in Chapter3, wounds with transplanted GFP+ vibrissae follicles were compared with wounds without hair follicles. Migration of hair follicle cells in the dermal and epidermal compartments of granulation tissue was assessed and the effects of transplanted hair follicles in wound closure, vascularity, innervation and infiltration of macrophages were investigated. Migration of cells from transplanted hair follicles was also assessed in distant wound, bone marrow and host whisker pad.

4.1.1 Participation of hair follicle cells in wound repair

As discussed in Chapter 1 (1.5.4 and 1.5.6) the hair follicle is considered a niche for epithelial and mesenchymal stem cells participating in skin wound healing. The contribution of hair follicle epidermal cells in wound re-epithelialization has long been noticed in humans. In superficial wounds where portions of hair follicles have remained intact, re-epithelialisation begins from the remnants of hair follicles, and wounds heal more quickly and re-epithelialize evenly across their entire surface compared to deep wounds where the hair follicles have been destroyed [297]. Participation of hair follicle epidermal
cells in wound re-epithelialisation has likewise been confirmed in several lineage analysis animal experiments [136, 298, 299]. These studies showed that hair follicle cells contribute up to 30% of cells in the healing epithelium [136, 299] (discussed in 1.5.4.)

It has also been suggested that hair follicle dermal cells participate in granulation tissue and might be the origin of pericytes and myofibroblasts in skin wounds. Jahoda et al. (2001) hypothesised that hair follicle dermal cells are reservoirs of fibroblasts and that mesenchymal stem cells participate in the repair of dermis [313]. These authors argued that the dermal sheath expresses α-SMA in vivo and in vitro, as do cultured dermal papilla cells. As α-SMA is considered a marker for pericytes and myofibroblasts (discussed in 1.5.6), these authors concluded that hair follicle dermal cells are capable of differentiation to myofibroblasts and suggested that hair follicles may be the origin of myofibroblasts in wounds [313]. In addition, cultured hair follicle dermal cells when transplanted into skin wounds were incorporated into the granulation tissue [321]. However, this hypothesis was not confirmed in a lineage tracing experiment by Driskell et al. (2013) [50]. As discussed earlier, lineage tracing in 8 mm excisional wound healing experiments in mice, showed that granulation tissue myofibroblasts have mainly originated from lower dermal fibroblasts, and the hair follicle dermal cells, like upper dermal fibroblasts, appear only at a later stage of wound healing (day 17) [50].

In this chapter, cells originating from transplanted GFP transgenic donor mice hair follicles were traced in the dermal and epidermal components of excisional wounds in recipient nude mice. By histological examination of wounds we investigated participation of GFP+ cells, originating from transplanted hair follicles, in dermal and epidermal components of healing wounds and also in wound vascularization.

4.1.2 Circulating mesenchymal stem cell

Mesenchymal stem cells have been identified in hair follicles and various other tissues. There is also some evidence for the existence of circulating mesenchymal stem cells which, like immune cells, migrate to injury sites and participate in wound vasculature, differentiate into myofibroblasts and even trans-differentiate into keratinocytes (discussed
in 1.6.1, ‘Bone marrow mesenchymal stem cells and circulating fibroblast, Page 87). These facts suggest the possibility of two-way cell trafficking between various niches of mesenchymal stem cells in both the hair follicle and the bone marrow.

According to this hypothesis, the hair follicle is a reservoir of stem cells which not only participate in skin wound healing, but may also migrate to bone marrow and be a part of the ‘stem cell pool’ in the body. This hypothesis is the rationale of one of the aims in this chapter, which investigates the migration of cells from the transplanted hair follicle to bone marrow, distant wounds and the host vibrissae.

4.1.3 **Hypothesis and aims**

This chapter will examine migration of hair follicle dermal and epidermal cells from transplanted hair follicles during the wound healing response and also investigate whether transplanting vibrissae hair follicles around an excisional wound would improve the wound healing process.

**Hypothesis:**
- Hair follicle transplantation improves wound healing
- Hair follicle stem cells as members of a wider stem cell population are involved in the healing process and migrate to adjacent wounds as well as distant wounds and bone marrow during the healing response.

**Study aims:**
To assess the migration of cells from hair follicles in:
- Local wounds
- Distant wounds
- Bone marrow
- Host whisker

To characterise the effect of hair follicle transplantation on wound healing with respect to:
- Wound size
- Vascularization
- Innervation
- Macrophage infiltration
4.2 Materials and methods

4.2.1 Experimental design

A series of experiments was performed where isolated vibrissae from GFP transgenic mice were transplanted as described in the previous chapter. Wounds were then created at the transplant site. Two sets of experiments were undertaken. The first aimed to optimise wound induction and management procedures. The second quantified the healing rates of full thickness wounds with and without transplanted follicles, and evaluated the contribution of follicle cells to wound tissues. The overall design is summarised below, followed by specific methods.

Optimisation of wound induction
Initial experiments aimed to optimise the wound induction and management procedures for the purpose of tracing the contribution of follicle cells to healing. In all cases, circular full thickness wounds were induced using a 6 mm biopsy punch. Two major variables were compared:

- The spatial arrangement of transplanted follicles, relative to an excisional wound: follicles were grafted in either a rectilinear matrix (Figure 4-1, A) or in a circular ring at the periphery of the intended wound site (Figure 4-1, B).
- The timing of wound induction relative to follicle grafting: Wounds were induced either on the day of follicle grafting (Figure 4-1, C) or 6 weeks later (Figure 4-1, D).

![Figure 4-1](image)

**Figure 4-1** Optimising the wound induction experiments. A: spatial arrangement of transplanted follicles, relative to excisional wounds. B and C: timing of wound induction relative to follicle grafting. B: hair transplantation and wound healing at the same time. C: hair transplantation prior to wounding.
**Effect of follicles on wound healing**

The optimised experimental design (Figure 4-1, D) was used to assess the effect of hair follicle transplant on wound healing. Hair follicles (25-30) were micro-dissected from whisker pads from each of six GFP transgenic mice. Isolated follicles from each GFP positive mouse were transplanted in a circular pattern in the right upper back of each nude mouse (n=6). As shown in Figure 4-2, six weeks after hair transplantation, full thickness wounds were applied to the right side (within the circle of transplanted hair follicles) and the left side (away from transplanted hair follicles) of the upper backs of the animals. The edge of the wound on the right side was within 1-2 mm from the transplanted hair follicles. Wounds were photographed on days 3, 5 and 7. Animals were sacrificed on day 7 by cervical dislocation and wounds and bone marrow were harvested (for full experimental details refer to 4.2.7and 4.2.8).

![Experimental design of hair transplantation and excisional wound in nude mice.](image)

Healing was assessed in wounds with and without hair transplants by measuring the area of wounds in images from live animals as well as wound diameter by Masson’s trichrome staining of tissue. Innervation, angiogenesis and infiltration of macrophages were compared in right and left wounds by histology analysis.
**Hair follicle cell migration**

Migration of cells from hair follicles in wound dermis and epidermis was investigated in histology studies seven days after wounding. At the same time bone marrow was also harvested and evaluated for migrating GFP+ cells.

Migration of follicle-derived cells expressing GFP was firstly examined in wounds close to hair transplants. Wounds surrounded by transplanted hair follicles (right wounds) were examined in frozen tissue as well as in paraffin embedded tissue by IHC (using an anti-GFP antibody).

To detect any possible migrating cells in distant tissue, bone marrow specimens were examined by flow cytometry analysis. Half of the wounds away from hair transplantation (left wounds) and the left whisker pads were also frozen for analysis. Serial sections at 18-20 µm throughout the frozen tissue were examined by direct fluorescence to detect GFP+ cells.

4.2.2 **Animals**

All animal experiments were conducted with the approval of St Vincent’s Health Human Research Ethics Committee (AEC number 45/10). Animals were maintained in PC2 animal housing room with a 12-hour light/ dark cycle. Mouse food and water were available ad libitum.

**Nude mice**

4- 6 week-old male athymic nude mice (BALB/c Foxn1nu/Arc) were obtained from the Animal Resource Centre in Western Australia. Nude mice were used as the recipient for hair follicle transplantation and wound healing experiments.
**GFP transgenic mice**

Vibrissae from 4-6-week-old male GFP transgenic mice (GFP under the control of a chicken beta-actin promoter as described in 2.1.1.) were used as donors of hair follicles for hair transplantation into nude mice.

4.2.3 **Aseptic technique and anaesthesia methods**

All surgical instruments were sterilised at 125° C for 15 min and all surgical procedures were carried out in a sterile environment. Mice were anaesthetised by inhalation of 2% (v/v) Isoflurane (Baxter Healthcare Ltd. Qld Toongabbie, NSW, Australia) during all hair transplantation procedures, wounding, and *in vivo* wound imaging. The depth of anaesthesia and the respiration rate were monitored throughout the procedure with the animals secured on the operating table by taping down all limbs.

4.2.4 **Vibrissa hair follicle micro-dissection and transplantation**

4-6-week-old male GFP transgenic mice were killed by cervical dislocation, and their vibrissae follicles were excised as described in 3.2.4. Isolated GFP⁺ follicles were transplanted into nude mice, as described in detail in Chapter 3, 3.2.5.

As described in 4.2.1 a series of experiments were performed to optimise a protocol to study the role of hair follicle transplantation in wound healing.

4.2.5 **Optimising the excisional wound experiment**

Initial experiments were aimed at optimising the wound induction and management procedures for the purpose of tracing the contribution of follicle cells to healing. Two major variables were the spatial arrangement of transplanted follicles at the wound site and the timing of wound induction relative to follicle grafting.

The results are summarised in Table 4-1. Isolated follicles were transplanted either in four rows, five vibrissae follicles in each row (Table 4-1, A), around the wound immediately after an excisional wound (Table 4-1, C), and then in the optimised protocol, micro-
dissected follicles were transplanted in a circular pattern and the excisional wounds applied six weeks after hair follicle transplantation (Table 4-1, B and D).

**Table 4-1 Optimising the excisional wound experiment**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Technique</th>
<th>Result</th>
<th>Final exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Linear hair transplantation ((n=3))</td>
<td>Hair follicles were transplanted in 3-4 horizontal rows, 5 HF in each row in an area of approximately 1 cm² in right upper back of the animal. (Figure 4-1, A)</td>
<td>Some hair follicles were harvested during the wounding and few hair follicles remained around the wound.</td>
<td>✗</td>
</tr>
<tr>
<td>B) Circular hair transplantation ((N=7)) Used for the optimized wound experiment</td>
<td>Hair follicles were transplanted around a 6mm circle in the right upper back of nude mice. (Figure 4-1, B)</td>
<td>Maximum number of hair follicles surrounded the wound</td>
<td>✓</td>
</tr>
<tr>
<td>C) Wound applied at the time of transplantation ((3 \text{ animals with dressing and 3 animals without dressing).} \ (N=6))</td>
<td>6 mm punch biopsy applied to the back of the 7 nude mice on right and left side and hair follicles transplanted with a 1 mm margin around the wound (Figure 5 1, C)</td>
<td>Hair follicles came off without dressing. Dressing interfered with contracture of the skin around the wound. This experiment was excluded from final results</td>
<td>✗</td>
</tr>
<tr>
<td>D) Wound applied after establishment of hair follicle transplantation in animals from experiment B. ((N=7)) Used for the optimized wound experiment</td>
<td>6 weeks after hair follicle transplantation two 6 mm punch biopsies applied to the back of the nude mice on right (with transplanted follicles) and left side (without transplanted follicles). (Figure 4-1, D and Figure 4-2)</td>
<td>Transplanted hair follicles were successfully established at the time of wounding.</td>
<td>✓</td>
</tr>
<tr>
<td>E) Wound splinting with a silicone sheet ((N=4))</td>
<td>Silicone sheets were sutured on 6 mm excisional wounds</td>
<td>Sutures cut the skin and silicone sheets came off</td>
<td>✗</td>
</tr>
</tbody>
</table>

When hair follicles were transplanted in a matrix of three or four rows of five follicles, a large number of the transplanted hair follicles were removed during the punch biopsy, and very few hair follicles remained around the wound.
Following these procedures, the circular pattern of transplantation was designed where the follicles were grafted in a ring. The punch biopsy was applied in the middle of a circle and the wound therefore had the maximum contact with all transplanted hair follicles.

When wounds were applied at the same time as transplantation, the hair follicles needed a protective dressing during the first six days (3.3.1). However, this dressing interfered with wound healing as it kept the wound moist and physical contact of the dressing with the wound prevented the contraction of the skin surrounding the wound (C, Table 4-1). In contrast, when wounds were applied six weeks after transplantation, the grafted hair follicles had already been established and there was no need for dressing.

An excisional wound mouse model which prevented skin contraction by splinting the wound with a silicone sheet (Table 4-1, E, Figure 4-3) has also been tried in a total of four animals, as described by Galiano et al. [608]. In brief, a 0.5mm-thick silicone sheet with a 6 mm punch biopsy cut was glued and sutured onto the excisional wounds in such a way that the wound was centered within the splint. Silicone sheets were cut either in a square or circular shape and fixed to the skin either by four interrupted sutures (Figure 4-3, A) or with continuous sutures (Figure 4-3, B) respectively. However, the thin and fragile skin in nude mice could not tolerate the splints sutured into the skin and the splints separated from skin on the second and third day after wounding.

Figure 4-3  Excisional wound with silicone splint experiment.
4.2.6 Optimised protocol for excisional wound surrounded by transplanted follicle

As a result of the above experiments, it was determined that the optimal wound model for assessment of the hair follicle contribution to healing was as follows: hair follicles were transplanted in a circular pattern (B in Table 4-1) and after establishment of the transplants a wound was created in the middle of the transplanted area using a 6 mm punch biopsy tool. Consequently the wound created maximum contact with the transplanted hair follicles (D in Table 4-1). A total of seven animals were used in the optimised excisional wound protocol.

Circular pattern of hair follicle transplantation

Between 25-30 vibrissae hair follicles from GFP transgenic mice were transplanted in a circular pattern in the right upper back of each nude mouse. As shown in Figure 4-4, hair follicles were transplanted at an acute angle facing toward the animal’s tail, parallel to the host pelage. A circular patch of punched sticky tape with a diameter of 6 mm was placed on both right and left sides with 1cm spacing as a guide for both transplant procedures (Figure 4-4, green arrow) and mock (control) procedures (Figure 4-4, red arrow).

![Figure 4-4](image)

Figure 4-4 Circular pattern of transplanted follicles used in excisional wound experiments. Transplanted follicles on day 0 (A), and after six weeks (B). Patches of punched sticky tape, with a diameter of 6 mm, were placed on both right and left sides to guide both transplant (green arrow) and mock procedures (red arrow).

Vaseline gauze dressing was applied to keep the hair follicles in place, followed by surgical bandage tape as described in 3.2.5. Baytril 25 (25mg/ml Bayer 20 mg / kg /day)
was added to the animal’s drinking water for five days after hair transplantation to prevent infection and the dressing removed after three days.

**Full thickness excisional wound experiment**

After six weeks, the hair shafts of transplanted follicles were cut 3-4mm above the skin surface, immediately before full thickness excisional wounds were applied. Under general anaesthesia (Isoflurane inhalation), two full skin thickness excisional wounds were created on the dorsum of the mouse extending through the panniculus carnosus using a 6 mm biopsy punch (Biopsy punch Stiefel). Wounds on the left and the right sides were at the level of the animal’s shoulder and were spaced 1cm apart. To ensure equal depth of wounds on both sides, dorsal skin was first marked with a surgical marking pen. A 23G needle was inserted at the midline of the dorsum, the animals were then turned to lie on their left side and the dorsal skin was folded. Two full thickness punch biopsies were applied with a 6 mm punch biopsy needle (Figure 4-5).

![Figure 4-5 Full thickness wound technique.](image)

Temgesic (SC.05 mg/kg SC) was administered in mice subcutaneously for pain relief. Wounds were left open and animals were put in individual cages without sawdust to minimise contamination of the open wounds. Animals were reviewed after one hour and then daily.

On days 3, 5 and 7 the mice were anesthetized (by Isoflurane inhalation) and the wounds were photographed (Cannon EOS 500D), with a measuring tape at the level of the wound to be used as a scale in image analysis. Animals were sacrificed by cervical dislocation on day 7 and left and right wounds, as well as animals’ vibrissae and bone marrow, were harvested.
4.2.7 **Bone marrow harvesting and FACS sorting**

To identify and quantify migrating GFP^+^ cells in bone marrow, collected bone marrow were examined using fluorescence-activated cell sorting (FACS).

After cervical dislocation and harvesting of the excisional wounds, bone marrow was collected from the animals’ femurs as described in 2.3. Cells were sorted using a BD FACS Aria cell sorter (BD Biosciences) as described in 2.4.

4.2.8 **Harvesting wounds from nude mice**

At day 7 the mice were anesthetised by inhalation of 2% (v/v) Isoflurane and wounds were collected with a margin of at least 5 mm. The animals were then sacrificed by cervical dislocation after harvesting of excisional wounds to avoid disruption of the weak wound tissue.

To prepare frozen and paraffin embedded tissues, each wound was cut in half (across the shortest diameter of the wound in circular excisional wound, Figure 4-6), placed in histology cassettes and fixed with PFA. In each wound the medial pieces were prepared to freeze and the lateral pieces prepared for paraffin embedded tissue as described below.

![Figure 4-6](image)

**Figure 4-6**  Wound harvesting technique from nude mice on day 7. Harvested skin cut in half across the narrowest diameter of the wound.

**Paraffin embedded tissue**

The lateral halves of the bi-sectioned wounds were fixed in PFA for 24 hours and then stored in PBS at 4°C prior to processing. Tissues were dehydrated through a graded alcohol series and infiltrated with paraffin in a six hour program using an automated tissue processor (Shandon Hypercentre XP). Immediately following processing, the tissues were
embedded in paraffin blocks with the section side facedown to allow vertical sectioning of skin. Paraffin embedded tissues were cross-sectioned at 5μm (Biocut 1130 microtome) and sections were mounted on poly-lysine coated glass slides (Thermo scientific), then dried at 37°C overnight, to be used for histology staining.

**Frozen tissue**

One half of the bi-sectioned wounds (medial part) were placed in 10%(w/v) PFA for six hours, followed by 15% (v/v) sucrose for 24 hours at 4°C. They were then placed in OCT (Tissue-Tek, Optimal Cutting Temperature Compound) with the section side facing down and frozen with liquid nitrogen as described in 2.4.

Frozen tissues were cut at 18μm sections (Reichert-Jung Cryocut 1800), dried at room temperature overnight and stained the next day or stored at -80 °C.

4.2.9 **Histology studies**

**Direct detection of GFP-Expressing cells**

Cells containing GFP can be easily visualised in frozen sections [609]. To detect GFP+ cells, slides with frozen sections were simply washed in distilled water and counterstained with DAPI (1μg/ml) for 1 min, then washed in PBS and mounted and covered with a coverslip.

**Haematoxylin and Eosin staining**

Haematoxylin and Eosin staining were used in paraffin-embedded wound sections, as described in Table 2-2.

**Masson’s trichrome staining**

Masson’s trichrome staining was used to study wound size in paraffin-embedded wound sections as described in Table 2-3. Mature collagen in undamaged dermis appears as
intense blue in Masson’s trichrome staining and can be discriminated from a light, and diffuse blue staining of poor collagen bundles in the granulation tissue.

**Immunohistochemistry staining**

Slides from paraffin embedded wound tissue were stained using Diaminobenzidine chromogen (DAB) to study and compare wounds with and without hair transplantation.

Antibodies prepared against GFP (Abcam, AB 290) were used to detect migrating GFP+ cells, and antibodies against CD31, PGP9.5 and F4/80 were used to measure angiogenesis, innervation and the presence of macrophages respectively. The antibodies and their dilutions are summarised in Table 2-4 and Table 2-5. Protocols of staining with antibodies against CD31, F4/80, PGP9.5 and GFP are given in Table 2-6, Table 2-8, Table 2-9 and Table 2-10 respectively.

Mouse tissue was used for a positive and negative control and to validate each antibody, primary antibodies were replaced with matching isotype pre-immune serum to determine the level of nonspecific binding.

**Anti PGP9.5 staining**

The cutaneous nerve distribution in wounds with and without hair transplantation was examined using IHC with antibodies against the general nerve marker PGP 9.5, as described before [610].

PGP 9.5 is a cytoplasmic neuronal marker commonly used to investigate small sensory fibres in skin biopsies. Anti-PGP 9.5 targets ubiquitin carboxyl-terminal esterase L1, an enzyme found specifically in neurons [611, 612].

Innervation can be localised in skin, especially around the hair follicles. The localised nerve distribution around the follicles makes it difficult to assess the skin innervation in 2D histology slides. To obtain a better estimation of wound innervation, two sections, 500 µm apart (100 consecutive sections apart), were taken from each wound and IHC was done with anti PGP9.5 as described in Table 2-9.
**Anti GFP staining**

Although cells containing GFP can be easily visualised in frozen sections, the fluorescent activity of GFP protein is eliminated during the processing of the paraffin-embedded tissue. Anti-GFP staining was used to detect transplanted GFP positive cells in the paraffin embedded tissue using the immune peroxidase staining as described in Table 2-10.

**Immunofluorescence staining**

**CD31 and anti GFP double Immunofluorescence staining**

Double fluorescent staining of sections from paraffin-embedded tissue was done for GFP and CD31 antigens, (protocol in Table 2-11). In this staining the secondary antibody of goat anti rabbit IgG Alexa Fluor 488 (Jackson Imm 111-545-144) is a specific antibody against rabbit IgG with a greater affinity to the Rabbit anti GFP (IgG Isotype, AB 290) and not to the Biotinylated Rabbit anti Rat antibody (Polyclonal, DAKO E0468). Negative controls were clear (using Isotype control for each individual antibody).

**Cytokeratin immunofluorescence staining**

Anti pan-cytokeratin antibody was used in immunofluorescence staining to show the epithelium and to detect migrating GFP+ epithelial cells (expressing both GFP and pan-cytokeratin) in frozen tissue sections. A detailed protocol of pan cytokeratin immunofluorescence staining is described in Table 2-12.

**4.2.10 Photography and image analysis**

Bright-field and fluorescent images were taken using a digital fluorescence microscope (Olympus BX-61). Macroscopic images of wounds on mice were taken with a Cannon EOS 500D camera.
4.2.11 **Wound diameter measurement**

Wound diameter was measured in images from Masson’s trichrome histology staining (Table 2-3) of paraffin block sections. Photos were taken with an Olympus BX-61 microscope with x4 magnification. ImageJ software was used to analyse the wounds.

The borders of the wounds were considered the points where the normal collagen colour was apparent on the wound borders in dermis (white arrow in Figure 4-7 A &B) or where normal epithelium appeared in the epidermis. Normal epithelium appears as a thin epithelium with host hair follicles (red arrow in Figure 4-7 A &B).

The minimum diameters measured either in dermis or in epithelium were compared between wounds with and without hair transplantation in each animal using the paired Student’s t-test.

![Figure 4-7 Wound size measurement techniques in the excisional wound experiments. A and B: wound diameter measured in Masson’s trichrome histology staining. C: wound area measurement using ImageJ program.](image)

4.2.12 **Wound area measurement**

Individual wounds on the right and left sides were photographed (Cannon EOS 500D) on days 3, 5 and 7, with animals under general anaesthesia.

The border of the wound scab was considered the wound border (Figure 4-7, C) or, where there was no scab, the normal epithelium. Wound area was measured in mm². Measurements were done doubly blinded and wounds were considered completely closed.
when the wound area was equal to zero [608]. Measurements were done, using digital image analysis, ImageJ software.

4.2.13 Border of granulation tissue in Masson’s trichrome staining

Masson’s trichrome (MT) staining was used to define the GT area for histology staining on subsequent slides.

The border of granulation tissue in Masson’s trichrome stained slides was defined as epidermis on surface, sub-dermal fat or muscles at the base and the presence of host hair follicles in the lateral border of granulation tissue (Figure 4-8).

4.2.14 Capillary density measurement and analysis

Capillary density was assessed morphometrically by examining at least 200 points in granulation tissue per section of the wound which was immunostained for endothelial cells with an anti-CD31 antibody.

Immunostained slides were examined in an Olympus BX-61 microscope and images were taken with ×10 magnifications. Multiple images were taken to cover all granulation tissue area in each slide. Blood vessels were counted with the grid generated by ImageJ software.

The border of granulation tissue in the histology images was defined as described in 4.2.13. The percentage of blood vessel tissue in the granulation tissue was calculated as:
Vascularity of granulation tissue was compared in two groups of excisional wounds with and without hair transplant using the Student’s t-test.

4.2.15 Wound innervation and macrophage density measurement

Innervation and macrophage infiltration of wounds was compared in two groups of wounds with and without hair transplantation using Aperio scan and image analysis. PGP9.5 and F4-80 immunostained sections were scanned with the Aperio ScanScope system (Vista, CA, USA) and analysed with ImageScope (Aperio). Times 10 magnifications were selected in each section.

One slide per wound was assessed for macrophage infiltration using F4-80 antibody. For staining with PGP9.5, two slides 500µm apart (100 consecutive sections apart) were selected in each wound and the average percentages for each wound were compared in wounds with and without hair transplantation.

The border of granulation tissue was defined as described in (4.2.13). Epidermis was not included in macrophage infiltration assessment; however, it was included in innervation assessment.

Results are expressed as the ratio of positive pixel stained nerves or macrophages to the total pixel of granulation tissue (or positive pixel stained nerves or macrophages relative to the total pixel of granulation tissue) multiplied by 100.

\[
\frac{\text{Positive pixel in granulation tissue}}{\text{Total pixel of granulation tissue}} \times 100 = \text{Percentage of macrophage infiltration in granulation tissue}
\]
4.2.16 **Statistical analysis**

The data was measured in percentage (innervation, vascularity and macrophages infiltration) shown as mean ± SEM (n=6). Measurements in wounds with and without hair transplant were compared and statistical significance was analysed on GraphPad Prism Software and the GenStat program using one- or two-way ANOVA or paired Student’s t-test, and subsequent Bonferroni post-hoc test. A value of P<0.05 was considered significant.

\[
\frac{\text{Positive pixel in granulation tissue}}{\text{Total pixel of granulation tissue}} \times 100 = \text{Percentage of nerves in granulation tissue}
\]
4.3 RESULTS

4.3.1 Excisional wound experiment

As described in 4.2.1 and 4.2.5 a series of experiments were performed to optimise a protocol to study the role of hair follicle transplantation in wound healing. The optimised protocol (circular pattern of follicle transplantation and excisional wound 6 weeks after follicular transplantation) was used for all the excisional wound experiments described in this chapter, in a total of seven animals.

One of the animals was excluded on day 0 of wounding as the skin was folded during the wounding and the left wound increased in size. A total of six animals were sacrificed on day 7, and right and left wounds, bone marrow and whisker pads were harvested (as explained in 4.2.7 and 4.2.8).

4.3.2 Quantitative analysis of wounds with and without hair transplant

*In vivo wound area measurement*

In one out of six animals the scab of right and left sides were joined together. This animal was excluded from wound area analysis, as it was difficult to define the borders of right and left wounds on images (Figure 4-9, red arrow).
Figure 4-9  Images of right and left wounds in six mice at day seven. Animal #6 was excluded from wound area analysis because the scabs of right and left wounds were joined together and it was difficult to define the border of right and left wounds in the images.

The hair fibres which were cut short just before the wounding, were observed to be growing in all of the transplanted follicles during the seven days after wounding, suggesting that the follicles were in the anagen phase of the hair cycle.

Figure 4-10  An example of excisional wounds on day 3, 5 and 7 in two animals.
Wound area was measured in images from day 3, 5 and 7 in a total of five animals. Figure 4-10 is an example of wounds at day 3, 5 and 7 in two animals.

![Figure 4-10](image)

**Figure 4-11** Comparing wound area in wounds with (right wound) and without (left wound) hair transplant on day 3, 5, and 7. The wounds with hair transplant had smaller area, and this difference was statistically significant on day 3 and 5. Results are shown as mean ± SEM.

Analysis of wound area at different time points revealed a reduction of wound size on the right side with hair transplant. As is shown in Figure 4-11, wounds on the right side were smaller than those on the left. The average difference between right and left wounds was about 4 mm² on day 3 and 5, and 2.25 mm² on day 7. Using analysis of variance (ANOVA), overall this difference was significant (P value = 0.012). However, ANOVA suggested that the effect of hair transplantation was not time dependent and the difference in wound size did not change at different time points (P= 0.595). Further experiments need to be done to determine whether the effect of hair transplantation is time dependent. When the left- and right-sided wounds were compared at each time point independently, using paired Student’s t-tests, the differences were significant on days 3 and 5 only (P values of 0.0003, 0.0274 and 0.2576 at days 3, 5 and 7, respectively).

**Wound diameter measurement**

Wound diameters were measured in right and left wounds from six animals. Each wound biopsy was cut in half across the shortest axis of the wound after harvesting. Wound
diameter was measured in images from Masson’s trichrome histology staining of 5 μm paraffin sections (Figure 4-12).

![Figure 4-12](image)

**Figure 4-12**  Wounds diameter measured in Masson’s trichrome staining on day7 of wounds with (RT) and without (LT) hair transplant.

As discussed in 4.2.11, mature collagen in undamaged epidermis appeared as intense blue in Masson’s trichrome staining. This appearance can be compared with granulation tissue containing blood vessels, invading inflammatory cells and poor collagen bundles shown in less intense and diffuse blue in Masson’s trichrome staining. The thick healing epithelium
is located above the granulation tissue, has no hair follicles, and is distinguished from the thin, normal epithelium containing host peladge hair follicles (Figure 4-7).

The borders of the wounds were considered the points where the normal collagen is visible as intense blue in dermis (white arrow in Figure 4-7) or where there is normal epithelium on the epidermis (red arrow in Figure 4-7). The shortest diameters, measured either in dermis or epithelium, were compared between wounds with and without hair transplantation in each animal (Figure 4-12).

On average the diameters of wounds with hair transplant were 30% smaller than control wounds. As is shown in Figure 4-13, this difference was significant (p=0.024 t-test).

![Figure 4-13](image-url)

**Figure 4-13** Comparing the diameter of wounds with (RT) and without (LT) hair transplant on day 7. Wounds with hair transplant (HT) were 30% smaller than wounds without transplant (P=0.0236). Error bar: standard error.

### 4.3.3 Hair follicle cell migration

Migration of cells from transplanted hair follicles were assessed in recipient animals in the right (adjacent) and left (distance) wounds, as well as animals’ vibrissae and bone marrow, seven days after wounding. Flow Cytometry Analysis was used to detect GFP+ cells in the bone marrow and migration of GFP+ cells in wounds and whisker pad was assessed by histology.
Migration of cells from transplanted hair follicle to distant tissue

Harvested bone marrow specimens were examined by Fluorescence-activated cell sorting (FACS) as described in 2.3 and 2.4. One of the harvested bone marrow samples from nude mice with hair follicle transplant was lost (spilled) during the experiment. FACS was performed on the bone marrow samples harvested from 5 nude mice with hair transplantation, one GFP transgenic mouse (positive control) and one nude mouse without hair follicle transplantation (negative control). A total of 50,000 cells were studied by Flow Cytometry in each animal and no GFP cells were detected in bone marrow.

Migration of cells from transplanted hair follicles was also assessed in distant wounds (left wounds) and recipient left whisker pads in all six animals. Left wounds were harvested, cut in half and medial parts from each wound were frozen and left whisker pads were also harvested and frozen as per (4.2.8). More than 70 frozen sections (18µm thick sections) from each left wound or whisker pad tissue were examined for GFP+ cells under the fluorescence microscope. In this experiment no GFP+ cells were detected in any slides.

In the case of presence of GFP+ cells in bone marrow, distant wounds or recipient whisker pad, migrating cells would have been characterized for the expression of CD markers (as recommended by the International Society for Cellular Therapy [613]) by FACS and by immunohistochemistry. However, CD marker expression was not evaluated in these experiments, as no GFP-positive cells were detected.

Migration of cells from transplanted hair follicle to adjacent wound epidermis and dermis

25 to 30 vibrissae follicles were located within 1-3 mm from the edge of right-sided excisional wounds. The presence of GFP+ cells in the wound area was examined in right-sided wounds harvested on day 7.

All transplanted hair follicles were recognisable from host hair follicles in histology slides, as a vibrissae follicle is more than 10 times bigger than normal host pelage follicles. GFP+ cells were detected in all transplanted hair follicles, either in frozen sections (as GFP+...
cells) or in immunostained paraffin sections (using anti GFP antibody). GFP+ cells appear brown in colour after immunoperoxidase staining.

As shown in Figure 4-14 and Figure 4-15, GFP+ cells were detected in healing epithelium (Figure 4-14, B, red arrow, and Figure 4-15, A and B). Individual GFP+ cells were also found in normal epithelium (Figure 4-14, D), around the host hair follicle (Figure 4-14, E, double arrow head and Figure 4-15, C) and sebaceous gland (Figure 4-14, red asterisks).

**Figure 4-14** Representative photomicrographs of GFP immunostaining showing migration of cells from transplanted hair follicle in excisional wounds. GFP+ cells appear brown in colour in immunoperoxidase staining. GFP+ cells in the healing epidermis (B and C), in host hair follicle (E red double arrowhead) and in sebaceous gland (E, red Asterisks). Yellow arrow: transplanted hair follicle. Red arrows: migrating GFP+ cells.
Figure 4-15  Representative photomicrographs showing migration of GFP+ cells from transplanted hair follicle in epidermis (18 µm cryosections). Wound epithelium (A, white arrow). GFP+ cells in normal epidermis (A, B, C green arrow), and around host hair follicle (C, red arrowhead).

Figure 4-16 shows transplanted follicles detected within 1-2.5 mm distance from the excisional wound. As shown in the schematic picture of excisional wound surrounded by transplanted follicles (Figure 4-16, C), in histological tissue sections it is difficult to know from which follicle the migrating GFP positive cells have been derived. The origin of the migrating GFP positive cells in the healing epithelium could be adjacent follicles that have not appeared in the histology section. This will be discussed in 4.4.2. The migration of the hair follicle epithelial cells through intact epithelium will be discussed in in chapter 5.

Figure 4-16  Position of transplanted hair follicles and migrating cells in 2D histology images. A: Excisional wound surrounded by transplanted follicle. B: Migrating GFP positive cells in wound epithelium (with higher magnification). C: Schematic diagram of excisional wound surrounded by transplanted follicles showing migrating GFP positive cells (black arrow) in wound epithelium detected in histology sections (dotted line). It is difficult to know from which follicle the migrating GFP positive cells have been derived.
In just one out of six wounds, GFP\(^+\) cells were localised in healing dermis with a disorganised structure (Figure 4-17, red asterisks), probably due to damage and cross-sectioning of a GFP\(^+\) hair follicle during the wounding.

![Figure 4-17](image)

**Figure 4-17**  Representative photomicrographs showing migration of GFP\(^+\) cells from transplanted follicle in healing dermis (red asterisks), (18 µm cryosections).

### 4.3.4 Wound vascularization

Double immunostaining with CD31 and anti GFP antibody was performed on paraffin embedded sections from wounds close to the hair transplants, to detect participation of GFP\(^+\) cells in blood vessel formation. Wound vascularisation was also compared by CD31 immunohistochemistry (IHC) of wounds close to hair transplants and wounds away from hair transplants.

**Involvement of cells originated from transplanted hair follicle in blood vessels**

In the immunofluorescence double anti CD31 and anti GFP staining (described in Table 2-11), no cross-reaction between goat anti-rabbit IgG and Biotinylated Rabbit anti-rat antibodies was detected in the negative controls.

In double IHC staining with anti-CD31 and anti-GFP of wounds with transplanted hair follicles, GFP\(^+\) capillaries were detected around the intact hair follicles (Figure 4-18 A&B). GFP\(^+\) cells did not directly participate in granulation tissue vasculature (5 out of 6 wounds) (Figure 4-18, C), unless a disorganised and damaged hair follicle appeared within the granulation tissue (as in 1 out of 6 wounds), in which case GFP\(^+\) cells (Figure 4-17, red
asterisks) and several GFP+ blood vessels (Figure 4-18, D, white arrows) were localised within the granulation tissue.

Figure 4-18  Representative photomicrographs of CD31 and anti GFP double staining, showing involvement of hair follicle cells in blood vessel formation on day 7 after wounding. Transplanted hair follicle: white asterisks. GFP+ cells were found in blood vessels around the hair follicles (A and B, white arrow). No GFP+ vessels were found in the granulation tissue (C), except in one animal (D, white arrow).

**Capillary density**

Wounds with and without hair transplant were assessed for capillary density in six animals. Paraffin embedded slides were stained by CD31 antibody and assessed for capillary density in granulation tissue. Capillary density was assessed morphometrically by examining at least 200 grid points in granulation tissue in two groups of wounds with and without hair transplantation. Figure 4-19 shows wound area vascularization in right and left wounds in one animal as an example. All the granulation area was examined and all
points were counted. Between one and four images were needed to cover the whole wound area in each section.

![Image of histological sections showing CD31 immunostaining and histomorphometry assessment of vascular density of wounds.](image)

**Figure 4-19** Representative photomicrographs of CD31 immunostaining and histomorphometry assessment of vascular density of wounds. A and C: Left wound; B and D: right wound. C and D: Histomorphometry assessment of granulation, showing the grids and area quantified. Red arrow: Healing epithelium.

![Image of bar graph showing vascular density of wounds with and without follicular transplant.](image)

**Figure 4-20** Comparison of the vascular density of wounds with and without follicular transplant using histomorphometry analysis of the granulation tissue stained by anti CD31. The bar graph shows percentage area of blood vessels in granulation tissue. No significant difference of vascular density was observed between the granulation tissues of right and left wounds. Error bar: standard error.

As is shown in Figure 4-19 and Figure 4-20, granulation tissue in wounds was highly vascularised in both groups; however, there was no significant difference in wound vascularity between the two groups (N=6, P=0.768).
4.3.5 **Macrophage infiltration**

Wounds with and without hair transplant were assessed for macrophage infiltration in a total of six animals. F4/80 immunostained slides were quantified to assess macrophage infiltration in granulation tissue. Only one slide per wound was analysed, as macrophages were spread throughout the granulation tissue and were not localised (Figure 4-21).

![Figure 4-21](image)

**Figure 4-21** Representative photomicrographs and image analysis of F4/80 immunostaining showing macrophage infiltration of granulation tissue. Yellow line shows defined granulation tissue in excisional wounds with (right wound) and without (left wound) hair transplant. Images of scanned slides (A&B). Images by Aperio ImageScope (C & D). Negative pixel is shown in blue and the positive pixel in red.

Sections were scanned and analysed as per (4.2.15). As shown in Figure 4-22 there was an increase in macrophage infiltration in granulation tissue in wounds with hair transplantation (an average of 17.88 % in wound without hair transplants, compared to
22.1% in wounds with hair transplants); however, this result was not significant (p = 0.3028).

![Diagram showing macrophage density comparison](image)

**Figure 4-22** Comparison of macrophage density in F4/80 immunostained wounds with and without hair transplants. The bar graph shows percentage area of F4/80 positive tissue in granulation tissue. No significant difference of F4/80 positive areas were observed in right and left wounds’ granulation tissue. Error bar: standard error.

4.3.6 Wound Innervation

Cutaneous nerve distribution was examined by IHC (using anti PGP9.5, a general nerve marker [610]). Immunostained nerve bundles were detected around hair follicles. Figure 4-23 shows the nerve bundles (stained by PGP9.5, green arrow) around the transplanted hair follicles (red asterisks) and in the hypodermis.

Innervation can be localised in skin (discussed in 4.2.9), thus for more reliable assessment of nerve distribution in granulation tissue, two sections spaced 500µm apart (100 consecutive sections apart) were selected in each wound for staining with PGP9.5. The average percentage of two sections of each wound was compared in wounds with and without hair transplantation.
Immunostained slides were scanned by Aperio ScanScope. The border of granulation tissue in the wound images was defined using Masson’s trichrome staining on subsequent slides (as described in 4.2.13). The positive immunostained cells were quantified using Aperio ImageScope as per 4.2.15. Data was expressed as the percentage of pixels within granulation tissue that were positively stained for PGP9.5, which represents the percentage area of nerve tissue within the granulation tissue in these sections.

Figure 4-24 is representative histology images in one animal showing innervation of granulation tissue (IHC, by anti PGP9.5) in wounds with and without hair transplantation (two sections per wound, 500 µm apart). The green line is the defined granulation tissue. As shown in Figure 4-24, in images analyzed by Aperio ImageScope, negative pixels are shown in blue, and the positive pixels of nerve bundles are shown in red in the defined granulation tissue.

Wounds with and without hair transplant were assessed for innervation in a total of 6 animals. The average percentages across two sections for each wound were compared for wounds with and without hair transplantation (described in 4.2.15). As shown in Figure 4-25, wounds with hair transplant had four times greater nerve density than the wounds without hair transplant, and this difference was significant (P=0.0154, paired t-test).
Figure 4-24 Representative photomicrographs and image analysis of PGP9.5 immunostaining in one animal. Innervation of granulation tissue in wounds with and without hair transplantation (two sections per wound). Green line is defined granulation tissue. Negative pixel is shown in blue and the positive pixel of nerve bundles shown in red in images analysed by Aperio ImageScope.

Figure 4-25 A comparison of innervation in wound with and without transplanted follicles. Wounds with transplanted follicles had four times greater nerve density (percentage of the volume of nerve fibres in granulation tissue) than wounds without follicular transplantation.
4.4 DISCUSSION

The connection between wound healing and the hair follicle is well established. Hair follicle bulge cells have been shown in several studies [136, 298, 299] to participate in wound re-epithelialisation. Wounding induces anagen in surrounding follicles [582] and the growth phase of the hair cycle is associated with increased vascularization [10] and innervation [11]. Vascularization and skin innervation play critical roles in wound healing [534, 535] [536] (see detail in 1.6.2). Taken together, these findings suggest that hair follicle transplantation may be used to accelerate healing.

In this chapter, an animal model has been established and optimised to study the role of hair follicle transplantation in wound healing and to investigate the participation of cells from the hair follicles in wound epidermal and dermal repair.

Hair follicles were micro-dissected from whisker pads of GFP transgenic mice and transplanted in a circular pattern in the right upper back of six nude mice. Six weeks after transplantation full thickness wounds were applied on both sides of the animals’ upper backs, on the right (close to the transplanted hair follicle) and the left (away from transplanted hair follicle).

In this study, wound depth was controlled by folding the skin from the midline using a 23G needle, whereupon the sharp edge of the punch biopsy passed through the right and the left side of the dorsal skin simultaneously. This suggests that wounds on the left and right side were comparable and equal in depth and size at day 0 of the experiment.

Migration of cells from the transplanted hair follicles in the right-sided wound dermis and epidermis was investigated in histology studies seven days after wounding. Healing rates were assessed in full thickness wounds with and without transplanted follicles. To detect possible migration of cells to distant tissue, bone marrow specimens were examined by FACS, and half of the left-sided wounds, and also host whisker pad were examined for any fluorescent detection of GFP⁺ cells.
4.4.1 Effect of hair follicle transplant on wound size

Histological analysis of wounds on day 7 showed a smaller wound diameter (shortest diameter) in wounds with hair transplant, and results were confirmed by examining wound area at different time points. This difference was found to be greater on day 3 and 5 compared to day 7; however, the analysis of data did not confirm whether the effect of hair transplantation is time dependent.

To test whether the effect of hair transplantation on wound healing is time dependent and to exclude the effect of side (Rt vs. Lt), experiments involving larger numbers of animals and alternate side controls will be required.

4.4.2 Participation of cells from transplanted follicles in re-epithelialization

Previous studies have shown that bulge epithelial cells from hair follicles at the wound edge migrate to wound epithelium. Taylor et al. (2000) showed that during wound healing, slow-cycling hair follicle cells labelled by BrdU moved upward and participated in wound epithelialization [298]. Several lineage analysis animal experiments have since confirmed the participation of hair follicle cells in healing wounds [136, 224, 284, 292, 295, 299, 301, 302] (reviewed in 1.5.4).

My current study has supported previous experiments and shown that transplanted hair follicles participate in wound re-epithelialization in a similar manner to normal hair follicles. This study does not have the limitation of a previous lineage tracing experiment by Ito et al., in which the minor expression of the label (K15 positive cells in Krt1-15–CrePR1 R26R mice that had been previously treated with RU486) in normal interfollicular epidermis [136] raises doubts about whether all labelled cells come from follicles. The result of this chapter, therefore, strengthens the conclusions of the lineage tracing experiments. This study is also the first report showing the migration of epithelial cells from transplanted hair follicles in wound re-epithelialization.
Histology examination of the transplanted follicles in Chapter 3, showed no GFP positive cells in the surrounding host tissue in the absence of a skin wound (Figure 3-10). In the presence of excisional wounds however, GFP+ cells that had originated from transplanted hair follicles were detected in healing epithelium. GFP+ cells were also found in the surrounding normal epithelium and host follicles. These findings indicate that GFP+ cells in the interfollicular epidermis migrated from transplanted hair follicles after wounding. However, as shown in Figure 4-16, this experiment cannot determine from which transplanted follicle the migrating GFP+ cells originated.

The origin of migrating GFP+ cells may be either from a damaged hair follicle that spreads cells, or from an intact hair follicle affected by surrounding damaged tissue. In addition, migrating cells could originate from hair follicles away from the wound edge. Although at the transcriptional level, changes in wound edge epithelium extend back to 70 rows of cells from the cut wound edge [614-616], previous lineage analysis animal experiments have not shown whether follicles away from the wound edge also participate in wound re-epithelialization. In this case, migrating cells would have to travel through the normal epithelium to reach the wound edge.

As all the cells in the transplanted follicle were GFP labelled, this experiment cannot determine from which part of the transplanted follicle the migrating GFP+ cells have originated. Migrating hair follicle cells can be further characterised using follicles from donor mice that have a specific genetically labelled epithelial cell population such as K15, CD43 or Blim1.

The characteristics of migrating epithelial cells, the microenvironment that promotes cell migration and the distance they travel to reach the wound edge all have important implications not only for wound healing itself, but also for epithelial cancer cell migration. These details will be discussed in Chapter 6. In addition, the role of damaged hair follicles in migration of hair follicle epithelial cells and migration of epithelial cells through normal epithelium will be further investigated and discussed in Chapter 5.
4.4.3 Participation of cells from transplanted follicles in wound dermis

The animal experimental model used by Gharzi et al. (2003) showed that isolated dermal sheath cells participated in the dermal component of granulation tissue when transplanted into skin wounds [321]. Transplanted labelled dermal papilla cells also induced new hair follicle development (hair induction assay) and participated in granulation tissue from reconstituted hair follicles during skin-wound healing. In this experiment, labelled dermal papilla cells persisted in the healing dermis in a similar manner to the normal skin fibroblasts [321]. Furthermore, when hair follicles were transplanted into an incisional wound, hair follicle cells form blood vessels that were connected to the host vasculature after transplantation [323].

This result however, was not confirmed in the lineage tracing experiment performed by Driskell et al. (2013) using normal hair follicles (not reconstituted hair follicles) [50]. As discussed earlier, in the case of lineage tracing in 8 mm excisional wounds in mice, the hair follicle dermal cells appeared in healing dermis only at a later stage of wound healing (day 17) and the fibroblasts in reticular dermis were the main participants in granulation tissue [50].

In the 6mm excisional wounds described in this chapter, only 1 out of 6 animals showed the GFP+ cells in the wound dermis at day 7, participating in wound vascularisation (Figure 4-17 and Figure 4-18, D) In this histology study GFP+ cells were localised in the dermal component of healing wound; however, organised structures of transplanted hair follicles were not found, and the hair follicle seems to have been damaged during wounding. The appearance of remnants of hair follicles suggests that hair damage at the site of skin injury may release hair follicle dermal cells to participate in the dermal component of wound.

Based on this new hypothesis, intact hair follicle cells do not participate in the dermal component of wound healing until a late stage, unless the hair follicle is trans-sectioned, in which case cells that originated from hair follicles contribute to the dermal component of granulation tissue.
The hypothesis that cells from damaged hair follicles participate in the dermal component of granulation tissue is justified by the experiment by Gharzi et al. [321], as isolated dermal sheath cells may behave similarly to dermal cells from damaged hair follicles.

It should be noted that, because of the relatively large size of transplanted vibrissae follicles they might be more susceptible to damage during the process of obtaining a punch biopsy compared to the pelage hair follicles in the experiment described by Driskell et al. [50].

However, in the excisional wound experimental design in the present chapter there was no control over damage to the hair follicle. Therefore, it has not been possible to determine whether damaged hair follicles promote participation of hair follicle cells in dermal and epidermal components of wound. As shown in Figure 4-26 while follicle ‘A’ escapes damage, follicle ‘B’ may be trans-sectioned by an excisional wound.

![Figure 4-26](image)

**Figure 4-26** Schematic illustration of transplanted follicles in excisional wound experiments. There is no control over damaging the hair follicles in this experimental design, as follicle ‘A’ escapes damage, while follicle ‘B’ might be transected.

There is a possibility that hair follicle damage may indeed have an important role in wound healing and scar formation. Therefore, this hypothesis may have clinical implications, as damage to the hair follicles can be controlled in surgical cuts by making incisions parallel or perpendicular to hair follicles. The role of hair follicle transection in wound healing will be discussed in Chapter 5.
4.4.4 Effect of hair follicle transplant on wound vascularity

It is presumed that angiogenesis plays a critical role in the wound healing process (reviewed in [366]). As mentioned in 1.3.6 and 1.8, the hair follicle’s anagen phase is associated with an increase in vascularity [10, 200]. On the other hand, it has been shown that skin wounds in mice shift the surrounding hair follicle from telogen to anagen [582]. Based on these observations it was hypothesised that hair follicles may promote healing by shifting to anagen and increasing wound vasculature.

Previous studies have shown the participation of cells from transplanted hair follicles in incisional wound vascularization. Amoh et al. (2005) showed that nestin positive cells originating from transplanted hair follicles in an incisional wound, formed blood vessels connected to the host vasculature and expressed endothelial cell markers (CD31 and VWF) [323].

Histological examination of excisional wounds in this chapter supports the previous finding, to the extent that transplanted follicles were connected to host vasculature and GFP⁺ blood vessels were found around the transplanted follicle. GFP⁺ cells formed CD31⁺ blood vessels in the granulation tissue in one animal. Despite participation of hair follicle cells in wound vasculature when capillary density of granulation tissue was assessed morphometrically in CD31 immune stained slides, there was no significant difference in the volume of blood vessels observed in wounds with and without hair transplant.

However the presence of remnants of transplanted follicles close to GFP⁺ vessels suggests that trans-sectioning of the follicle may promote involvement of hair follicle cells in granulation tissue vasculature. This hypothesis may have clinical applications, as in surgical incision damage to hair follicles can be controlled by the angle at which an incision is made.

In our excisional wound experiments there was no significant difference in the capillary density of wounds with and without hair transplants. However, participation of cross-sectioned follicles in wound vasculature may be more significant in a clinical setting and in rodent experiments. This is because the follicle density in a human scalp and mouse pelage
is higher than that of the transplanted hair follicles used in this experiment. Damaged hair follicles in human scalp, for example, may therefore significantly influence the wound vasculature and subsequently the wound healing process.

As discussed earlier, trans-sectioning of the follicle was not controlled in this experiment (shown in Figure 4-26). Overall this study is unable to assess if damage to hair follicles would influence wound vasculature and the healing process. Modified experiments with higher density of transplanted follicles and larger numbers of animals are needed to assess the role of hair follicle damage in wound vasculature.

4.4.5 Effect of hair follicle transplant on wound innervation

As discussed in 1.4, the hair follicle is a sensory organ that represents one of the most densely innervated organs in mammals [215, 216]. Hair follicles may also have an important trophic influence on nerve fibres. Studies conducted in the 1980s, and 1990s have shown that hair follicles in grafted hairy skin [245] and even those transplanted into the anterior chamber of the eye in animal experiments [246] become an active target for nerves. Recent experimental studies have also shown that when hair follicle buds from new-born mice were incorporated in an in vivo tissue engineered skin, innervation was localised around hair follicle buds [248, 617].

In addition to its neurotrophic effect, as mentioned in 1.3.6, hair follicles undergo dramatic changes in innervation during the hair cycle. Increased innervation during anagen also appears within the skin, in the dermis and subcutis, as is shown in rodent experiments [11, 220]. The additional fact that wounding alters the hair cycle and induces anagen (discussed in 1.8) suggests that innervation may be one of the mechanisms by which the hair follicle induces healing.

Although the neurotrophic effect of the hair follicle has long been observed, this is the first study which shows transplanted hair follicles can increase nerve fibre density within the healing granulation tissue. In this study granulation tissue innervation is compared in
wounds with and without hair follicle transplant by IHC using PGP9.5 antibody (a pan-neural marker).

Histology examination of wounds from six animals showed a higher percentage of area stained with PGP9.5 within the granulation tissue of wounds with vibrissa follicle transplant. This difference was significant, and wounds with hair transplant had a nerve density four times greater than the wounds without. In this experiment nerve fibres observed in the granulation tissue were located mainly in the dermis and lower dermis, on top of the sub-dermal fat or regenerated muscles in the Panniculus Carnosus.

As shown in Chapter 3 (3.3.5) vibrissae follicles in intact skin were innervated six weeks after transplantation. The granulation tissue nerve fibres may originate from the nerves surrounding transplanted hair follicle at the dermal or sub-dermal level.

It is believed that cutaneous innervation is important for wound healing and several neuropeptides have been shown to increase proliferation of fibroblasts and keratinocytes and to stimulate extracellular matrix synthesis (reviewed in 1.6.2). A higher percentage of the area stained with the pan-neural marker (PGP9.5) in granulation tissue may therefore play a role in accelerated wound closure in wounds after hair transplant.

Recent studies emphasize the important role of lower dermal fibroblasts in dermal repair. As discussed in 1.5.6, dermal fibroblasts in reticular dermis are the main source of myofibroblasts in the granulation tissue [50]. Several neuropeptides have been shown to stimulate synthesis of the extracellular matrix and also to stimulate differentiation and proliferation of fibroblasts (reviewed in1.6.2) during wound healing. From this discussion it can be concluded that the higher density of nerve fibres in the lower level of the dermis found in our study, might also contribute to activation of fibroblasts in the lower dermis, and act as the source of neurotransmitters involved in wound healing.

From this experiment it is not clear whether vibrissae follicle transplantation promotes skin innervation in unwounded skin. Further investigation is needed to explore if transplantation of hair follicles alters innervation in intact skin or in pathologic conditions
such as nerve injury, diabetes or herpes zoster infection. The clinical application of hair follicle transplantation in skin neuropathies will be discussed in chapter 6, 6.3.3.

4.4.6 Clinical trial of hair follicle transplantation in chronic wounds

While the experiments of this thesis were in progress, Jimenez et al. (2012) [618] conducted a clinical trial in 10 patients with chronic wounds, using scalp hair follicle transplantation into the wound bed, (20 follicles transplanted in an area of 2x2 cm²). Hair follicle transplantation was found to reduce wound size when compared to control wounds without hair transplantation (27% vs. 7% at 18 weeks). Histology study at 18 weeks showed that transplanted wounds had higher rates of re-epithelialization, formation of blood vessels and maturation of dermis when compared with wounds without hair transplant.

Another clinical trial was conducted in 2015, with fourteen patients suffering from chronic wounds for periods of six months to thirteen years who were non-responsive to conventional treatment [619]. In this study hair follicles from 6 x 1.5 cm² of scalp tissue were micro-dissected and transplanted into the wound bed (4 units/cm²). The authors reported improvement of healing by re-epithelialization (clinically observed by 5 weeks), and reduction of wound size. Histology analysis from punch biopsy after sixteen weeks showed that the epidermis and papillary dermis were regenerated within the recipient square. They also reported more elasticity in the recipient area compared to scar tissue [619]. However, this measurement was not quantified and there was no control wound without hair transplant.

The reduction of wound size and participation of hair follicle cells in wound re-epithelialization shown in this chapter are supported in both of these recent studies. In the study by Jimenez et al. [618], the likelihood of vascular organisation and formation of papillary dermis was higher in the hair transplanted area than in the un-transplanted area. In the present study; however, there was no difference in the volume of blood vessels in the wound with follicular transplant compared to controls. The reason could be that wound healing in our animal model was not impaired and by day 7 all wounds, including the
control wounds, were fully epithelialized and the vascular plexus had already formed. My experiment therefore did not show any significant difference in wound vascular volume, and for better assessment of the pro-angiogenic effect of hair follicles, earlier assessment of wound vascularization or an experimental model of impaired wound healing is needed.

None of these studies assessed the skin innervation. Considering the neurotrophic effect of transplanted follicles in wounds seen in this chapter, the role of grafted follicles in promoting innervation of human wounds would warrant further investigation.

4.4.7 **Migration of cells from transplanted hair follicle to distant tissue**

As discussed before (4.1.2 and 1.6.1, page 87), there is some evidence for the existence of circulating mesenchymal stem cells, which migrate to injury sites and participate in wound vasculature, differentiate into myofibroblasts and even trans-differentiating into keratinocytes. Circulating mesenchymal stem cells are believed to derive from bone marrow. However, the existence of mesenchymal stem cells in the hair follicles and other tissues suggests the possibility of two-way cell trafficking between various niches of mesenchymal stem cells in both the hair follicle and the bone marrow. Based on this hypothesis, the hair follicle is a reservoir of stem cells which not only participate in adjacent skin wound repair, but may also migrate to bone marrow and be a part of the circulating mesenchymal stem cells involved in distant tissue repair.

Based on this rationale, the bone marrow and whisker pad of recipient animals were harvested at the same time that wounds were collected on day 7. The wound without adjacent transplanted follicles (left wound), left whisker pads and bone marrow were searched for the presence of GFP$^+$ cells originating from the transplanted hair follicles.

Examination of bone marrow samples from five animals with transplanted GFP follicles on day 7 after wounding by FACS showed no migrating GFP$^+$ cells in bone marrow. In addition, no GFP$^+$ cells were found in the wounds without adjacent transplanted follicles (distant wound, left wound), and left whisker pads.
Limitations of distant migration experiments

This study did not show migration of hair follicle cells to distant tissues (whisker pad, bone marrow and distant wounds) and did not support the hypothesis of cell trafficking from the hair follicle niche to the bone marrow. It should be noted that this study has very limited capacity to test this hypothesis as only 25-30 GFP+ vibrissae follicles were transplanted in each animal, and the tissues were examined for migration of GFP+ cells only on day 7 after wounding.

The hypothesis of migrating cells from hair follicles to distant tissue perhaps could be examined in experimental models with greater numbers of transplanted follicles and with more severe tissue injury at a greater distance from transplanted hair follicles, without wounds adjacent to transplanted hair follicles.

The addition of a PCR assay would provide a more sensitive test to detect the GFP sequence of migrating cells derived from transplanted hair follicles in distant wounds and bone marrow. Unfortunately, this experiment was not possible in the present study due to time constraints. It should finally be considered that even if GFP+ cells are found in distant tissue or bone marrow, they should still be characterised based on the presence of mesenchymal stem cell markers [613], since migrating GFP+ cells may in this instance be derived from any of the donor cells, such as immune cells or melanocytes, located in the transplanted hair follicles.

4.4.8 Conclusion

In conclusion, this chapter shows vibrissae follicle transplantation promotes wound healing in nude mice excisional wounds. This is the first study showing direct participation of cells from transplanted hair follicles in wound re-epithelialization. In addition, it shows higher nerve density in the granulation tissue in wounds with hair transplant. The involvement of hair follicles in re-epithelialization and promotion of innervation are possible mechanisms by which transplanted hair follicles contribute to wound healing.
Although GFP + cells from hair follicles participated in wound vasculature, there was no significant difference in the volume of blood vessels within the granulation tissue of wound with and without hair transplant. Further studies are required to investigate possible pro-angiogenic effects of transplanted hair follicles during wound healing using an impaired wound healing model as well as assessment of wounds at earlier stages of healing.
5.1 Introduction

Hair follicles have an exceptional regeneration capacity and they are able to recover from significant damage (reviewed in [116]). Follicles are able to regenerate in hair induction experiments where only dermal or epidermal parts of hair follicles [113, 114, 120, 590] or even isolated and cultured cells [114, 291, 591, 592] are implanted into skin.

In excisional wounds, damage to the hair follicle depends on the depth of the wound. In superficial skin wounds, the lower parts of the hair follicle remain in the wound bed, while full thickness skin wounds, may lead to complete loss of hair follicles (Figure 5-1).

![Figure 5-1](image)

**Figure 5-1**  Schematic illustration of HF damage during incisional and excisional wounds. Damage to the HF depends on the depth of the excisional wound (partial thickness or full thickness excisional wounds), and the angle of incision, perpendicular (A, B) or parallel (C) to hair follicle (picture modified from [1]).

The axis of the hair follicle is oblique relative to the skin surface, which results in its transection in any perpendicular skin cut (Figure 5-1, A&B). In skin incisions, surrounding hair follicles are damaged unless the angle of incision is parallel to the axis of the hair follicles (Figure 5-1, C), as it is recommended for scalp surgeries.
5.1.1 Hair damage and the healing response

As discussed in Chapter 1 (1.5.4), hair follicles have an important role in wound re-epithelialization. Several animal experiments have shown that in an excisional wound, epithelial cells from surrounding intact hair follicles migrate to wound epithelium and participate in wound re-epithelialization. In these studies hair follicle epithelial cells contribute up to 30% of cells in the healing epithelium [136, 299] (discussed in 1.5.4.).

In both clinical and experimental studies, damage to the hair follicles does not prevent the participation of hair follicle epithelial cells in the wound area; however, the exact process is not known.

It was assumed that in a skin wound, if stumps of hair follicles remained intact, then a large contribution to the healed epidermis derived from these hair follicle remnants [376]. Clinical observation in superficial skin damage has shown that the re-epithelialization starts from the edge of hair follicle stumps (Bishop in 1945) [297] (Figure 5-2, A). This observation was supported in animal experiments by Ito et al. (2005), which showed that labelled Bulge (K15+) cells from residual hair follicles in shallow wounds (from tape stripping epidermis) migrate to the newly formed epidermis (Figure 5-2) [136].

These clinical and experimental findings allow one to speculate that the damage to hair follicles in superficial wounds may facilitate participation of hair follicle cells in epidermal and possibly the dermal component of wounds (Figure 5-2, C).
Despite this clinical and experimental evidence for participation of epithelial cells from cross-sectioned follicle in wound re-epithelialization, the role of hair follicle damage in incisional wounds and their potential effect on scar appearance for the cosmetic outcome is not known. No wider scar or hypertrophic scar has been reported due to damage to hair follicles in surgical incisions. In hair restoration surgery, the donor site incisions are made parallel to the hair follicles, mainly to avoid a hairless scar and also to preserve the maximum intact hair follicle to be used for transplantation. In the ‘trichophytic closure technique’ (reviewed in [620]), a narrow rim of epidermis from the inferior border of the donor site is excised (Figure 5-3, B&C, black arrow) along with the upper part of the hair follicles (infundibulum) located in the border of wound. This technique is widely used in hair transplantation and scalp surgery. Also, in the case of ‘transdermic trichophytic incisions’, used at the hair line in scalp surgery, the surgical incision is perpendicular to the follicles at the hair line (black arrow in Figure 5-3, D) (reviewed in [621]). Both these techniques allow the hair follicles to grow into and anteriorly to the scar, which results in an improved scar and better cosmetic outcome (reviewed in [620, 621]). However, it is not
clear if damage to hair follicles in these surgical incisions accelerates wound re-epithelialisation.

![Figure 5-3 Schematic illustration of trichophytic technique in hair restoration surgery (A, B, and C) [620] and transdermic trichophytic incision at hair line in scalp surgery(D) [621].](image)

5.1.2 Participation of hair follicle cells in the dermal component of skin wounds

There are several controversies surrounding the participation of hair follicle cells in the dermal component of wound granulation tissue. It has been hypothesised that in addition to bulge epithelial cells, hair follicle dermal cells also participate in the dermal component of a healing wound [313].

This hypothesis has been supported by previous studies showing that isolated hair follicle dermal cells participated in granulation tissue when transplanted at the wound site (detected on day 7-10 after wounding) [321, 322]. Furthermore, when hair follicles were transplanted into an incisional wound, hair follicle cells formed blood vessels, which connected to the host vasculature by day 3 after transplantation [323]. However, as discussed in (1.5.6) participation of cells from intact hair follicles in the dermal component of excisional wound healing was not confirmed in a lineage tracing experiment in mice [50]. Driskell et al. [50] showed that the fibroblasts in the reticular dermis were the main participants in granulation tissue, and the hair follicle dermal cells appeared in healing dermis only at a later stage (day 17 in 8 mm excisional wound) [50].

In the experiment reported in Chapter 4 of this thesis, in one out of six animals with excisional wound, GFP labelled follicle cells appeared in the granulation tissue, and participated in vascularisation (see 4.4.3 for discussion). Histology showed localised GFP⁺ cells in wound dermis, but no organised structures of the transplanted hair follicles were
found. This finding suggests that the hair follicles were damaged during wounding and the GFP+ cells in wound dermis originated from the damaged follicles.

Based on this observation and previous clinical and experimental findings discussed in 6.1.3, I hypothesized that the damage to hair follicles may release the follicular cells and magnify the signals required for their migration in dermal and epidermal components of skin wounds.

According to this hypothesis, cross-sectioning of hair follicles may release their cells and facilitate their participation in the dermal and epidermal components of granulation tissue. By contrast, the cells from intact hair follicles may have limited contribution in wound re-epithelialization or may not participate in the dermal component of the wound until a late stage.

This hypothesis may have several clinical implications. Damage to the hair follicle can be controlled in surgical cuts by incisions parallel or perpendicular to the hair follicle. Cross-sectioned follicles may influence cell migration in dermal or epidermal components of incisional wounds and subsequently alter healing outcomes. Involvement of hair follicle cells in wound re-epithelialisation may facilitate wound closure. Participation of hair follicle cells in the dermal component of wounds may also promote healing by improving the vascularisation and regenerating tissue and replacing the dermal defect. On the other hand patients predisposed to hypertrophic or keloid scar participation of hair follicle cells in the dermal component of incisional wounds may lead to scar formation.

5.1.3 **Hypothesis and aims**

Despite the clinical and experimental evidence for the participation of hair follicle cells in wound re-epithelialisation, participation of hair follicle cells in the dermal component of wound granulation tissue remained a point of discussion. In addition there have not been any studies investigating the role of damage to the hair follicle in incisional wounds and their potential effect on cell migration in the dermal and epidermal components of the granulation tissue.
This chapter will examine migration of cells from intact and cross-sectioned follicles in incisional wounds and investigate if cross-sectioning of hair follicles releases their cells and facilitates their participation in the dermal and epidermal components of granulation tissue.

**Hypothesis:**

Hair follicle damage is a precursor to the release and migration of cells from the hair follicle to regenerate wound epidermis and dermis.

**Study aims:**

- To investigate the migration of cells from intact hair follicles into the dermal and epidermal component of incisional wounds
- To investigate the migration of cells from cross-sectioned hair follicles into the dermal and epidermal component of incisional wounds
5.2 Materials and methods

5.2.1 Experimental design

Participation of hair follicle cells from cross-sectioned follicles could not be studied in Chapter 4 because in the excisional wound experiment it was not possible to control damage to the hair follicle (as discussed in 4.4.3, Figure 4-26).

In order to control hair follicle cross-sectioning, vibrissae follicles from GFP transgenic mice (2.1.1) were transplanted in a linear pattern. Then after six weeks, the incisional wounds were applied either parallel or perpendicular to the transplanted hair follicle. As presented in image A, Figure 5-4, incisional wounds on the right side were perpendicular to the axis of the transplanted follicle and cross-sectioned the follicle. On left sides, incisions were parallel to the transplanted hair follicles leaving the transplanted follicle intact (Figure 5-4, B).

![A) Right wound](image1.png)

![B) Left wound](image2.png)

**Figure 5-4 Schematic illustration of hair follicle damage in incisional wounds. A) Right side incisions are perpendicular to transplanted follicles (injured follicle). B) Left side Incisions are parallel to transplanted follicle leaving the hair follicles intact.**

As shown in Figure 5-5, 40 vibrissae follicles from GFP transgenic mice were transplanted into each of two nude mice (in four rows on both right and left sides, with five hair follicles in each row). Incisional wounds were applied to each row on different days (days 0, 2, 4 and 6). All wounds were harvested on day 8 (from the time of the first wound) for histological examination to study the presence of GFP+ cells in the wound area.
5.2.2 Hair follicle transplant

All animal experiments were conducted with the approval of St Vincent’s Health Animal Ethics Committee (AEC, St Vincent’s Hospital, Melbourne) according to the Code of Practice for the Care and Use of Animals for Scientific Purposes developed by the Australian National Health and Medical Research Council (NHMRC, 7th Edition, 2004) as described in sections 2.1 and 2.2.

Vibrissae from transgenic mice (act-EGFP) were transplanted into nude mice under general anaesthesia, as described in detail in Chapter 3, 3.2.5. A total of 40 micro-dissected vibrissae follicles were transplanted in to each nude mouse parallel to the host pelage and the axis of the hair implants was at an approximate angle of 30° to the vertical directed dorsally. Hair follicles were transplanted into the right (20 follicles) and left (20 follicles) upper back of each nude mouse in four rows (five hair follicles in each row) on each side. There was a minimum distance of 0.5 cm between rows.

5.2.3 Linear wound experiment

As described in Figure 5-4 and Figure 5-5, six weeks after hair follicle transplantation, under general anaesthesia (Isoflurane inhalation), linear wounds were applied on the right and left sides using a No.15 blade and the depth of the wound was controlled subjectively.
Temgesic (SC.05 mg/kg SC) was administered in mice subcutaneously for pain relief and animals were reviewed one hour after wounding and then every day.

On day 0 of wounding, two linear wounds were applied parallel to the top row of transplanted hair follicles on the right and left sides. More incisional wounds were applied under general anaesthesia, on the second, third and fourth rows of transplanted follicles on days 2, 4 and 6 respectively, as described in Figure 5-5.

On the right side a blade was positioned perpendicular to the hair follicles within 1-2 mm of the hair follicle orifice, and the skin including the transplanted follicles was cut down to the dermal level (Figure 5-4, B). The transplanted follicles were cross-sectioned at the level of mid- or lower dermis.

On the left side a blade was positioned parallel to the hair follicle shaft within 1-2 mm of the hair follicle orifice, and the skin was cut without direct damage to the hair follicle, as shown in Figure 5-4, A.

5.2.4 Harvesting linear wounds from nude mice

Eight days after the first wounds, the mice were anesthetised by inhalation of 2 % (v/v) Isoflurane and four pairs of wounds were collected from each animal. Wounds were harvested under general anaesthesia to avoid disruption of the weak wound tissue. Animals were then sacrificed by cervical dislocation. Each wound was cut in half (at a 90° angle to the linear wounds) and frozen tissue specimens were prepared as described in 2.5.2. Wounds were embedded in frozen blocks with the section side face-down to allow vertical sectioning of skin.

5.2.5 Histology studies

For each incisional wound, 40 serial frozen sections counterstained with DAPI, and were observed by optical microscopy and the location of all GFP-positive cells was examined.
Direct detection of GFP-Expressing cells

Cells containing GFP can be easily visualised in frozen sections [609]. To detect GFP+ cells, slides with frozen sections were simply washed in distilled water and counterstained with DAPI (1µg/ml) for 1 min. Slides were then washed in PBS, mounted and covered with a coverslip. The cell nuclei were stained with DAPI to visualise the tissue structure. For each wound, the location of all GFP-positive cells was examined in approximately 50 frozen sections.

Cytokeratin staining Immunofluorescence staining

Anti-pan-cytokeratin antibody was used in immunofluorescence staining on frozen sections as described in Table 2-12.

| Table 2-12 Detailed protocol for anti pan-cytokeratin immunofluorescence staining |
|------------------------------|---------------------------------|-----------------|
| Reaction                      | Reagent                          | Time            |
| 3 washes with dH2O            | 10 dips per wash                 |
| Wash sections in buffer (TBS) | 3× 5’/wash                       |
| Antigen retrieval             | Proteinase K                     | 6 min           |
| Blocking serum                | DAKO blocking reagent            | 30 min          |
| Primary antibody              | Rabbit Anti cytokeratin Ab.1/1000| 1h              |
| Wash sections in buffer (TBS) | 3× 5’/wash                       |
| 3 washes with dH2O            | 10 dips per wash                 |
| 2nd Ab                        | Goat anti Rabbit Ab, Cy™3, 1/2000| 1h              |
| Counterstaining               | DAPI                            | 30 sec          |
| Mount + coverslips            | Fluorescence mounting medium     |                 |
Anti-pan-cytokeratin antibody was used to differentiate the epithelium from dermis and to determine whether the GFP$^+$ cells that appeared in the dermis or epidermis were epithelial cells.
5.3 Results

As described previously, forty hair follicles were transplanted into both the right and the left upper back of two nude mice in four rows on each side, each row containing five hair follicles. Six weeks after transplantation, linear wounds were applied. The incisional wounds were applied on days 0, 2, 4 and 6 (a total of four pairs in each mouse) perpendicular or parallel to transplanted hair follicles on the right and left side respectively. Wounds were harvested on day 8.

5.3.1 Migration of cells from intact follicles

Similar results were seen in both mice. In days 4, 6 and 8 left-sided wounds (with incisions parallel to transplanted follicles, uninjured follicles), histological analysis showed migrating GFP$^+$ cells in the healing epidermis. As shown in Figure 5-6 GFP$^+$ cells appeared in the wound epithelium on days 4, 6 and 8. Figure 5-7, shows sections of the wounds in which migrating GFP positive cells presented around the orifice of the transplanted follicle.

In the skin dermis however, scattered GFP$^+$ cells were detected around the transplanted follicles (Figure 5-6, C&D, red arrow), but outside the dermal component of the granulation tissue (Figure 5-6, orange circle). These observations imply that the GFP$^+$ cells are not participating in the dermal healing. The presence of scattered GFP$^+$ cells in the dermis (Figure 5-6, C&D, red arrow) may suggest a transplanted follicle nearby.
When the incision was parallel to the hair follicle, GFP$^+$ cells were present in healing epithelium in wounds on day 4, 6 and 8 (B, C and D, white arrow). Transplanted follicles (red asterisk) present in image A, C and D. Transplanted follicle is not present in image B; however, GFP$^+$ cells participate in healing epithelium (image B, white arrow). The dermal component of granulation tissues are shown by orange circles and characterised by epidermal defect (A) or thick healing epithelium (B, C and D) that is detected on top, or the presence of red blood cells and bleeding in dermis (B). GFP$^+$ cells are not present in dermal component of wound; however, scattered GFP$^+$ cells present are seen around the transplanted follicle, outside the border of the granulation tissue (C and D, red arrow).

It should be noted that 3D images are required to study the migration of GFP$^+$ cells into the epithelium. The GFP$^+$ cells found in the wound epidermis (Figure 5-6) could be the migrating cells or the continuation of a transplanted follicle, which is not otherwise discernible in the 2D tissue section under examination. However, in the experiment, incisions were applied at a 1-2 mm distance from the orifice of transplanted follicles. Besides, when the orifice of the vibrissa follicle was present in the tissue section (Figure 5-7, A&C), GFP$^+$ cells were still detected within 1 mm of the orifice in the epithelium (Figure 5-7, white and red arrow), suggesting that cells had migrated from the hair follicle through the epidermis, toward the wound epithelium.
Figure 5-7 Representative photomicrographs of incisions with intact follicles showing migration of GFP+ cells through the epithelium (18 µm cryosections). When the incisions are parallel to hair follicles, GFP+ cells pass through intact epithelium and the orifice of transplanted follicles. Red asterisk: transplanted hair follicle. Red arrow: Migrating GFP+ cells within the healing epithelium on day 6 (A) and day 8 (C and D (high power)). Images A and B (high power) show migration of GFP+ cells passing through the intact epithelium (white arrow).

5.3.2 Migration of cells from damaged follicles

As shown in Figure 5-8, GFP+ cells appeared in the dermal component of right-sided wounds, in which the incision was perpendicular to the transplanted hair follicle and the follicle was cross-sectioned. In all wounds on days 4, 6, and 8 migrating GFP+ cells were located in the upper part of the dermis just beneath the dermo-epidermal junction.

In the right side incisions (wounds with hair damage), in one of the two wounds on day 4, migrating GFP+ cells were detected in the epidermis, around the orifice of the cross-sectioned follicle (Figure 6-9, B, red arrow). While in all wounds on days 2, 6, and 8 and one of the wounds on day 4, the GFP+ cells were confined to the cross-sectioned follicles and did not migrate to the interfollicular epidermis (Figure 6-9).

Figure 6-9 shows wounds with cross-sectioned follicles in one of the animals. As shown in these histology slides, migrating GFP+ cells were observed in day 4 wounds (Figure 6-9, B, red arrows); however, the GFP+ cells detected in epidermis were confined to the follicle in wounds on day 2, 6 and 8 (Figure 5-9, A, C&D, white arrow).
Figure 5-8 Representative photomicrographs of incisions with cross-sectioned follicles showing migration of GFP$^+$ cells in wound dermis (18 µm cryosections). GFP$^+$ cells were detected in wound dermis and beneath the dermo-epidermal junction.

Figure 5-9 Representative photomicrographs of incisions with cross-sectioned follicle showing migration of GFP$^+$ cells in wound epidermis (18 µm cryosections). Red arrow: migration of GFP$^+$ cells appeared in the epidermis around the orifice of the transplanted follicle on day 4 (Image B). GFP$^+$ cells were confined around the orifice in wounds on day 2, 6 and 8 (images A, C, and D, white arrows).
5.3.3  **Cytokeratin expression in migrating cells**

Immunostaining of incisional wounds with pan-cytokeratin antibody showed that migrating GFP\(^+\) cells in interfollicular epidermis and healing epithelium express pan-cytokeratin (Figure 5-7, C, and Figure 5-10, A). This observation suggests that migration GFP\(^+\) cells in healing epithelium are epithelial cells.

However, as shown in Figure 5-10, the GFP\(^+\) cells observed in the dermal component of granulation tissue in wounds with cross-sectioned follicle did not express pan-cytokeratin (Figure 5-10, B), suggesting that they were not epithelial cells.

![Figure 5-10](image)

**Figure 5-10**  Representative photomicrographs of pan-cytokeratin immunostaining in incisional wounds with and without hair damage on day 6 (18 µm cryosections) A: incisional wounds parallel to transplanted hair follicles (intact transplanted hair follicle). GFP\(^+\) cells appeared in the healing epidermis and expressed pan-cytokeratin (white arrow). B: incisional wounds perpendicular to transplanted hair follicles (cross-sectioned hair follicle) GFP\(^+\) cells appeared in the dermis beneath the dermo-epidermal junction and did not express pan-cytokeratin (white arrow).
5.4 Discussion:

5.4.1 Damage to hair follicle may affect cell migration during skin wound healing

I hypothesised that damage to the hair follicle is the mechanism by which hair follicle cells are released into wounds and participate in both the dermal and epidermal components of wounds, while intact hair follicles only participate in wound re-epithelialisation.

The result of this experiment showed that with intact hair follicles, GFP+ cells only migrate to the epidermal component of a wound, and not to the healing dermis. When the follicle was cross-sectioned, hair follicle cells mainly migrated to the healing dermis, and they did not participate in wound re-epithelialisation except in one of the wounds on day 4 where the GFP+ cells were expanded around the orifice of the cross-sectioned follicle.

Cell migration from undamaged hair follicle

As discussed in Chapter 1 (1.5.4) several previously published animal experiments have shown that in an excisional wound, epithelial cells from surrounding intact hair follicles participated in wound re-epithelialization. In this chapter, incisional wound experiments with undamaged hair follicles in two animals showed that GFP-expressing hair follicle cells participate in wound re-epithelialisation (from day 4), but do not migrate to the dermal component of granulation tissue. These results also suggest that the GFP+ cells pass through the orifice of the transplanted follicle and migrate through the intra follicular epidermis toward the healing epithelium (Figure 5-11).

Figure 5-11 Schematic illustration of migration of cell from an intact hair follicle in healing epithelium.
These results support previous studies showing that when follicles are intact, GFP\textsuperscript{+} cells originating from the transplanted follicles migrate to healing epithelium [136, 299]. In previous studies, migration of hair follicle epithelial cells was detected after 24 [299] and 48 hours [136]. In my experiments, GFP\textsuperscript{+} cells did not appear in day 2 wound epithelium and were only detected in wounds on day 4, 6 and 8. The main reason for this difference is probably that the transgenic mice used in these studies allow tens of hair follicles to be examined histologically. In comparison, in our follicle transplantation model not more than one hair follicle was examined in each histology examination, which would decrease the chance of detecting migrating cells at an earlier time point.

Another possibility is that the vibrissae follicles or transplanted follicles in our experiment may participate differently to the pelage follicles examined in previous studies [136, 299]. For example, the size of the hair follicles could be a factor, where cells may take longer to migrate out of a large follicle. In this case stem cell niche might be deeper, particularly if they migrate through the outer root sheath and exit via the orifice of an intact follicle.

In my intact hair follicle experiment, individual GFP\textsuperscript{+} cells were found to be in the intact interfollicular epidermis between the transplanted hair follicle and wound, which suggests that GFP\textsuperscript{+} cells migrate through the epithelial layer from the hair follicle to the healing epithelium. Migration of epithelial cells within the epithelium and its clinical translation in cancer metastasis will be discussed in Chapter 6.

**Cell migration from damaged hair follicles**

Clinical observation in superficial skin damage shows that re-epithelialization starts from the edge of hair follicle remnants in the wound bed (Figure 5-2, A) [297]. This observation was supported in animal experiments by Ito et al., which showed labelled bulge (K15\textsuperscript{+}) cells from residual hair follicles in the tape stripping model of epidermal injury migrated to the newly formed epidermis (Figure 5-2, B) [136].

In this chapter, it was hypothesised that hair follicle damage results in the release and migration of cells from the follicle to both epidermal and dermal components of wound
repair. Therefore, it was expected that GFP$^+$ cells from damaged follicles would participate in both healing epidermis and dermis (Figure 5-12, A).

When the transplanted vibrissae follicles were cross-sectioned, GFP$^+$ cells from the follicles appeared in the healing dermis in all wounds on day 4, 6 and 8. This result supports the idea that damage to hair follicles promotes their participation in the dermal component of wound repair. The GFP$^+$ cells released from the hair follicle into the healing dermis did not migrate beyond the dermis, but stayed at the border of the dermo-epidermal basement membrane. These results suggest that GFP$^+$ cells in the wound dermis are released from where the follicle was cut (Figure 5-12, B).

![Figure 5-12](image)

**Figure 5-12** Schematic illustration of migration of cell from a cross-sectioned follicle in the dermal and epidermal components of incisional wounds.

However, in just one of the wounds with cross-sectioned follicles, migration of GFP$^+$ cells was also detected in the epithelium. In this wound, GFP$^+$ cells were found around the orifice of the cross-sectioned follicle. This observation suggests that GFP$^+$ cells in the epidermis migrated along the upper part of the hair follicle and passed through the orifice of the cross-sectioned follicle (Figure 5-12,C) rather than migrating directly through the skin defect (Figure 5-12, D). In other words, if an incision is perpendicular to the hair follicle, the released hair follicle cells in the cross-sectioned area participate in the dermal component of the incisional wound (Figure 5-12, B). However, these released cells do not
migrate through the skin defect to the wound epithelium. To get to the wound epithelium, hair follicle cells migrate through the hair epithelium and pass through the orifice of the hair follicle (Figure 5-12, C).

Detection of migrating GFP\(^+\) epithelial cells around the orifice of cross-sectioned follicles in only one wound suggests that damage to hair follicles may weaken epithelial cell migration in incisional wounds. Considering the limited number of animals in this study a greater number of experiments is required to explore the possible influence of hair follicle damage on migration of hair follicle epithelial cells in incisional wounds.

Further experiments are needed to investigate whether migration of epidermal cells from hair follicles depends on the extent of skin damage or the exact position of damage in the hair follicle. This will be discussed in 5.4.2.

**Participation of hair follicle cells in the dermal component of wounds**

As discussed earlier (5.1 and 1.5.6) there are controversies regarding the participation of hair follicle cells in the dermal component of wound granulation tissue.

It has been hypothesised that in addition to bulge epithelial cells, hair follicle dermal cells also participate in the dermal component of the healing wound [313]. In previous studies when hair follicles were transplanted into an incisional wound, hair follicle cells formed blood vessels, which were connected to the host vasculature after transplantation [323]. Gharzi *et al.* [321] and Biernaskie *et al.* [322] also showed that isolated hair follicle dermal cells formed myofibroblasts in granulation tissue when transplanted at the wound site. These results however, were not confirmed in a lineage tracing experiment on intact hair follicles [50]. Driskell *et al.* (2013) showed that the main participants in granulation tissue were the fibroblasts from the reticular dermis, and the hair follicle dermal cells appeared in healing dermis only at a later stage of wound healing (day 17 in 8 mm excisional wound) [50].

The experiments described in this chapter showed that hair follicle cells participated in the dermal component of wounds when the follicle was cross-sectioned, while GFP\(^+\) cells
migrated to the healing dermis when the incision was parallel to the follicle, leaving the follicle intact.

A reasonable assumption is that in the experiments of Driskell et al. [50], most pelage follicles around excisional wounds remained intact, as the small pelage follicles are mobile and can easily slip away in the loose dermis from the cutting edge of the punch instrument during application of an excisional wound. The behaviour of intact hair follicles in my experiment is similar to that described by Driskell et al.; therefore, it is possible to interpret these results as supporting the results of the present experiment.

On the other hand, the behaviour of damaged hair follicles supports previous studies which showed that isolated hair follicle dermal cells participated in the dermal component of granulation tissue when transplanted at a wound site [321, 322], if one assumes that isolated cells behaved in a similar manner to those of damaged hair follicles.

These results suggest that damage to hair follicles may affect the migration of cells from the hair follicles during wound healing. However, further studies are needed to explore the exact parameters involved in these epithelial and dermal cell migrations.

Participation of hair follicle cells in wound microvasculature has not been studied in this chapter and more experiments are required to explore whether the hair follicle is involved in wound microvasculature.

5.4.2 Proposed mechanism for epithelial cell migration from cross-sectioned follicles

Only one of the wounds with damaged follicles showed migration of epithelial cells around the orifice of a cross-sectioned follicle (one of the wounds on day 4). In other wounds with cross-sectioned follicles no GFP+ cells were detected in wound epidermis or interfollicular epidermis, and GFP+ cells were confined to the hair follicle orifice in the epithelium.
These results suggest that hair follicle damage may inhibit migration of follicular cells into the wound epithelium, allowing one to speculate about several mechanisms which may influence the behaviour of damaged hair follicles during wound healing (Figure 5-13).

### Proposed mechanism for hair follicle epithelial cell migration in cross sectioned follicle:

- Damaged hair follicles may behave differently during skin wound healing.

- Cell migration may depend on the exact position of damage in relation to stem cells.

- An intact epithelium or basement membrane is required for migration of epithelial stem cells.

- Cell migration may depend on the extent of skin damage.

- Cell migration may depend on the anatomical location of the incisional wound.

**Figure 5-13** Schematic illustration of proposed mechanism for HF epithelial cell migration in incisional wounds. Damaged hair follicles may behave differently during skin wound healing. Cell migration may depend on the exact position of follicle damage in relation to stem cells (A). Hair follicle intraepithelial migration may be interrupted in damaged hair (B) Cell migration may also depend on the extent of skin damage in incisional and excisional wounds (C and D). Cell migration may depend on the anatomical location of the incisional wound in relation to arrector pili muscles and the angle of the hair follicle (E).

### The healing response in damaged hair follicle

As discussed in 1.8, the hair follicles respond to skin wounds by shifting to the anagen phase, increasing skin innervation and vascularization, and participating in wound re-epithelialisation. However, damaged hair follicles may behave differently during skin wound healing. The normal response of an intact hair follicle during skin wound healing—including participation in wound re-epithelialisation may be disrupted until the hair follicle is fully recovered from the damage.
**Level of damage**

In our experiment the follicle was transected at the level of mid- or lower dermis; however, the exact point of hair follicle damage was not known. This may be the reason that migration of hair follicle cells was detected in wound epidermis in only one wound with damaged follicles (day 4), while in the other wounds (seven wounds at various time points; day 2, 4, 6, and 8), GFP+ cells did not migrate to the epidermis.

The level of hair damage may influence the recovery of the hair follicles and the behaviour of the follicles during wound healing, depending on the position of the transection relative to the level of the bulge stem cells. This may influence the participation of hair follicle cells in the epidermal components (Figure 5-13, A).

There is some clinical evidence that cells from damaged hair can participate in healing epithelium (as discussed in section 5.1.1). In shallow wounds for example in the donor sites of split-thickness skin grafts, where the epidermis and part of the dermis are removed, the epidermis is reconstituted from the hair follicle remnant. However, the results of the present experiment did not support this notion. As only one of the wounds showed migrating GFP+ cells in epithelium, and the rest of the wounds (a total of seven wounds on day 2, 4, 6, and 8) did not show any migrating GFP+ cells in the epithelium. The GFP+ cells in the wound dermis in these wounds did not migrate beyond the basement membrane.

Compared to the animal model presented here, the epidermis in human skin is thick and the hair follicles are long and thin; therefore, damage to hair follicles in shallow wounds or trichophytic incision tends to occur only at the epidermis and infundibulum of the hair follicle. The epidermis in nude mice; however, is very thin, and the incisions in this experiment were at the lower level of the dermis, and certainly not limited to the epidermis.

This comparison suggests that shallow wounds to human skin probably tend to damage the more superficial level of the hair follicle; and therefore, do not prevent migration of hair follicle cells to healing epithelium. However damage at the level of mid to low dermis,
may interrupt epithelial cell migration within the hair follicle. The mechanism of any impaired hair follicle epithelial cell migration in deep skin wounds could be explained by the location of the bulge stem cells in hair follicles relative to the location of follicle damage. As well as directly damaging the bulge, lower follicle injury might also disrupt communication between the bulge, arrector pili muscles, and dermal papilla or secondary hair germs in deep dermis, which may be involved in the signalling required for migration of hair follicle epithelial cells.

In our experiment the follicle was trans-sectioned at the level of mid- or lower dermis; however, the exact point of hair follicle damage was not known. Further experiments are needed to investigate if migration of cells from hair follicles depends on the exact position of damage in relation to stem cells. For example, a micro-surgery approach using an incision parallel to the follicles and then reaching into the wound with a pair of micro scissors to cut the follicle at different levels would address this question.

**Intraepithelial cell migration**

Hair follicle epithelial stem cells may need an intact epithelium and basement membrane to migrate up to the interfollicular epidermis. As discussed in 1.6.1 several cell membrane proteins such as integrins and their interaction with basement membrane matrix have been found to be involved in the process of keratinocyte migration (reviewed in [385]).

In my intact hair follicle experiment, individual GFP+ cells were found in intact interfollicular epidermis between the transplanted hair follicles and the wound edge, which suggests that GFP+ cells migrate through the epithelial layer from the hair follicles to the healing epithelium.

These results suggest that hair follicle epithelial cells may need an intact epithelial layer between the stem cell niche and the orifice, to migrate to wound epithelium. In this case, when the basement membrane or epithelial layer is breached in a cross-sectioned follicle, follicular epithelial cells may not be able to participate in wound re-epithelialisation.
**Extent of skin damage**

The extent of skin damage may influence the migration of hair follicle cells. Participation of hair follicle cells may be different in small incisional wounds (Figure 5-13, C), in which the skin gap is easily closed compared to wide superficial wounds (Figure 5-13, D), in which more time and also more cells are required to repair the epithelial gap. This rationale may explain the unexpected result in our experiment, namely, that damaged, hair follicles did not participate in wound re-epithelialisation, while experimental studies as well as clinical observation suggested that epithelialization starts from the edges of cross-sections of hair follicle stumps [136, 297].

In addition, similar trauma may damage human and mouse skin differently because of differences in their anatomy. Cell migration through epidermis may be interrupted even in shallow wounds in mouse skin, as the epidermis is thin, while shallow wounds in thicker human skin may leave the lower level epidermis or infundibulum intact for migration of hair follicle epidermal cells.

**Anatomical location of incisional wound**

The hair follicles and their surrounding tissues are not symmetrical. The hair follicle is not perpendicular to the surface of the skin, and the arrector pili muscle is located at the side that forms an obtuse angle with the lower surface of the epidermis (Figure 5-13, E) [622]. To date there have been no previous reports of any alteration in the wound healing response and cell migration in epithelium in relation to asymmetrical follicle position.

In my experiments, the incisions perpendicular to hair follicles were located at the side of the follicles that forms an acute angle with the lower surface of the epidermis (α angle in Figure 5-13, E). The incisions parallel to hair follicles however, were located at the side where the arrector pili muscle is located (β angle in Figure 5-13, E).

From the experiments described in this chapter, it is not clear whether the arrector pili muscles have regenerated and reattached to the transplanted vibrissae follicles. However, Sato et al. showed that after pelage follicular unit transplantation, the arrector pili muscles
were regenerated in their normal anatomical location [593]. If the arrector pili muscles are regenerated following vibrissae follicle transplantation, similar to pelage follicles, then based on these findings, a new mechanism for hair follicle cell migration can be proposed as follows: damage to the arrector pili muscle in incisional wounds parallel to and at the obtuse side of the hair follicle may affect the epithelial stem cell niche at the insertion of the arrector pili muscle and provide the starting signal for epithelial cell migration. Further investigation into hair follicle cell migration in epithelium in relation to the angle of the hair follicle may reveal new mechanisms and signals involved in wound healing.

5.4.3 Further characterization of hair follicle cells involved in wound healing

Using GFP+ follicles in wound experiments in chapter 4 and 5 made it possible to trace the migration of follicle-derived cells. However, in these experiments, it was not possible to determine exactly from which part of the transplanted follicle –e.g. the bulb, dermal papilla, dermal sheath, isthmus or bulge– the migrating cells originated.

Several specific markers have been characterized in dermal and epidermal components of hair follicles (Chapter 1, 1.5.3 and 1.5.5). Previous studies have characterised some populations of hair follicle epithelial stem cells that migrate to wound epithelium. However, the origin of hair follicle derived cells appearing in the dermal component of granulation tissue seen in this chapter is not clear. Further investigation is required to show whether the dermal component of hair follicle migrates to the dermal component of wounds or whether the hair follicle epidermal cells ‘trans-differentiate’ into fibroblasts and myofibroblasts in the granulation tissue.

An in vivo experiment, using donor follicles from a mouse where a specific cell population is genetically labelled, would show whether cells from different parts of the follicle have different propensities for migration and differentiation into dermal or epidermal components of healing wound.
5.4.4 **Further characterization of signalling molecules involved in cell migration**

There are numerous signaling molecules, including adhesion molecules, cytokines and growth factors, involved in cell migration during cancer spreading, morphogenesis, and wound healing [2]. Examples are stromal cell-derived factor 1 (SDF1) and platelet-derived growth factor (PDGF). Similar signaling molecules are also involved in epithelial mesenchymal transition (EMT) or mesenchymal epithelial transition (MET) [347, 378, 379]. These molecules may also be required for migration of hair follicle derived cells. It would be interesting to identify the signaling molecules involved in hair follicle cell migration in the dermal and epidermal components of wounds.

Examination of signaling molecules in injured and uninjured hair follicles may reveal the mechanism involved in hair follicle cell migration during wound healing. Perhaps different concentrations of these molecules would explain the different patterns of cell migration in an injured follicle and an uninjured one. In addition, the concentration of signaling molecules in dermal and epidermal components may determine the direction of migration.

Further characterization of hair follicle cells participating in skin repair and the mechanism involved would provide better understanding of the complex process of cell migration in skin wounds and malignancies, and may provide new candidate therapeutic targets.

5.4.5 **Limitations**

The main shortcoming of this experiment was the limited number of experimental wounds and sample animals (four pairs of wounds at different time points in each of two mice). In addition, there is a significant difference between mouse and human skin, and their respective healing processes. Besides this, one must consider the innate abnormality of nude mouse skin. A final limitation is that vibrissae are a specialized follicle with a thick capsule containing cavernous blood vessels, and there are no comparable follicles in humans. These limitations will be discussed in Chapter 6.
5.4.6 Conclusion

The results of these incisional wound experiments suggest that intact hair follicles participate only in the epidermal component of granulation tissue and cells migrate from transplanted hair follicles to the healing dermis only if the hair follicle is cross-sectioned. Intraepithelial migration of hair follicle cells was detected only in one of the two wounds on day 4 and no intraepithelial migration was found in healing epidermis in the remaining wounds with cross-sectioned follicles on days 2, 6 and 8. These results suggest that damage to hair follicles may have a negative effect on epithelial cell migration during wound healing. More experiments are required to confirm these results and to reveal the exact mechanisms involved in hair follicle cell migration in skin wounds. Migration of hair follicle cells may depend on the extent of skin damage, the depth of the incision, and the location of damage to the follicular unit in relation to the stem cell niche in the bulge or the arrector pili muscles.

If participation of hair follicle cells in healing dermis or epidermis alters the healing process or scar formation, these results may have important clinical applications, as damage to the hair follicles can be simply controlled in surgical incisions.
Chapter 6
General Discussion and Future Direction

6.1 Experimental findings

Adult stem cells were first identified in bone marrow. It has been shown that bone marrow mesenchymal stem cells are able to migrate to the site of an injury and participate in tissue repair [538, 623-626]. Adult stem cells have also been isolated from various tissues including the hair follicle (reviewed in 1.5).

Populations of stem cells for epithelial and mesenchymal lineages are found in hair follicles and are responsible for generation of hair fibres during the growth phase of the hair cycle (reviewed in [627]). Hair follicle epidermal stem cells also participate in re-epithelialization during skin damage. In addition, hair follicles respond to injury by shifting to the rapid growth phase of the hair cycle (anagen), which is associated with better healing, and increased skin innervation and vascularity (1.3.6 and 1.8).

To take advantage of the hair follicle’s strong response to injury, the whole hair follicle was micro-dissected and then transplanted into injured skin while keeping the follicle structure intact. Vibrissae follicles from GFP transgenic mice were transplanted into nude mice. The excisional wounds with and without vibrissae follicle transplants were compared in six nude mice, to investigate whether the hair follicle transplantation improves vascularity, innervation and wound closure.

Using hair follicles from GFP transgenic mice also provided an opportunity to study migration of hair follicle cells in the dermal and epidermal components of adjacent excisional wounds. Left-sided wounds away from transplanted follicle, bone marrow and host whisker pad were also searched for the presence of GFP+ cells to determine whether there is two-way cell trafficking between bone marrow and peripheral mesenchymal stem cell niches in the body.
6.1.1 Optimising hair follicle transplantation in nude mice

In Chapter 3, an animal model of hair follicle transplantation, similar to human hair follicle transplantation, was established using the vibrissae hair follicles from green fluorescent protein transgenic mice, transplanted into the back skin of nude mice. The technique was optimised by a series of experiments done in order to keep the transplanted hair follicle in place during the first week after transplantation. In the optimised protocol transplanted follicles were covered with Vaseline gauze, a non-adherent absorbent dressing, and then secured with an elastic adhesive bandage for three days.

In the optimised technique, the graft survival rate was similar to human scalp hair restoration surgery (about 90%). GFP⁺ follicles were readily identified in histological sections and integrated normally into GFP negative skin. Transplanted follicles were innervated and their cavernous blood vessels appeared to be connected to the host circulation system. GFP⁺ dermal and epidermal cells remained confined to the site of implantation and did not expand within the host tissue. The follicle epidermal cells did not participate in homeostasis of the interfollicular epidermis. Similar to normal hair follicles, the transplanted vibrissae can be induced to cycle by plucking of their hair shafts. Their growth rate was similar to normal vibrissae in mice at about 1mm/day during the anagen phase [605].

This technique has the advantage of being simpler and quicker than other protocols for animal hair transplantation developed by Amoh et al. [323] in which the vibrissae follicles are transplanted into an incisional wound and secured by nylon stitches. Transplanted vibrissae follicles did not contain any surrounding donor tissue from the interfollicular epidermis. This precise micro-dissection makes it possible to differentiate migration of hair follicle cells from interfollicular epidermis in lineage tracing experiments using genetically labelled donor follicles. In contrast, it would be very difficult to exclude surrounding interfollicular epidermis from the hair follicles when micro-dissecting pelage hair follicles. This disadvantage was seen in the transplantation experiments performed by Sato et al. where GFP⁺ interfollicular epidermis was also transplanted into the nude mice [593]. The normal anatomical relationship of the hair follicle and surrounding tissue as well as the
minimal damage to transplanted follicles and recipient skin make this a suitable model for studying the normal physiological function of hair follicles and skin.

6.1.2 Hair follicle transplantation improves wound healing

In Chapter 4, an animal model was established and optimised to study the role of hair follicle transplantation in wound healing and also to investigate the participation of cells from hair follicles in wound epidermal and dermal repair.

Hair follicles were micro-dissected from whisker pads of each GFP transgenic mouse and transplanted in a circular pattern in the right upper back of each nude mouse in a total of six nude mice. Six weeks after hair transplantation, full thickness wounds were applied on the right (close to transplanted hair follicle) and left (away from transplanted hair follicle) side of the upper back of animals.

Migration of cells from hair follicles in right-sided wounds was investigated in histology studies seven days after wounding and these showed migration of GFP+ cells in healing epithelium. GFP+ blood vessels were also detected in the granulation tissue in one animal where the structure of the hair follicle was disorganised.

Histology analysis of wounds on day 7 showed a smaller wound diameter in wounds with hair transplant (right wounds) compared to wounds without hair transplant (left wounds), and results were confirmed by looking at the wound area at different time points. This difference was more noticeable on days 3 and 5 than on day 7. The average difference between the right and left wound areas was approximately 4 mm² on day 3 and 5 and 2.25 mm² on day 7. Overall this difference was significant (P value = 0.012). However, ANOVA suggested that the effect of hair transplantation was not time dependent and the difference in wound size did not change at different time points (P= 0.595). Measuring the wound diameter by histology examination of wounds on day 7 also showed that the diameter of the wounds with hair transplants was 30% smaller on average than control wounds (p=0.024 t-test).
Wounds with transplanted vibrissae follicles also showed higher nerve density in the granulation tissue. Although GFP+ cells expressing the endothelial cell marker CD31 were detected within the granulation tissue vasculature in one of the wounds, there was no significant difference in the volume of blood vessels within the granulation tissue of wounds with and without hair transplants.

Participation of hair follicle epithelial cells in wound re-epithelialization has been shown previously [136, 299]. However, the present work is the first experimental study in rodents showing the healing effect of follicular transplantation. From these observations, one can conclude that involvement of hair follicles in re-epithelialization and promoting innervation are possible mechanisms by which transplanted hair follicles have contributed to wound healing.

While I was undertaking the experiments detailed here, Jimenez et al. (2012) [618] and Liu et al. (2015) [619] conducted clinical trials in two cohorts including 10 and 14 patients respectively. In their studies, patients with chronic wounds were transplanted with scalp follicles (4-5 follicular units/cm²). They reported higher rates of re-epithelialization, formation of blood vessels and maturation of dermis when compared to wounds without hair transplant [618]. Liu et al. (2015) reported more elasticity in the recipient area compared to scar tissue; however, this measurement was not quantified, and their study did not incorporate control wounds without hair transplants [619].

The reduction of wound size and participation of hair follicle cells in wound re-epithelialization shown in Chapter 4 is supported in both of these recent studies [618, 619]. In my study, there was no difference in the volume of blood vessels in wounds with follicular transplant and control wounds. The reason could be that wound healing in my animal model was not impaired and by day 7 all wounds (including the control wounds) were fully epithelialized and the vascular plexus was already formed. Therefore, my experiment did not show any significant difference in wound vascular volume. For better assessment of the pro-angiogenic effect of hair follicles, earlier assessment of wound vascularization or an experimental model of impaired wound healing is needed.
To reveal whether the effect of hair transplantation on wound healing is time dependent and to exclude the effect of side (right vs. left), experiments involving larger numbers of animals and alternate side controls would be required. Further studies using an impaired wound healing model as well as assessment of wounds at an earlier stage of healing would be required to investigate a possible pro-angiogenic effect of transplanted hair follicles during wound healing. Furthermore, histology analysis of wounds at an earlier stage of skin wound healing (e.g. day 3-4) or using transplanted follicles in an animal model of impaired wound healing, may reveal a pro-angiogenic effect of transplanted hair follicles during skin repair.

6.1.3 Migration of hair follicle stem cells to distant skin wound and bone marrow

It has been hypothesised that circulating fibrocytes, like inflammatory cells, enter the injured tissue and contribute to granulation tissue (reviewed in [397]). The idea of bone marrow derived circulatory cells contributing to wound myofibroblasts, was supported by several experiments using intravenous injection of labelled bone marrow mesenchymal stem cells (BM MSC) [409-411]. These experiments showed migration of the labelled cells in the wound granulation tissue [409-411]. There is also some evidence that bone marrow derived cells migrate to wounds and differentiate into endothelial cells, pericytes [411, 412] (reviewed in [369]) or even trans-differentiate into keratinocytes [413] and participate in wound re-epithelialisation [411, 412] (reviewed in [415]) [412, 414].

Considering the similarities between mesenchymal stem cells from bone marrow and other tissues, I hypothesised that there is two-way cell trafficking between bone marrow and peripheral mesenchymal stem cell niches in the body and that mesenchymal stem cells migrate from peripheral tissues, including hair follicle, to bone marrow and distant injured skin.

In the excisional wound experiment discussed in Chapter 4, after harvesting tissue on day 7 post-wounding, the left wounds which were away from the transplanted follicles (in two animals), host whisker pad (in two animals) and bone marrow (in five animals, as one
sample was lost during the experiment) were also examined for any fluorescent detection of GFP+ cells. The histological study showed no GFP+ cells in wounds on the left side (examined in six animals) and host left whisker pad (examined in six animals), and FACS analyses were negative for GFP+ cells in host bone marrow (for five animals).

This study however, has very limited capacity to test the hypothesis, because only 25-30 GFP+ vibrissae follicles were transplanted in each animal and the tissues were examined for migration of GFP+ cells only on day 7 after wounding. The hypothesis of cell migration from hair follicle to distant tissues perhaps could be examined in experimental models using larger numbers of transplanted follicles, without wounds adjacent to the transplanted hair follicle, and with more severe tissue injury within a significant distance to the transplanted hair follicles. The application of \textit{in vivo} real time imaging [628] would also provide an opportunity to detect any possible migration at different time points after tissue injury.

6.1.4 \textbf{Damage to hair follicle may affect cell migration during skin wound healing}

In Chapter 4, in one of the excisional wounds with transplanted follicles, GFP+ cells originating from hair follicles appeared in the healing dermis. The GFP+ cells were localised in dermis, but no organised structure of transplanted hair follicle was found, and the hair follicle seemed to have been damaged during wounding. Based on this observation and those of previous studies, which showed wound re-epithelialization started from the edge of the hair follicle stump in shallow wounds [136, 297], I hypothesised that hair damage at the site of skin injury may promote participation of hair follicle cells in the dermal and epidermal components of wounds.

To investigate the role of damaged hair in follicular cell migration (Chapter 5), 40 hair follicles were transplanted into both the right and left upper back of each nude mouse (N=2) in four rows on each side, each row containing five follicles. Six weeks after transplantation, linear wounds were applied. The incisional wounds were applied on days
0, 2, 4 and 6 (a total of four pairs) perpendicular or parallel to the transplanted hair follicles on the right and the left side respectively, and the wounds harvested on day 8.

In the right-sided wounds without hair damage, histology analysis showed that GFP\(^+\) cells had migrated to the healing epidermis. This migration was observed at days 8, 6 and 4. This finding is supported by previous animal experiments, which showed migration of labelled epithelial cells from intact hair follicles [136, 299]. GFP\(^+\) cells however, were not found in the wound dermis in wounds with intact follicles.

By contrast, in left-sided wounds with hair damage, GFP\(^+\) cells from damaged hair follicles spread into the healing dermis. However, migration of GFP\(^+\) cells was detected in epidermis around the follicle orifice in only one of the two wounds with cross-sectioned follicles, on day 4. GFP\(^+\) cells were not detected in the wound epidermis in the rest of the wounds with cross-sectioned follicles, on days 2, 6 and 8.

These results support the idea that damage to hair follicles promotes participation of hair follicles in the dermal component of wounds. However, migration of hair follicle cells in the wound epidermis may be independent of hair damage. In fact, these observations suggest that damage to follicles may even have an inhibitory effect on hair follicle epithelial cell migration.

These results contradict clinical observations and experimental animal studies, which show islands of new epithelium around the edge of a cross-sectioned follicle in shallow wounds [136, 297]. The contradiction may be related to the anatomical location of incision and cross-section in the hair follicle relative to the bulge stem cells or arrector pili muscle, which may influence recovery of the hair follicle and hair follicle epithelial cell migration during wound healing. In addition, the extent of skin damage may influence migration of hair follicle cells and participation of hair follicle cells may be different in small incisional wounds when the skin gap is easily closed compared to wide superficial wounds where more time and more cells are required to repair the epithelial gap.
Our preliminary experiment suggests that damage to hair follicles may be a starting signal for migration and participation of hair follicle cells in the dermal component of hair follicles. However, more extensive experiments are needed to prove whether hair damage alters epithelial cell migration and, if so, the exact mechanism involved.

The hair follicle transplantation technique would be a valuable technique to investigate whether the exact position of damage in relation to hair follicle stem cells, arrector pili muscles and the extent of skin damage influence epithelial cell migration during wound healing. It would also be interesting to identify the role of hair follicle trans-section on wound healing properties like angiogenesis, inflammation and innervation.

6.2 Limitations

6.2.1 Wound healing in Nude mice

It is known that the immune system and inflammation response have an important role in wound healing (discussed in 1.6.2). The nude mouse is a well-established model in wound healing studies and recent studies show that nude mice have almost normal healing with less scar formation after wounding [531, 629]. As discussed in 3.1.1, nude mice exhibit the same number of hair follicles as normal mice and the relevant genetic mutation does not directly interfere with the hair cycle. However, the immune deficiency and abnormal skin and hair follicle phenotype of nude mice can potentially alter the wound healing experiments.

In Chapter 4, healthy follicular tissue transplanted around the wound promoted wound closure compared with the control wound (with no normal tissue). However, these considerations do not impact on the neurotrophic effect of transplanted follicles or the participation of cells from transplanted follicles in the re-epithelialization seen in this experiment. Therefore, the healing-promoting effect of the vibrissae follicles transplantation seen in the nude mice experiment is still a valid result and may have clinical applications.
Another limitation of this study is the difference in skin structure and wound healing properties of humans and rodents. As mentioned in 1.6.4, skin contraction is important in wound healing in nude mice, while in humans it is mainly wound re-epithelialisation that leads to wound closure, not skin contraction as such. Other animals with similar wound healing properties to humans, such as pigs, were not accessible in the present project. In another rodent model of wound healing, skin contraction can be controlled by a silicone sheet splint sutured on excisional wounds [608]. As described in Chapter 4 (4.2.5), this technique was tested in nude mice; however, it was not successful because of the thinness and fragility of their skin.

In excisional wound experiments in chapter 4, all wounds were eventually fully epithelialized within one week, which differs markedly from what is seen in chronic wounds in humans. Participation of transplanted hair follicles in wound re-epithelialisation suggests that hair follicle transplantation may have an even more significant effect in patients with chronic wounds. The effect of hair follicle transplantation in wound re-epithelialisation could be further investigated in human clinical trials, and also in swine and primate models of wound healing, as these animals have similar wound healing properties to human.

6.2.2 Immune reaction to GFP protein and graft-versus-host reactions

Another potential limitation of transplantation experiments is the possibility of immune reaction to GFP proteins mediated by T lymphocytes in an immune competent animal [530]. This reaction has not been reported in immune deficient nude mice and it is less likely in our animal model of hair transplantation as nude mice have been the host for tumours expressing GFP proteins in cancer research [630].

Donor immune cells such as T cells, macrophages and Langerhans cells from transplanted tissue may also attack the host tissue and induce ‘graft-versus-host’ disease. Graft-versus-host’ disease has mainly been reported in cases of bone marrow transplant; however, it may also occur in solid organ transplants [631]. In hair follicle transplants in this study, no clinical inflammation was detected during the six weeks after transplant. Considering the
limited number of immune cells within the transplanted tissue, the occurrence of a graft-versus-host reaction seems unlikely. Donor immune cells could be quantified and characterised by immunohistochemical examination of transplanted follicles. Characterisation of donor immune cells may also show their migration during the healing process and a potential ‘graft-versus wound’ effect. This experiment was not done because of the limited time available during this study.

6.2.3 Vibrissae follicles

Vibrissae follicles are considered proprioceptive sensory receptors with special anatomical characteristics such as blood sinuses, rich innervations and muscle attachments, and there is no comparable follicle in humans. Vibrissae follicles may not represent candidate hair follicles available for transplantation in a clinical setting, such as the human scalp hair follicle.

Transplanted vibrissae follicles may also behave differently during wound healing. The neurotrophic characteristic, participation in wound re-epithelialization and the effect of follicle cross-section in incisional wound may be different in vibrissae and human hair follicles. The healing property of human hair follicles could be examined using human hair follicle transplantation in nude mouse and the excisional and incisional wound protocols described in Chapters 4 and 5.

6.2.4 Effect of side dominance

In Chapter 4, all control wounds were on the left side and all wounds with hair transplantation were on right side of the animal, while in Chapter 5 all incisional wounds with damage to transplanted follicles were on the right and all incisional wounds with intact follicles were on the left side of animals. This experimental design may potentially have biased the result due to an unknown side-dominance effect. Further experiments are required to control for this effect and test wounds on both the left and right side in different animals.
6.2.5 **Wound size measurement in histology and image analysis**

In Chapter 4, both measurements of wound area and diameter from images of wounds and from histology examination have their own limitations. In histology analysis the wound diameter may not represent the overall wound size as only the smallest diameter of wounds, rather than the whole size is measured. Measuring the area of the whole wound area in images from an animal gives us a better estimation of wound size; however, when there is a scab, analysis of wound images may show a larger wound area when compared to images of healing epithelium without a scab. Images of excisional wounds (Chapter 4) showed scabs remained on wounds in 2 out of 6 animals on day 7. One animal had a scab on a right-sided wound, and one animal had a scab on a left-sided wound.

These two complimentary techniques, namely the wound diameter in histology examination and wound area measurement in images, were used in order to achieve a better estimation of wound size in this experiment, and they gave consistent results.

6.2.6 **Assessment of wound innervation**

Innervation can be highly localised in skin, and thin 5μm paraffin sections are therefore not suitable for quantitative assessment of wound innervation. In the experiments described in chapter 4, to obtain a more reliable assessment of nerve distribution in granulation tissue, two sections 500 μm apart (100 consecutive sections apart) were selected in each wound for staining with anti PGP9.5. The average percentages of areas stained for two slides in each wound were compared in wounds with and without hair transplantation. Although a significant difference was found, very few nerve fibres were identified in the normal epidermis with this staining method, and no nerve fibres were detected in the healing epithelium.

The effects of hair follicle transplant on skin and wound innervation could be further investigated by quantitative assessment of nerve fibres in dermis and epidermis, in thick 50 μm frozen sections in both intact skin and in excisional wounds. The intraepidermal nerve fibres could be quantified as the number of nerve fibres crossing the basement membrane or the number of isolated nerve fragments in epidermis that do not cross the basement
membrane (reviewed in [612]). Thick histology sections would allow three-dimensional visualization of the morphological features of dermal innervation. In addition, the epidermal component of skin could be separated and subjected to protein and mRNA analysis (reviewed in [612]).

Further characterisation of nerve fibres and neurotransmitters within the wound may reveal possible mechanisms and neurotransmitters involved in healing of wounds after hair transplant.

6.3 Clinical applications and future directions

6.3.1 Follicular transplantation technique in skin and hair biology and wound healing studies

In Chapter 3, I described a refined hair follicle transplantation protocol that uses vibrissae from GFP transgenic mice for transplantation into the back skin of recipient nude mice. Transplanted vibrissae follicles integrated normally into the host epithelium and GFP+ cells were confined to the transplanted hair follicles and were not found in the interfollicular epidermis. Wound experiments in Chapter 4 and 5 showed that cells migrated from hair follicles and participated in healing epithelium and dermis.

Follicle transplantation models have several applications in skin and hair biology research. As discussed in 5.4.4, there are numerous signaling molecules involved in epithelial cell migration. Examples are stromal cell-derived factor 1 (SDF1) and platelet-derived growth factor (PDGF) [2]. These molecules may also be required for migration of hair follicle-derived cells. The use of vibrissae follicles from transgenic mice with fluorescent or luminescent labelled cells makes it possible to study the migration of specific cell populations from hair follicles during skin injury. In addition, transplanting vibrissae follicles from knock-out or knock-in transgenic mice allows investigation of signalling pathways and genes of interest, specifically in follicle cells. Investigation into the role of specific genes, signalling molecules or pharmacologic agents with a focus on the hair follicle, may reveal specific regulatory molecules and may provide wider possibilities for
exploitation of hair and skin biology in health and disease. Such investigation may also provide new candidate therapeutic targets. Investigating the signaling molecules involved in hair follicle cell migration may also reveal the mechanisms involved in cell migration during cancer spreading and morphogenesis. This will be further discussed in 6.3.5.

6.3.2 Therapeutic strategy to enhance skin repair

My results showed a reduction in the size of wounds close to the hair transplanted area (p=0.053). Histological studies also showed that transplanted follicles participated in wound reepithelialisation and improved wound innervation. These data suggest that hair transplantation may promote healing in a clinical setting. Transplanted hair follicles in chronic wounds would introduce a tissue rich in epidermal and dermal stem cells, and an organ responsive to injuries. Follicular transplantation might be considered an alternative treatment in difficult-to-heal wounds, especially neuropathic wounds.

As discussed in 1.6.3, in stem cell therapy it is believed that stem cells improve healing mainly through paracrine factors (reviewed in [508]), by reducing inflammation [511], recruiting endothelial cells [510], promoting angiogenesis [512, 516], and reducing fibrosis [409, 526]. It is interesting to explore whether follicular transplantation in skin specifically influences the physiologic response during inflammation proliferation, or during the remodeling stage of the healing process.

Future experiments could focus on the role of the transplanted follicle in each specific physiological function of the healing process. For example, it would be interesting to investigate whether hair follicle cells, in addition to migration, also proliferate or whether transplanted follicles influence the proliferation of host tissue. Proliferation of migrating follicle cells or host tissue could be histologically examined using proliferation markers such as Ki67. This experiment was not conducted because of the limited time available during this study.

In human scalp the follicles are long and thin and are able to be transplanted into the wound bed easily. In addition, the long term effect of hair transplantation may be even
more pronounced in a clinical setting as the human scalp hair follicle has a long period of anagen, which is the phase that promotes healing [78].

Hair follicle transplantation is an established hair restoration technique with high success rates and low rates of infection in human scalp. These advantages suggest that hair follicle transplantation may also have higher success rates for engraftment into wounds together with a higher resistance to infection compared to full thickness skin grafts.

Hair follicles can be conceptualised as an epidermal fold extending deep into the dermis. The transplantation of a human scalp hair follicle would provide approximately 18 times the volume of epidermal tissue per unit of skin surface area (depth of the the follicle divided by depth of the interfollicular epidermis, as shown in Figure 6-1). Human hair follicle transplantation would introduce extra epithelial tissue deep into the skin and may be considered a “deep” or “vertical skin graft”, and thus an alternative to skin grafts in difficult chronic wounds.

![Figure 6-1](image.png)

*Figure 6-1 A comparison of a hair follicle’s epidermal tissue volume relative to the skin surface area. Transplantation of one hair follicle introduces approximately 18 times more epidermal tissue per unit of skin surface area.*
6.3.3 Improving sense of touch

The neurotrophic effect of hair follicles has been shown in previous studies. In an animal experiment when skin from hairy occipital scalp was transplanted into the frontal scalp of stump-tailed macaques (Macaca arctoides), hair follicles in the skin graft were active targets for nerves [245]. Hair follicles also had a specific neurotrophic effect when transplanted into the anterior eye chamber of adult rats [246]. In another study, when hair follicle buds from new born mice were added to an in vivo skin tissue engineered model, innervation was localised around hair follicle buds [248, 617].

Although no sensory alteration has been reported following hair follicle transplantation or hair follicle laser removal in humans, there is still a potential for therapeutic benefit if transplanted hair follicles increase the presence of nerves in chronic wounds where healing is compromised by a background of skin neuropathy.

In the histology studies (Chapter 4), nerve bundles (stained with a pan-neural marker, PGP9.5 antibody) were found around the transplanted hair follicles after six weeks, suggesting that transplanted hair follicles had been innervated. The innervation of intact skin following hair transplantation was not measured in my study. However, the observation of thick nerve bundles around hair follicles suggests that hair follicles may have a neurotrophic effect and increase skin innervation. The neurotrophic effect of hair follicles was supported in histology examination of excisional wounds in chapter 4, which showed wounds with hair transplant had four times greater nerve density than the wounds without hair transplant.

These results suggest that, in addition to improving the healing of chronic wounds, manipulation of follicles may influence skin innervation and sense of touch in clinical situations, such as hypersensitivity in shingles, or hyposensitivity in neuropathy or nerve damage. Patients could use their own hair follicles transplanted to improve the sense of touch and to heal wounds. For example, patients with diabetic neuropathy and chronic wounds may benefit from direct participation of transplanted follicle epithelial cells in the
wound epithelium. Hair transplantation may also exercise an indirect effect by increasing innervation.

A systematic study is needed to explore if hair follicle transplantation influences skin innervation and whether follicular transplantation could be considered as a novel therapeutic strategy for managing skin disorders that are directly or indirectly affected by skin innervations.

6.3.4 Damage to hair follicle may affect migration of cells in skin

The incisional wound experiment on two animals described in Chapter 5 suggests that migration of epithelial cells from hair follicles to the healing epithelium may need intact hair follicles. On the other hand, damage to hair follicles promotes participation of hair follicle cells in wound dermis.

In trichophytic incision and wound closure in scalp surgery, which is a common technique in scalp surgery, incision is perpendicular to the follicle. The better cosmetic outcome in this technique was related to the growth of hair through and in front of the scar, so it is less visible, and there is no report of its effect on wound closure or healing.

This is the first study suggesting that damage to the hair follicles may affect migration of cells in skin. As damage to the hair follicles can be controlled in surgical cuts by incisions parallel or perpendicular to the hair follicles, these results may have important clinical applications. If participation of hair follicle cells in healing dermis or epidermis alters the healing process or scar formation, there might be potential to improve healing of surgical wounds by controlling the orientation of cuts relative to the adjacent follicles. In addition, if transplanted follicles were used to enhance healing of difficult wounds, those follicles could be deliberately damaged or left intact to enhance the contribution of hair follicle dermal and epidermal cells respectively, which may influence the healing outcome.

Further experiments are needed to investigate whether migration of dermal or epidermal cells from hair follicles depends on the extent of skin damage or the exact position of damage in relation to stem cells, arrector pili muscles and the angle of the hair follicle, and
if participation of hair follicle dermal or epidermal cells influences wound repair or scar formation.
6.3.5 **Intraepithelial migration: a possible route for distant metastasis**

This study and other previous studies have shown that epithelial cells migrate within the epithelium during wound healing. Similar cell migration is also important in epithelial malignancy. In light of these similarities, the results of this study suggest a novel hypothesis of intraepithelial metastasis as a mechanism of cancer cell dispersal.

As discussed in Chapter 1, normal keratinocytes from skin and hair follicle adjacent to the wound edge migrate through the epithelium and participate in re-epithelialization [136, 299]. Normal epithelial cells from the hair follicles that are located at the wound edge travel from the bulge up to the interfollicular epidermis to participate in wound re-epithelialization.

Previous studies also showed that, at the transcriptional level, changes in wound edge epithelium extend back to 70 rows of cells from the cut wound edge [614-616]. However, it has not been previously reported whether the epithelial cells from follicles away from the wound edge also participate in wound re-epithelialization. It is unclear at what distance from the wound edge hair follicle cells can be involved in healing. In Chapter 5, epithelial cells migrated from transplanted hair follicles through intact epithelium and participated in epithelialization of incisional wounds at least 1-2 mm from transplanted follicles (Figure 6-2). Migration of keratinocytes over these distances may have implications for the aetiology and management of skin cancers.

![Figure 6-2](image_url)

**Figure 6-2** A representative photomicrograph showing intraepithelial migration of keratinocytes during wound healing (18 µm cryosections). The transplanted GFP positive follicle is marked by red asterisk. GFP positive keratinocytes can be seen migrating through intact epithelium (white arrow) toward the wound area (red arrow), 6 days after induction of an incisional wound parallel to transplanted follicles. Scale bar: 100 µm.
Local invasion occurs either when malignant cells spread within the epithelium contiguously or migrate within the nearby epithelium adjacent to the primary tumour (Figure 6-3, A). In malignancy, intraepithelial migration of cancerous cells is considered the initial step of tumour expansion and the prerequisite for stromal invasion. Distant metastasis is believed to include sequential steps of vertical migration of malignant epithelial cells, including passage through the basement membrane (trans-epithelial migration), trans-endothelial migration, and finally dissemination through lymphatic and blood vessels (Figure 6-3, B). These sequences of events spread malignant cells to discontinuous organs [632].

Figure 6-3  Schematic illustration of cancer cell dispersal routes in epithelial malignancies.
A) Local spread: malignant cells spread within the epithelium contiguously, or migrate through the epithelium but stay within a short distance from the primary tumour.
B) Classical route of metastasis: malignant cells pass through the epithelial basement membrane (trans-epithelial migration), and then endothelial basement membrane (trans-endothelial migration) and spread through lymphatic and blood vessels to distant tissue.
C) Intraepithelial route of metastasis: malignant cells migrate within the epithelium to distant epithelium without passing through the basement membrane.
However, clinical examples of multiple, spatially distinct, clonal, malignant lesions within epithelium with intact basement membrane and without any evidence or suggestion of vascular or lymphatic metastasis infers that there are other routes of dissemination for malignant cells. Examples include carcinoma of the oesophagus, [633] lungs, [634] vulva, colon, [635] bladder, [636] squamous cell carcinoma of the head, neck [637] and oral cavity [638], and basal cell carcinoma. [639]

Observations of intraepithelial cell migration in wound healing suggests an intraepithelial route for distant metastasis, which would involve malignant cells migrating exclusively within the epithelium and without passing through the basement membrane (Figure 6-3,C).

**Alternative routes for metastasis**

Alternative routes of distant metastasis (as opposed to local invasion) have been postulated in a limited number of malignancies. Specific patterns of cell distribution in histology studies have suggested the spreading of malignant cells along anatomical tracks such as nerves (neurotropism) in lymphoma [640] and prostatic adenocarcinoma [641] or blood vessels (angiotropism) in melanoma [642] and glioma. [643]

The concept of malignant cell migration along the epidermis has been used to explain the pathogenesis of mammary [644] and extra-mammary Paget’s disease, [645] in which breast cancer metastasis is restricted to the epithelium (in situ), but is separated from the primary tumour. This theory proposes the migration of breast cancer cells along the basal membrane of the epithelium and infiltration of malignant cells into the epidermis.

Lugassy et al. suggested that Beta 2 laminin promotes malignant melanocyte migration along the external surface of blood vessels, and postulated a similar mechanism for metastatic migration along other anatomical structures such as nerves, and epidermal appendages including the hair follicle and eccrine sweat glands [642, 646]. The migration of hair follicle melanocytes in wound healing [333] also supports the concept of malignant melanocyte migration along epidermal appendages such as the hair follicle as suggested by Lugassy et al. [642, 646].
**Intraepithelial migration in field cancerization**

The “field cancerization theory,” introduced by Slaughter in 1953, explains the presence of multiple tumours surrounded by tissue of normal appearance [647]. According to Slaughter, exposure to a carcinogen results in a genetically altered field of tissue containing multiple primary mutations, which subsequently lead to multiple tumours. However, clonality is sometimes seen in multiple malignant or pre-malignant lesions [633-639]. This scenario implies that the distinct neoplasias are derived from a single primary mutant cell. This observation led to the concept of intraepithelial spreading of pre-malignant cells as suggested in the field cancerization theory, either in the form of contiguous spreading [633, 636, 637] or migration of individual cells [28, 634].

Alt-Holland et al. [28] showed vertical and lateral migration of individual cells from implanted E-cadherin deficient malignant squamous cell carcinoma in an *in vitro* three dimensional human skin tissue equivalent and suggested that this model represents a mechanism for squamous cell carcinoma dispersal, recurrence and field cancerization.

Pipinikas et al. [634] showed the clonal spread of carcinoma *in situ* within the airway epithelium leads to multiple spatially distinct, *in situ* bronchial squamous cell carcinomas. These authors concluded that mutated cells with migratory as well as proliferative characteristics would be able to disperse discontinuously through the normal airway epithelium without disrupting its normal morphology and function.

**Intraepithelial migration in wound healing**

As discussed in chapter 1, normal keratinocytes and melanocytes migrate along the normal epithelium during wound healing. Experiments with labelled populations of hair follicle cells in transgenic mice show that hair follicle keratinocytes as well as melanocytes migrate within the epidermis, from follicles, to participate in wound repair [136, 299, 333].

The distance that an epithelial cell from a hair follicle at the wound margin travels (from the bulge to midway between two hair follicles) is over 3 mm [298]. Intraepithelial migration of cells also has been observed in several other epithelial
tissues. For example, the enterocytes of intestinal epithelium, originating from stem cells located in the crypt [648], are able to migrate along the basement membrane for 0.5 mm toward the tip of the villi at an estimated rate of 200–400 μm/day [649]. Corneal epithelial cells, derived from the limbal stem cell zone, are known to migrate centripetally at a rate of 32 μm/day toward the central cornea that can be up to 5.5 mm away [650, 651], (reviewed in [298]).

The intraepithelial migration of un-transformed epithelial cells and melanocytes in a wound healing microenvironment—without their passing through the basement membrane—suggests that intraepithelial migration is an innate property of these cells and is not the consequence of transformation. Thus, intraepithelial migration may be relevant to any epithelial malignancies and is not confined to specific cancer types.

**The intraepithelial route of metastasis hypothesis**

The theories of individual cell migration in field cancerization and intraepithelial route of metastasis both postulate a similar mechanism for the development of multifocal clonal malignancies. The difference between these two concepts lies in the pre-existing spread of precancerous cells in field cancerization versus the later migration of malignant cells from the primary malignant lesion in intraepithelial metastasis. This distinction gives a completely different clinical perspective in malignancies. Compared to the spreading of normal looking precancerous cells in field cancerization, intraepithelial metastasis emphasizes the spread of fully transformed epithelial malignant cells from one location in the body to another without passing through the basement membrane. This concept may also be relevant to tumours, which invade through the basement membrane, as it is possible that distant epithelial metastasis occurs through the epithelial route and that invasion across the basement membrane is coincidental and not a prerequisite step for the metastasis.

An intraepithelial route for metastasis would mean that malignant cells could also migrate within the epithelium without passing through the basement membrane. Secondary epithelial tumours, which have lost their connection to the primary malignant lesion, could
occur through the migration of malignant cells along the epithelium. This concept would represent intraepithelial metastasis rather than local expansion, as there would be no local continuity of cellular spread. Hence the postulated mechanism could explain the emergence of multiple *in situ* and clonal tumours surrounded by normal tissue and early dissemination of malignant cells in patients with *in situ* malignant lesions for example, in breast cancer. [652] In contrast to the current convention, an intact basement membrane in a histology examination would not necessarily be a sign of an “*in situ*” lesion, as cells may migrate to distant epithelium without passing through the basement membrane.

The hypothesis of an intraepithelial route for distant metastasis would also explain the observation of malignant cells within epithelium at some distance from the main lesion in routine pathology examinations, which are often described as ‘satellite lesions’, ‘in-transit metastasis’, ‘Pagetoid infiltration’, or the ‘Borst-Jadassohn phenomenon’. It would also explain rare but interesting cases of secondary lesions, which are genetically related but spatially distinct from primary cancers. Reported recurrence of cutaneous squamous cell carcinoma (SCC) at a distance from the original tumour [653], or cancerization of a cutaneous flap after reconstruction surgery of an oral *in situ* SCC [654], are examples that involve significant spatial and chronological separation from the primary cancer. The fact that malignant cells can migrate at the speed of 0.1–2 μm/min [21], which equates to distances of 5.2–105 cm/year, is consistent with the distance of metastasis that occurs in these clinical situations.

**Testing the hypothesis**

Migration of malignant cells within the epithelium in different malignancies could be studied using an *in vitro* three-dimensional tissue model introduced by Alt-Holland *et al.* [28]. Real time *in vivo* imaging of cutaneous malignancies in animal models could be used [628], so that malignant cell migration could be visualized and any distant metastasis occurring by this intraepithelial route detected. Considering the similarity between wound healing and cancerous processes [655], experimental animal models of wound healing may also provide opportunities to investigate the characteristics of migrating neoplastic keratinocytes and their micro-environmental requirements.
The contiguity versus dispersal of individual cells or cell clusters in cutaneous malignancies, as well as in pre-cancerous fields, could be tested by in situ hybridization using histology specimens from patients. Genetic studies could determine whether there is a common origin of multiple cancerous or pre-cancerous lesions. Such studies would ideally include lesions at a significant distance from the primary neoplasm, and should compare multiple time points during disease progression.

Considering the complexity of the genomic change in malignant cells and the heterogeneity of tumours, complex genetic studies would be needed to identify early and late mutations and reconstruct a phylogenetic lineage.

**Clinical significance of intraepithelial metastasis**

When multiple cutaneous lesions are seen in the clinic, they may arise from the intraepithelial metastasis of a single primary tumour. The concept of field cancerization may thus be transformed to one of intraepithelial metastasis of epithelial lesions with an intact basement membrane that could potentially be prevented by early treatment of the primary lesion. In contrast, migration of premalignant cells in field cancerization would mean that the cells had already spread by the time the primary lesion was detected, so "early" treatment would not prevent secondary lesions.

Because the expansion of malignant cells in cutaneous cancers may be broader than detected by current pathological tests, wider histology examinations as well as new diagnostic tests may be required for early detection of migrating malignant cells in healthy-looking epithelium.

Based on this hypothesis, the occurrence of cancers may in fact indicate dissemination of malignant cells across a wide area of epithelium and distant metastasis via the intraepithelial route, which would in turn suggest that the epithelium should be the focus of treatment. The development of multiple malignancies may be prevented by early treatment, and by targeting a wider area of epithelium than only local treatment. Possibilities include expanded surgical resection margins, systemic adjuvant chemotherapy and
immunotherapy, radiotherapy, or targeting the epithelium by wide topical chemotherapy, for example, in skin cancer and bladder cancer, and intraductal chemotherapy in breast cancer.

The “seeds and soil” theory introduced by Paget in 1989 entailed the concept that metastasis results when tumour cells (the “seeds”) colonize a specific organ microenvironment (the “soil”). [656] Incorporating an epithelial route of migration into the seeds and soil theory gives a new insight into cancer metastasis. The physical and biochemical properties of epithelium, which determine the ability of malignant cells to migrate, may contribute to carcinogenesis. The similarities between wound healing and cancer [655] suggests factors involved in epithelial cell migration in wound healing may also be important in the pathogenesis and spreading of skin cancer, and may represent new targets for treatment. Potentially relevant factors include cell surface proteins and receptors, cell polarity [657], interaction between adhesion molecules, intracellular filaments and the extracellular matrix, and inflammation. [658-661] Non-steroidal anti-inflammatory drugs, which have a known chemo-protective effect in cancers of several tissues [662-668], may block epithelial cell movement by reducing inflammation. Risk factors such as ultra-violet radiation and inflammation may alter the epithelium to facilitate cell migration.

Patients who develop BCC are known to be at increased risk of subsequent BCC. Clinicians working in the field regularly see patients who have 10-20 BCC removed each year. Subsequent BCC is generally considered to be de novo primary BCC due to exposure to oncogenic ultraviolet radiation; however, in some cases, multiple 'sporadic' BCC might in fact be distant metastases that arise through intraepithelial migration of tumour cells over large distances in the skin.

In summary, the hypothesis of an ‘intraepithelial route’ for metastasis provides a new insight into the pathogenesis of malignancies. Investigating the micro-environmental properties that control cell migration in the epithelium might improve our understanding of cancer protective or risk factors, and subsequently provide new targets for prevention and treatment in epithelial malignancies.
Conclusion

Population aging is associated with an increase in chronic wounds. Despite several treatment options, chronic wounds remain a clinical challenge and socioeconomic burden to patients and the community. There is a need for new treatment modalities.

Multiple factors are involved in the pathogenesis of chronic wounds. Impaired innate healing response is the main characteristic of chronic wounds. Impaired healing may be related to poor stem cell pools in the skin of elderly patients [443-446].

As discussed earlier (1.5) hair follicles normally participate in wound healing and provide epithelial cells that contribute to repair of the skin defect. Hair follicles also respond to skin wounding by initiation of the anagen phase of the hair cycle and promotion of angiogenesis and innervation. In this study, whole hair follicles were micro-dissected and then transplanted into nude mice, while keeping the follicle structure intact. Then experimental wounds were induced adjacent to the transplanted follicles, to investigate the hair follicle’s response to injury.

In Chapter 3, a vibrissa transplantation protocol was established in nude mice that was based on the human follicular unit transplantation technique. Vibrissae follicles were micro-dissected from GFP transgenic mice and then transplanted into nude mice. Histological examination after six weeks showed that the transplanted follicles were re-innervated and their cavernous blood vessels were connected to the host’s circulation. The transplanted vibrissae could be induced to cycle by plucking their hair shafts. The growth of vibrissae fibres was similar to that of normal vibrissae. Transplanting precisely micro-dissected vibrissae follicles makes it possible to identify migrating hair follicle cells from recipient interfollicular epidermis in lineage tracing wound experiments using genetically labelled donor follicles.

In Chapter 4, vibrissae follicles from GFP transgenic mice were transplanted to nude mice. The healing response after wounding of skin adjacent to the follicle transplants was
compared to excisional wounds without adjacent transplanted follicles in six nude mice. Wounds with adjacent hair transplants were 30% smaller on average than control wounds on day seven after wounding. Migrating GFP positive (GFP+) cells were detected in the healing epithelium of excisional wounds with adjacent hair transplants. In one of the six animals, the GFP+ cells, also appeared in the healing dermis at day 7.

Wounds with adjacent hair follicle transplants also had four times greater nerve density than the wounds without adjacent follicular transplants. There was no significant difference in capillary density in wounds with and without hair transplants and there was no significant difference in macrophage density within the granulation tissue. From these observations, I conclude that re-epithelialization and promotion of innervation are possible mechanisms by which transplanted follicles contribute to wound healing.

In Chapter 5, an incisional wound experiment was designed to investigate whether cross-sectioning of hair follicles releases their cells and facilitates their participation in the dermal and epidermal components of wound healing. In this experiment, incisions were made parallel or perpendicular to the transplanted follicles and four pairs of incisional wounds were examined in each of two nude mice.

Histological examination of incisional wounds with undamaged hair follicles in two animals showed that GFP-expressing hair follicle cells participated in wound re-epithelialisation (from day 4). GFP+ cells were not detected in the dermal component of granulation tissue of these wounds. These results suggest that, in intact hair follicles, GFP+ cells pass through the infundibulum of the transplanted follicle and then migrate through the interfollicular epidermis toward the wound edge.

In contrast, the GFP+ cells were detected in the dermal component of wound healing when perpendicular incisions were made to transect the transplanted follicles. In these wounds, the GFP+ cells did not migrate beyond the dermis and stayed at the border of the dermo-epidermal basement membrane.
These results showed that damage to the hair follicle affects migration of cells in skin. Based on these findings, I proposed a new mechanism for hair follicle cell migration, that is, damage to hair follicles might be a starting signal for migration and participation of hair follicle cells in the repair of dermis. There is a possibility that participation of hair follicle cells in healing dermis or epidermis alters the healing process or scar formation. Therefore, these results may have clinical applications, as damage to the hair follicle can be simply controlled in surgical cuts by making incisions parallel or perpendicular to the hair follicle.

In the intact hair follicle experiment, individual GFP+ cells were found in the intact interfollicular epidermis between the transplanted hair follicle and the wound edge. This observation suggests that GFP+ cells migrate through the epithelial layer from the hair follicle to the healing epithelium. Migration of normal epithelial cells within the epithelium led me to postulate that cancer cells could also migrate through the epidermis, and metastasis to distant sites could occur without passage through the basement membrane. The hypothesis of an ‘intraepithelial route’ for metastasis may alter our understanding of malignant cell metastasis and provide new targets for the prevention and treatment of epithelial malignancies.

The results of this dissertation suggest that follicular transplantation may be a potential treatment for difficult-to-heal wounds, especially neuropathic wounds. The neurotrophic effect of transplanted follicles observed in this study also suggests that follicular transplantation may be used as a novel therapeutic strategy for managing peripheral nerve damage, neuralgia and neuropathies. Furthermore, this study showed that damage to follicles affects the migration and participation of hair follicle cells in skin wounds, which may subsequently influence the healing outcome.
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