The effects of Ephrin-A2 & –A5 on cutaneous development and wound healing

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School of Surgery

This thesis is presented for the degree of Doctor of Philosophy at the University of Western Australia

2014
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Published


The work presented in the paper is included in chapter 3 of the thesis.

Student’s contribution

I was responsible for tissue collection, immunohistochemistry, sensory function testing and data analysis. I was also involved in writing and editing the manuscript (Contribution 80%).

Contribution of co-authors

Dr Maghami and Mr Skykes provided assistance with the sensory function test. Dr Wallace was involved in statistical analysis. Dr Fear and Dr Rodger were involved in the experimental design and editing of the manuscript. Prof. Wood was involved in the editing of the manuscript.

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The work presented in the paper is included in chapter 1 of the thesis

Student’s contribution

I was involved in writing and editing the manuscript (Contribution 80%).

Contribution of co-authors

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Manuscripts in preparation

The role of Ephrin-A2 and Ephrin-A5 in hair follicle morphogenesis and cycling


The work presented in the manuscript is included in chapter 2 of the thesis.
Student’s contribution

I was responsible for obtaining ethics approval, tissue collection and processing. I also performed immunohistochemistry, part of the gender determination experiment and histomorphometric analysis of all the data. I was also involved in writing and editing of the manuscript (Contribution 80%).

Contribution of co-authors

Ms Goh performed part of the gender determination experiment. Dr Wallace was involved in statistical analysis. Dr Fear and Dr Rodger were involved in the experimental design and editing of the manuscript. Prof. Wood was involved in editing of the manuscript.

The role of Ephrin-A2 and Ephrin-A5 in wound healing


The work presented in the manuscript is included in chapter 4 of the thesis.

Student’s contribution

I was responsible for ethical approval and all cell culture studies. I provided assistance for the in vivo excisional wounding, monitored mice and processed all tissues after euthanasia. I was responsible for data analysis and involved in writing and editing of the manuscript (Contribution 90%).

Contribution of co-authors

Dr Valvis (surgeon) performed the excisional wounds. Dr Wallace was involved in statistical analysis. Dr Fear and Dr Rodger were involved in the experimental design and editing of the manuscript. Prof. Wood was involved in the editing of the manuscript.

Presentations

The following presentations have arisen from the PhD work;

Conjoint Annual Wound and Tissue Repair Society and Australasian Society for Dermatology Research Conference. Sydney, Australia (Poster presentation)


Student Signature

…07/03/14………………………………………………………………………………………..

Coordinating Supervisor Signature.
Dedication

I would like to dedicate this thesis to my mother P.N Mantilake, my father Y.G Wijeratne, PhD supervisors Dr M Fear, Dr J Rodger and my MSc supervisor Dr P Day.
Abstract

Eph receptors constitute the largest subfamily of receptor tyrosine kinases, with 14 different receptors in humans. The ligands for these receptors are named Ephrins, of which 8 are present in humans. The importance of two specific Ephrin ligands, Ephrin-A2 and -A5, has been well documented in axonal guidance in selected central and peripheral nerves during development and regeneration. However, to date, despite evidence of expression of these ligands in skin, and more recent evidence of a functional role for these ligands in skin, there are few studies focused on the potential role of these ligands in this organ. Therefore, in this thesis, the effects of Ephrin-A2 and -A5 ligands on cutaneous development and wound healing have been investigated using Ephrin-A2\(^{+/+}\), Ephrin-A5\(^{+/+}\), Ephrin-A2A5\(^{+/+}\) transgenic and wild type mice.

Wild type mice were used to detect Ephrin-A2 and -A5 ligand expression in the epidermis and hair follicles at P1, P19 and P25 in the normal skin. The results confirmed the expression of Ephrin-A2 and -A5 ligands in the epidermis and hair follicles at all three postnatal stages. The effects of these ligands on hair follicle morphogenesis, cycling and density were also investigated using the four genotypes. Hair follicle morphogenesis was retarded in Ephrin-A5\(^{+/−}\) and Ephrin-A2A5\(^{+/−}\) at P1. Hair follicle cycling was also retarded in Ephrin-A5\(^{+/−}\) mice with a similar trend in Ephrin-A2A5\(^{+/−}\). The data therefore suggest the importance of Ephrin-A2 and -A5 ligands in hair follicle morphogenesis and cycling.

The same transgenic strains were also used to investigate the impact of Ephrin-A2 and -A5 ligands on dermal and epidermal innervation. Skin tissues were obtained from mice at P1, P19 and adult stage and pan neuronal marker Protein Gene Product 9.5 (PGP 9.5) was used to identify epidermal and dermal nerves using immunohistochemistry. Results confirmed that Ephrin-A2 and -A5 ligands both have an effect on cutaneous innervation during development and on innervation in adult mice. Sensory function was also tested on adult animals using Semmes-Weinstein filaments to investigate potential functional impairment due to loss of Ephrin-A2 or A5 ligands. Ephrin-A5\(^{+/−}\) mice showed a significantly impaired light touch sensory function. This data strongly supports a role for these ligands in both cutaneous neuroanatomy and function.
Finally, the effects of Ephrin-A2 and A5 on wound healing were assessed. *In vitro* wound healing assays were conducted using keratinocytes and fibroblasts isolated from the transgenic mice. Ephrin-A2 and –A5 ligands did not have any effect on dermal fibroblast migration, proliferation and keratinocyte migration. Analysis of *in vivo* excisional wounding in Ephrin-A2<sup>-/-</sup>, Ephrin-A2A5<sup>-/-</sup> and wild type mice showed no impact of Ephrin-A2 and –A5 ligands on the rate of wound closure. However, qualitative changes in the healed scar, potentially due to changes in wound contraction, were observed in transgenic mice.

This research shows that Ephrin-A2 and Ephrin-A5 play important roles in cutaneous development and wound healing. Further work will be essential to fully understand the mechanisms and importance of these ligands. However, ultimately it is possible that modulating the interactions of Ephrin-A2 and –A5 ligands with the cognate receptors could potentially be a useful therapeutic strategy to improve healing and reduce scarring in the future.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain–derived neurotrophic factor</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<tr>
<td>BRDU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>cAMP</td>
<td>Cyclic monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DLGN</td>
<td>Dorsal lateral geniculate nucleus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium nutrient mixture</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNTP</td>
<td>Deoxyribonucleotide</td>
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<tr>
<td>DRG</td>
<td>Dorsal root ganglions</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
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<tr>
<td>FGF-10</td>
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</tr>
<tr>
<td>FGF-7</td>
<td>Fibroblast growth factor-7</td>
</tr>
<tr>
<td>FGF-R2-IIIb</td>
<td>Receptor IIIb variant of FGF receptor 2</td>
</tr>
<tr>
<td>GJIC</td>
<td>Gap junction mediated intercellular communication</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>HB-EGF</td>
<td>Heparin binding epidermal growth factor</td>
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<td>HCS</td>
<td>Hair cycle score</td>
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<tr>
<td>HDMEC</td>
<td>Human dermal microvascular endothelial cells</td>
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<tr>
<td>HGF/SF</td>
<td>Hepatocyte growth factor/scatter factor</td>
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<td>HRMEC</td>
<td>Human renal microvascular endothelial cells</td>
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<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<tr>
<td>IBM- SPSS</td>
<td>International Business Machines</td>
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<tr>
<td></td>
<td>Corporation-Statistical Product and Service Solutions</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin like growth factor-1</td>
</tr>
<tr>
<td>IL-1</td>
<td>Insulin like growth factor</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LMW-PTP</td>
<td>Low molecular weight phosphotyrosine phosphatase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MLMC</td>
<td>Medial lateral motor column</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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</table>
| MTS          | 3-(4, 5-dimethylthiazol-2-yl)- 5
|              | -(3- carboxymethoxyphenol)-2-(4-
<p>|              | sulfophenyl)-2H-tetrazolium |
| NaCl         | Sodium chloride |
| NGF          | Nerve growth factor |
| NIH/3T3      | Immortalized mouse fibroblast cell line |
| NPY          | Neuropeptide Y |
| NTC          | No template control |
| NT-3         | Neurotrophin-3 |
| NT-4/5       | Neurotrophin-4/5 |
| PBS          | Phosphate buffered saline |
| PCF          | Preclinical facility |
| PCR          | Polymerase chain reaction |
| PDGF         | Platelet derived growth factor |
| PGP          | Protein gene product |
| PI3          | Phosphatidyl-inositol-3 |
| ROCK         | Rho-associated kinases |
| RT-PCR       | Reverse transcriptase polymerase chain reaction |
| SAM          | Sterile A motif |
| SC           | Superior colliculus |
| SDS          | Sodium dodecyl sulphate |
| SH           | Src homology |
| SHH          | Sonic hedgehog |
| TBE          | Tris/Borate/EDTA |
| TBSA         | Total body surface area |
| TGF-α        | Transforming growth factor alpha |</p>
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TPBS</td>
<td>Tris phosphate buffered saline</td>
</tr>
<tr>
<td>TrkA</td>
<td>Receptor tyrosine kinase A</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential cation channel subfamily V member 1</td>
</tr>
<tr>
<td>VAB-1</td>
<td>Variable abnormal</td>
</tr>
<tr>
<td>VACM-1</td>
<td>Vascular cellular adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>VLGN</td>
<td>Ventral lateral geniculate nucleus</td>
</tr>
</tbody>
</table>
Scope and outline of thesis

The Ephrin/Eph signalling pathway is known to be involved in neuronal patterning and neuronal projection in the central nervous system. Little work has been carried out to identify the effects of these molecules in the skin (Walsh and Blumenberg, 2011, Yamada et al., 2008, Genander et al., 2010). In addition, with over eight ligands in the Ephrin family, the specific roles of individual ligands in the skin are yet to be elucidated. Here, the effects of Ephrin-A2 and –A5 ligands on cutaneous innervation, sensory function, hair follicle morphogenesis, hair follicle cycling and wound healing have been investigated.

In Chapter 1, the structure, molecular pathways and functions of the Eph receptor/Ephrin ligand family is discussed. The chapter also highlights what is currently known regarding the roles of Eph receptors and Ephrins in the skin, with particular focus on the findings that relate to cutaneous innervation and hair follicles. A brief overview of wound healing is also presented, highlighting potential roles for Ephrin ligands and Eph receptors during each phase of the healing process and evidence for this. The healing process is discussed with relevant literature.

Chapter 2 presents evidence for the expression of Ephrin-A2 and –A5 ligands in hair follicles and epidermis at P1, P19 and P25 of C57BL/6 wild type mice. In addition the expression of Ephrin-A2 ligand in Ephrin-A5+/− mice and Ephrin-A5 ligand in Ephrin-A2−/− mice is identified at P1, P19 and P25. The role of Ephrin-A2 and –A5 ligands in hair follicle morphogenesis and cycling is investigated using Ephrin-A2+/+, Ephrin-A5−/−, Ephrin-A2A5−/− and C57BL/6 wild type mice by monitoring the initial morphogenesis and synchronized cycling phase in these mice.

Chapter 3 presents data from an investigation into the effects of Ephrin-A2 and –A5 ligands on cutaneous innervation. Innervation was analysed at P1, P19 and in adults using Ephrin-A2+/+, Ephrin-A5−/−, Ephrin-A2A5−/− and C57BL/6 wild type mice. The chapter also contains findings regarding the role of Ephrin-A2 and –A5 ligands in normal sensory function of the skin using the same transgenic mouse strains.
Chapter 4 presents findings regarding the effects of Ephrin-A2 and –A5 ligands on wound healing. Specifically, dermal fibroblast proliferation and migration as well as keratinocyte migration is assessed in vitro using the three transgenic genotypes and wildtype mice used in previous chapters. Furthermore, the chapter presents results from an in vivo study investigating the impact of the loss of Ephrin A2 and A5 ligands on wound closure and scarring using an excisional wound model and transgenic (Ephrin-A2−/−, Ephrin-A2A5−/−) and C57BL/6 wild type mice.

Chapter 5 summarizes the preceding chapters and highlights the interdependency and links between the findings in the preceding experimental chapters. In addition, the chapter discusses the clinical importance of the findings and future work.
Chapter 1
Chapter 1: Introduction

1.1 Introduction

Ephrin ligands and Eph receptors are cell membrane bound proteins involved in many developmental processes in the body. Ephrin ligands and Eph receptors act as guidance molecules in neuronal patterning and projection and are critical for retinogeniculate projection, auditory mapping, olfactory sensory mapping and motor axon projection (Reber et al., 2007). In addition to their effects on the nervous system, Ephrin/Eph signalling contributes to the regulation of angiogenesis, epithelialization during embryogenesis, differentiation of epithelial cells in adults and dense hair follicle formation (Adams et al., 1999, Duffy et al., 2006, Walsh and Blumenberg, 2011, Yamada et al., 2008).

There are 14 Eph receptors and 9 Ephrin ligands in humans which have both specific and overlapping roles (O'Leary and Wilkinson, 1999, Walsh and Blumenberg, 2011). To date, limited studies have been carried out on the role of specific Ephrins in the skin (Yamada et al., 2008, Samaka, 2010, Genander et al., 2010). In particular, studies examining the roles for specific Ephrin/eph receptor signalling in the development of cutaneous innervation, sensory function and wound repair are limited. Here, the roles of two specific Ephrins, A2 and A5, have been investigated to further our knowledge of their function and to better understand the importance of Ephrins in the skin.

1.2 Eph receptors and Ephrins

1.2.1 Introduction to Eph receptors and Ephrin ligands

The largest subfamily of receptor tyrosine kinases is the Eph receptor family, named after its expression in an Erythropoietin Producing Hepatocellular (EPH) carcinoma cell line. Ligands of the Eph receptor family are Ephrins (Eph family Receptor INteracting proteins)(Committee, 1997).

Ephrin ligands are divided into Ephrin-A and Ephrin-B groups on the basis of the structure that attaches them to the cell membrane. EphA and EphB receptor classes are defined based on their binding specificity to corresponding Ephrin ligand subgroups. Sixteen Eph receptors, EphA1 to A10 and EphB1 to B6 and nine Ephrin ligands, Ephrin-A1 to A6 and Ephrin-B1 to B3 have been identified (Committee, 1997, Menzel et al., 2001, Murai and Pasquale, 2003). Despite this classification into two distinct
groups, there is some cross-talk between the A and B groups. For example, EphA4 can interact with B class Ephrins, while EphB2 can interact with Ephrin-A5 (Figure 1.1) (O'Leary and Wilkinson, 1999, Himanen et al., 2004).

![Diagram of Eph receptors and Ephrins]

**Figure 1.1 Binding specificities of fourteen Eph receptors and eight (of 9) Ephrins**

Ephrin A ligands predominantly interact with EphA receptors, with B ligands interacting with the EphB receptors. However, some cross-talk has been demonstrated and the complete set of physiologically relevant interactions is not known (Reproduced from: O'Leary and Wilkinson, 1999)

### 1.2.2 Structure of Eph receptors and Ephrin ligands

Eph receptors are comprised of an extracellular, transmembrane and cytoplasmic region. The extracellular region contains the ligand binding domain, a cysteine rich region adjacent to the ligand binding domain and two fibronectin repeats. The cytoplasmic region of the receptor is comprised of a juxtamembrane segment, kinase domain, sterile a motif (SAM) domain and PDZ-binding motif (Himanen.J and Nikolov.D.B., 2003) (Figure 1.2a).

Ephrin-A (Figure 1.2b) and Ephrin- ligands B (Figure 1.2c) are both membrane bound ligands with extracellular receptor-binding domains. Ephrin-A ligands are bound to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage, whereas Ephrin-B
ligands traverse the membrane and possess a cytoplasmic domain and C-terminal PDZ-binding motif (Himanen, J and Nikolov, D.B., 2003, Flanagan and Vanderhaeghen, 1998). The presence or absence of the cytoplasmic domain distinguishes the two types of ligand, with additional differences also observed from crystal structures (Toth, 2001). The A and B classes of receptor are distinguished by a specific structural feature – the presence or absence of an H-I loop connecting the H and I strands of the amino terminal ligand binding domain (Himanen, 1998).

Many studies have been carried out to better understand the binding specificities, functions and interactions between the individual Eph receptors and the Ephrin ligands (Labrador, 1997, Himanen, 2001, Himanen, 1998). Binding specificity of the Eph receptors is dictated by the structure of the N-terminus of the globular domain and to a lesser extent, the adjacent cysteine-rich domain (Labrador, 1997). Eph receptors and Ephrin ligands bind to form heterodimers with high affinity and specificity in solution with a range of concentrations from 10nM to 20µM. In high concentrations dimer pairs form tetramers. Tetrameric complexes form ring-like structures with each ligand binding to two receptors and each receptor binding to two ligands (Himanen, 2001). The highly conserved kinase domain, which is present in the cytoplasmic region of the Eph receptor, catalyses tyrosine phosphorylation of protein substrates. Crystallography, size exclusion chromatography and ultracentrifugation studies have revealed isolated EphA4 SAM motifs form dimers in a crystal environment as well as in solution, suggesting the role of the SAM domain in Eph receptors is to promote clustering (Stapleton, 1999). Whilst the structural features of Ephrins and Eph receptors are well characterised, the specificity and functional redundancy in different tissues and time-points remains the subject of further investigation. The study of two specific Ephrins in the developing and adult skin is the focus of the research presented here.
1.2.3 Ephrin ligand/ Eph receptor signalling

Since both the Ephrin ligands and the Eph receptors are membrane bound, Ephrin signalling is juxtacrine (between cells in close proximity). Ephrin ligand/ Eph receptor signalling is also bidirectional. A forward signal is sent to the Eph receptor expressing cell upon binding to the ligand, which simultaneously sends a reverse signal to the ligand-expressing cell (Arvanitis and Davy, 2008) (Figure 1.3a). Eph receptors and Ephrin ligands can interact between different cells in trans – producing the bidirectional signal. Alternatively both the ligand and receptor can be expressed on the same cell, signalling in cis and inhibiting any interaction with other cells (Figure 1.3b) (Arvanitis and Davy, 2008)
Eph receptors and Ephrin ligands use both common and more specific signalling effectors including Src family kinases and RAS/Rho family GTPases that regulate the actin cytoskeleton (Pasquale, 2008). Some signalling pathways are confined to specific Eph classes. For example, EphA receptors can signal through Rho exchange factor Ephexin whilst EphB receptors are associated with the exchange factors Intersectin and Kalirin (Pasquale, 2008). Although multiple studies show that Ephrin/ Eph signalling in the skin is likely to be important (Lin et al., 2012, Walsh and Blumenberg, 2011, Genander et al., 2010), the roles of specific Eph-Ephrin signalling molecules and pathways in the skin remain poorly understood.

![Figure 1.3 Eph/Ephrin Trans and Cis signalling](image)

**Figure 1.3 Eph/Ephrin Trans and Cis signalling.** (a) Eph receptors and Ephrin ligands can interact between different cells in trans – producing a bidirectional signal. (b) Both the ligand and receptor can be expressed on the same cell, signalling in cis and inhibit trans interaction and/or signalling. (Reproduced from: Arvanitis and Davy, 2008)

**1.2.3.1 EphA receptor forward signalling**

EphA receptor signalling most commonly results in repulsion, although in a few cases attraction or adhesion is also observed (Flanagan and Vanderhaeghen, 1998). The different consequences are determined by the downstream pathways activated by the EphA receptor and are therefore different for different cell types and potentially at...
different time-points during development. Small GTPases of the Rho family, namely RhoA, Rac1 and Cdc42 link EphA receptors to the actin microtubules during signal transduction (Reber et al., 2007). During nervous system development the motile tip of the growing axon (growth cone) moves towards the precise target by regulating the actin cytoskeleton. Activation of RhoA and its downstream effector Rho-associated kinases (ROCK) leads to growth cone collapse and retraction (Dickson, 2001). In contrast activation of Cdc42 and Rac1 promote growth cone extension (Kozma et al., 1997). Activated EphA receptors upon Ephrin ligand binding promote the ability of ephexin 1 to activate RhoA and inhibit the ability of ephexin to activate Rac 1 and Cdc42 (Shamah et al., 2001), predominantly resulting in repulsion (or loss of the growth cone). Ephrin-A/EphA signalling can also stimulate or inhibit the RAS/MAPK pathway dependent on other cellular factors, contributing to the regulation of cell proliferation as well as axon outgrowth and cell migration (Reber et al., 2007).

1.2.3.2 Ephrin-A reverse signalling

Ephrin-A ligands are attached to the cell membrane by a GPI anchor rather than a transmembrane domain. For a long time, it was thought that these proteins were therefore not capable of reverse signalling into the cell expressing them but merely acted as ligands to signal to the cells expressing EphA receptors. However, more recently, Ephrin-As were found to be localized in membrane micro domains, putting them in close proximity to a range of signalling molecules (Davy et al., 1999). Through this proximity to signalling mediators, Ephrin-A ligands can effectively induce reverse signals upon binding to EphA receptors. Reverse signalling of Ephrin-A ligands is dependent on the membrane bound Fyn protein tyrosine kinase and the outcome is generally to increase adhesion of cells to fibronectin and alter cell morphology (Davy et al., 1999, Davy and Robbins, 2000)

1.2.3.3 EphB receptor forward signalling

Similar to the EphA receptors, EphB receptors also activate members of the Rho family GTPases, Rac1 and Cdc 42, via different exchange factors and can also regulate the Ras/MAPK pathway (Reber et al., 2007). Their signalling affects the cytoskeleton and results in a range of cellular behaviours including increased adhesion and repulsion.
Activated EphB receptors bind to SH2 domain proteins such as the cytoplasmic tyrosine kinase Fyn, Src, NCK, Crk, RasGAP protein, low molecular weight phosphotyrosine phosphatase (LMW-PTP) and the p85 subunit of phosphatidyl-inositol-3 (PI3) kinase. Some of these molecules are components of pathways that regulate de-polymerization of the actin cytoskeleton, causing repulsion of cells, (Mellitzer et al., 2000) while some of the molecules, such as LMW-PTP, enhance cell adhesion (Stein et al., 1998).

1.2.3.4 Ephrin-B reverse signalling

Unlike Ephrin-As, Ephrin-Bs possess a cytoplasmic domain, providing a substrate for intracellular signalling. The cytoplasmic domain of Ephrin-Bs is phosphorylated by Src family kinases and provides binding sites for Src homology (SH2/SH3) domain adaptor proteins such as GRB4, which in turn regulate actin cytoskeleton morphology (Murai and Pasquale, 2004, Cowan and Henkemeyer, 2001). Ephrin-B signalling also occurs in a phosphorylation independent manner via interaction of the C- termini of Ephrin-Bs with PDZ domain proteins such as GRIP1 and GRIP2. These signalling pathways can modulate cell migration (Cowan and Henkemeyer, 2002, Reber et al., 2007).

1.3 Skin and Ephrins

1.3.1 Skin

Skin is the largest organ of the body. The three main structural layers of the skin are the epidermis, dermis and the subcutaneous layer (Figure 1.4a). Skin also contains many specialized structures with specific functions, including hair follicles (discussed in detail in 1.4.2). The epidermis is the barrier layer of the skin. It is formed from layers of stratified keratinocytes that differentiate as they migrate to the surface, ultimately forming a lipid rich, mitotically inactive cells that have extruded the nucleus on the outer surface. These cells are constantly replaced and lost as part of normal skin homeostasis (Robbins, 1997). After injury, epithelial cell production and migration is rapidly enhanced over normal homeostasis as repair of the lost barrier surface is critical to reducing the chance of infection and restoring function (Singer and Clark, 1999). The epithelium does not contain blood vessels, relying on diffusion of nutrients and molecules from the underlying dermis. The dermis lies between the epidermis and the subcutaneous tissue. The main cells of the dermis are fibroblasts, which synthesize
collagen, reticulin, elastin, fibronectin and glycosaminoglycans. The dermis provides structural integrity and elasticity to the skin (Robbins, 1997).

The blood supply to the skin has two main horizontal layers both contained within the dermis. The deep plexus lies above the subcutaneous fat and the superficial plexus lies in the papillary dermis (Robbins, 1997) (Figure 1.4b).

Sensory function in the skin is maintained by both myelinated and unmyelinated nerve fibres. Most free sensory nerve endings lie in the dermis with only a few non-myelinated nerves penetrating into the epidermis (Section 1.4) (Hunter et al., 2002, Gardner, 2000). As well as providing a protective barrier between the organism and the environment, the skin provides an essential role in sensory function. The overlap between the ability of the skin to repair injury through cell proliferation and migration with the known functions of Ephrins, as well as the complex and extensive innervation of the skin, suggest there may be a role for Ephrins in skin maintenance, repair and innervation.
Figure 1.4 Structure of the skin.

(a) Illustrates the three main layers of the skin: the epidermis, dermis and subcutaneous tissue. Skin also contains hair follicles, blood vessels and nerve fibres.

(Source: http://skincancer.dermis.net/content/e01geninfo/e7/index_eng.html )

(b) Illustrates the blood supply to the skin. The deep plexus lies above the subcutaneous fat and the superficial plexus lies in the papillary dermis (Adapted and modified from: http://www.skin-care-forum.basf.com/images-/home/the-skin-s-blood-vessel-system/2006/01/19?id=9a7d8dcb-5f5d-4bce-92d8-4840cbd71a81&mode=Detail).
1.3.2 Expression of Ephrin ligands and Eph receptors in the skin

The expression of Ephrins and Eph receptors in the skin has previously been investigated in both mice and human skin. Hafner et.al. investigated mRNA expression of 21 Eph receptors and Ephrin ligands in human skin and compared them with 13 other human tissues. All 14 Eph receptors (both A and B family) and 8 Ephrin ligands were detected in human skin, including EphA8 and Ephrin-A2, which were not expressed in most other tissues tested. EphA1, EphB3 receptors and Ephrin-A3 ligand had the highest levels of expression in skin compared to other tissues (Hafner et al., 2006).

In adult mouse back skin RTPCR was used to detect Eph receptor and Ephrin ligand expression. EphB3, EphB4, EphB6, EphA1, EphA3, EphA4, EphA6, EphA7 receptors were all expressed in murine skin, with all the Ephrin ligands detected except for Ephrin-B3 (Genander et al., 2010).

The EphA1 receptor has also been shown to be down regulated in non-melanoma skin cancers (NMSCs) when compared to normal epidermis (Hafner et al., 2006). This suggests a possible role of Ephrin/ Eph signalling in both normal skin homeostasis and pathology. Therefore further work on the role of specific Eph receptors and their ligands is warranted in the skin.

1.4 Cutaneous innervation development and function

1.4.1 Cutaneous innervation

The peripheral nervous system is divided into somatic and autonomic divisions. The somatic nervous system comprises of somatic sensory neurons which innervate the skin, muscles and joints (Amaral, 2000) as well as motor neurons predominantly innervating skeletal muscles. Anatomically the autonomic nervous system is divided into sympathetic, parasympathetic and enteric nerves (Sternini, 1997). Sympathetic and parasympathetic fibres innervate cardiac muscles, smooth muscles and glandular tissue, while the enteric nervous system innervates the gastrointestinal tract (Iversen et al., 2000). Both somatic sensory nerves and autonomic sympathetic nerves innervate the skin (FitzGerald, 1996).

Cutaneous innervation is functionally divided into the afferent somatosensory innervation and efferent autonomic innervation (Hendrix et al., 2008). Sensory nerves
are receptive for nociceptive stimuli such as touch, itch and pain, whereas efferent autonomic nerves control glandular activity of exocrine glands and smooth muscle contraction (Paus et al., 1997, Hendrix et al., 2008).

1.4.1.1 Somatosensory nerves in the skin

Peripheral information is transmitted to the central nervous system via afferent sensory nerve fibres. Sensory fibre nerve endings are of two types. The nerve terminals can be a bare nerve ending or alternatively attached to special sensory structures such as Pacini, Ruffini, Meissner’s corpuscles or Krauses organs (Figure 1.5). Sensory nerve terminals without specialized structures possess nociceptive units responding to noxious (painful) stimuli as well as thermo-receptive units responding to cold or warmth (Sternini, 1997, FitzGerald, 1996, Gardner, 2000). Merkel cell-neurite complexes are expanded nerve terminals, which are attached to Merkel cells and respond to sustained pressure. These complexes are present at the basal epithelium of the epidermal pegs and ridges (Figure 1.5). Meissner’s corpuscles are rapidly adapting receptors detecting delicate work, while Ruffini endings are slowly adapting receptors responding to shearing stress. Pacinian corpuscles are rapidly adapting receptors responding to vibration (FitzGerald, 1996, Gardner, 2000). Bundles of afferent and efferent nerve fibres join to form peripheral nerves. Morphologically, sensory axons are classified as myelinated A-fibres and unmyelinated C-fibres. Myelinated A-fibre axonal diameter is wider than 1\(\mu\)m and unmyelinated C-fibre axon diameter is 1\(\mu\)m wide or less. Unmyelinated axons are present in the invaginations of Schwann cell plasmalemma. Myelinated and Schwann cell enclosed nociceptive nerve endings lose the myelin and Schwann cell sheath at the dermal-epidermal junction and enter the epidermis (Table 1.1) (Oaklander and Siegel, 2005).
Figure 1.5 **Major sensory receptors and their modalities in the skin**

The bare nerve endings and nerve terminals attached to special sensory structures such as Pacini, Ruffini, Meissner’s corpuscles and Krauses organs present at different levels within the skin. (Source: http://brewoods90.edublogs.org/files/2012/05/skin-receptors-10q5r53.jpg)

<table>
<thead>
<tr>
<th>Receptor type</th>
<th>Fiber group</th>
<th>Modality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meissner’s corpuscle</td>
<td>Aα/β</td>
<td>Light touch</td>
</tr>
<tr>
<td>Merkel disk receptor</td>
<td>Aα/β</td>
<td>Pressure/texture</td>
</tr>
<tr>
<td>Pacinian corpuscle</td>
<td>Aα/β</td>
<td>Vibration</td>
</tr>
<tr>
<td>Ruffini ending</td>
<td>Aα/β</td>
<td>Skin stretch</td>
</tr>
<tr>
<td>Thermal receptors</td>
<td>Aδ/C</td>
<td>Temperature</td>
</tr>
<tr>
<td>Nociceptors</td>
<td>Aδ/C</td>
<td>Pain</td>
</tr>
</tbody>
</table>

**Table 1.1 Types of receptors and nerve fibre subtypes with their sensory modalities.**

Large myelinated Aα/β nerve fibres with encapsulated terminals mediate somatic modalities of touch. Small myelinated Aδ and unmyelinated C nerve fibres with bare endings mediate painful or thermal sensation (Adapted and modified from: Gardner, 2000)
1.4.1.2 Neuropeptides

Sensory nerves conduct nociceptive stimuli to the central nervous system, which is named an orthodromic response. Sensory nerves can also signal efferently to the skin, which is named an antidromic response. As a consequence of the antidromic response, sensory neuron terminals release neuropeptides such as substance P, calcitonin gene related peptide (CGRP) and neuropeptide Y (NPY) (Altun et al., 2001, Holzer, 1988, Lembeck and Holzer, 1979, Hendrix et al., 2008). Neuropeptides are one of the largest subfamilies of extracellular messengers, which can act as neurotransmitters, hormones and paracrine factors (Schäffer et al., 1998). It is the antidromic response that creates a close link between the peripheral sensory nerves in the skin and the inflammatory response in wound healing.

For example, substance P, released by sensory fibres and known to have an impact on the inflammatory response also stimulates epidermal cell proliferation in vitro (Tanaka et al., 1988). Another sensory neuropeptide named vasoactive intestinal polypeptide (VIP) also has mitogenic activity in human keratinocytes (Haegerstrand et al., 1989), whereas CGRP can have an inhibitory effect on Langerhans cell antigen presentation, suggesting it has an immunomodulatory effect (Hosoi et al., 1993). Therefore, through the release of neuropeptides, the cutaneous nerves have an intrinsic link to wound repair, inflammation and the skin.

1.4.1.3 Effects of Ephrin ligands and Eph receptors on cutaneous innervation

Nerve growth factor (NGF), neurotrophin-3 (NT-3) and brain –derived neurotrophic factor (BDNF) have an impact on the innervation and density of the peripheral nervous system (Albers, 1994, LeMaster et al., 1999). Receptor tyrosine kinases, expressed on neonatal sensory fibres signal through these neurotrophic factors and regulate the innervation of upper dermis, epidermis, and the upper portion of hair follicles (Bennett, 1996, Rice et al., 1998, Moss et al., 2005). However, the interactions of Eph receptors and their ligands on cutaneous innervation is not known.

Effects of Ephrin/Eph signalling on motor axons have been reported in several previous studies. Differential expression patterns of EphA4 and its ligands Ephrin-A2 and Ephrin-A5 during motor axon sorting at limb plexus, initial outgrowth of axons and path finding to the avian hind limb have been identified (Eberharta and Krulla, 2000).
Also motor axons of the medial lateral motor column (MLMC) with ectopically expressed EphA4 receptors, project into the dorsal avian hind limb and do not project into Ephrin-A5 rich regions (Eberhart et al., 2002, Eberhart et al., 2004). EphA receptor expressing growth cones of both cutaneous and muscle afferent outgrowths are inhibited by soluble and membrane attached Ephrin-A5 ligand in vitro. In addition, ectopic expression of Ephrin-A5 in ovo shows inhibitory effects of Ephrin-A5 on cutaneous sensory nerves in the presence of other normal guidance cues (Muñoz et al., 2005). It has also been suggested in Munoz et al. (2005), that A class Ephrin/Eph signalling, impacts on sensory innervation directly as well as indirectly via inhibitory effects on EphA4 expressing motor axons having an impact on sensory axon growth. Therefore the effects of Ephrin/Eph signalling on motor axons as mentioned above might have an indirect effect on sensory innervation (Muñoz et al., 2005).

Supporting the suggestion that Ephrins are important in sensory as well as motor axon growth a recent study showed that a lack of Ephrin-A2 and –A5 ligands caused impaired dorsal (epaxial) sensory projections in chick embryos (Wang et al., 2011). In addition, interactions with EphA3/4 receptor expressing motor axons were necessary for precise Ephrin-A expressing sensory axonal projection. However, the sensory projection was not dependent on EphA3/4 forward signalling, suggesting the importance of bidirectional signalling is not universal to all processes influenced by Ephrin signalling (Wang et al., 2011).

Many studies have identified changes in cutaneous innervation during wound healing, with other studies showing changes in innervation in scars (Reynolds, 1995, Zhang and Laato, 2001). Neonatal skin wounding of rats resulted in sensory hyperinnervation and the majority of the innervations contained both A and C fibre sensory axons (Reynolds, 1995). In another experiment, sensory hyperinnervation was observed after two weeks of superficial skin wounding in rats (Aldskogius, 1987). Many studies have reported that sensory nerve fibres possess many biological activities on skin such as cell proliferation and inflammation via neurotransmitters and neuropeptides, in addition to their role carrying information between the periphery and the central nervous system (Hendrix et al., 2008). A consequence of this is that molecules that impact on innervation can often have indirect consequences on wound repair, not only in restoration of sensory function but also scar formation (Scott et al., 2007, Hamed et al., 2011). This is important to consider when investigating the effects of proteins such as
the Ephrins, which are known to be important in innervation and therefore may have much wider effects in the skin.

The epidermis of rat during late embryogenesis and postnatal days (E20-P7) has been shown to express the Ephrin-A4 protein. However by P21, only the most superficial layers of the epidermis express Ephrin-A4. Neonatal dorsal root ganglions (DRG) also express EphA4, EphA5 and EphA6 (Moss et al., 2005). In addition following dorsal foot wound surgery the dermis and the epidermis was hyperinnervated at the wound site due to the down regulation of Ephrin-A4 (Moss et al., 2005). This suggests the expression of Ephrin-A4 in the epidermis plays an inhibitory role in cutaneous innervation in rats and may contribute to altered innervation patterns during wound healing. Therefore it will be important to understand the role of other Ephrin proteins in this context to gain a clear picture of the role of the pathway and of individual members of the Ephrin family.

1.4.2 Ephrins and hair follicles

1.4.2.1 Hair follicles

Hair follicles share the same basic structure with limited variation in size and shape depending on their anatomical location (Figure 1.6a). Matrix cells in the hair bulb proliferate to generate the hair shaft. Melanocytes distributed among the matrix cells produce pigment in the hair shaft. Differentiated matrix cells move upwards through the rigid inner root sheath. The dermal papilla, composed of specialized fibroblasts, is present at the base of the hair follicle and is considered to be important in controlling the number of matrix cells (Paus and Cotsarelis, 1999).

Hair follicle morphogenesis is the process of formation of the whole hair follicle structure from a primitive epidermis (Stenn and Paus, 2001). Hair follicle morphogenesis occurs as a consequence of neuroectodermal and mesenchymal interactions, controlled by defined molecular mechanisms (Botchkarev et al., 1998, Schmidt-Ullrich and Paus, 2005), including WNT signalling and Sonic hedgehog(SHH) pathways (St-Jacques et al., 1998, Millar, 2002). Normal murine hair follicle morphogenesis is comprehensively classified from stage 0 to stage 8 (Paus et al., 1999) (Figure 1.6b). Hair follicle morphogenesis and the first postnatal hair follicle cycling in C57BL/6 mice follow a precise time scale (Muller-Rover et al., 2001) (Figure 1.6c).
However, different types of hair follicles such as vibrissae, tylotrich and non-tylotrich develop at slightly different time points during fetal and perinatal life (Schmidt-Ullrich and Paus, 2005).

After morphogenesis, mature hair follicles undergo a cycling process, which consists of a growth phase (anagen), regression phase (catagen), resting phase (telogen) and shedding phase (exogen). Several reasons have been postulated as to why hair follicle cycling occurs, including controlling the length of the hair according to the site, shedding fur periodically to cleanse the body surface, changing the body cover according to environmental changes and protection from improper formation of the follicle (Stenn and Paus, 2001). During the anagen phase, the lower cycling portion of the hair follicle regenerates. Since the anagen phase is limited to a precise time scale, the lengthening of the hair shaft is also limited to the length of the anagen phase. The anagen phase is comprised of six stages and it is the length of anagen stage VI that determines the length of the hair follicle at different regions of the body (Stenn and Paus, 2001, Muller-Rover et al., 2001, Trotter, 1924). After the anagen phase, hair follicle growth ceases and catagen begins. Catagen phase, which consists of 8 stages, goes through a series of events including the ceasing of cell growth, pigmentation, release of the papilla from the hair bulb, disappearance of the differentiated layers of the lower follicle and extracellular matrix remodelling. At the end of catagen, hair follicles reside in the upper dermis. Hair follicles in telogen are located in the dermis reaching their minimal length (Muller-Rover et al., 2001, Stenn and Paus, 2001).

Hair follicles have a significant impact on skin biology in both animals (which have dense hair coverings) and humans (which have much less dense hair follicles). Hair follicles contribute to injury repair, angiogenesis and skin immunity (Stenn and Paus, 2001). Hair follicles are also a major site of storage of keratinocyte stem cells that can form the epidermis of normal neonatal mouse skin as well as adult mouse skin with small full thickness wounds (Taylor et al., 2000).
Figure 1.6a-c. Structure of the hair follicle, hair follicle morphogenesis and hair follicle cycling.

(a) Structure of the hair follicle (Source: http://www.proprofs.com/flashcards/upload/a4914017.gif). (b) Schematic representation of the hair follicle morphogenesis from stage 0 to 8, and the developmental changes of the thickness of the skin and the hair follicle angle (Source: Paus et al. 1999). (c) Time scale of hair follicle morphogenesis and hair follicle cycling of C57BL/6 mice (Source: Muller-Rover et al., 2001).
1.4.2.2 Association of Ephrins with hair follicles

There is substantial evidence that the Ephrin family is important in hair follicle proliferation and formation. Intravenous injection of soluble recombinant Ephrin-A2-Fc or Ephrin-B2-Fc that can bind to receptors and block endogenous signalling led to increased proliferation of hair follicle keratinocytes and cells in the basal layer of the epidermis (Genander et al., 2010). The results suggest that Ephrins are negative regulators of adult hair follicle and epidermal stem cell proliferation of the skin, although the use of these recombinant proteins primarily blocks the reverse signalling from the ligand rather than the bidirectional forward and reverse nature of normal Ephrin signalling (Genander et al., 2010).

Transient expression of Ephrin-B2 ligands in keratinocytes of embryonic hair buds suggested a possible role of Ephrins in hair follicle formation (Egawa et al., 2009). However, conditionally deleting the Ephrin-B2 gene from the epidermis did not cause any significant defect in the skin or in hair follicles. Therefore Ephrin-B2 is probably not necessary for the development of epidermal tissues (Egawa et al., 2009). In other studies high expression levels of EphA4, EphB4 and Ephrin-B1 in hair follicle bulge stem cells have been identified, again suggestive of a role for Ephrin signalling in hair follicles (Genander and Frisén, 2010).

Ephrin-A3 and its receptor EphA4 have been shown to be involved in the formation of new hair follicles and increased expression of Ephrin-A3 was associated with dense follicle formation and accelerated hair follicle maturation in neonatal mice (Yamada et al., 2008). EphA2 expression has also been observed in vascular endothelial cells surrounding the hair follicles at all stages of hair cycling, suggesting a possible role of EphA2 in vascularisation in the area surrounding the hair follicles (Yamada et al., 2008).

However, despite previous studies identifying an association of Ephrins with hair follicles, there is still limited functional information regarding the relationship between specific Ephrins and hair follicle morphogenesis and cycling. In addition, the role of Ephrin-A2 and Ephrin-A5 in hair follicle cycling and morphogenesis is yet to be examined. Hence in this project the role of Ephrin-A2 and Ephrin-A5 in hair follicle cycling and morphogenesis was investigated.
1.5 Wound healing and Ephrins

1.5.1 Introduction to wound healing and scar formation

Wound healing is a complex process involving a wide range of factors such as soluble mediators, formed blood elements, extracellular matrix and parenchymal cells. Wound healing has 3 major phases: early/late inflammation, reepithelialisation and granulation tissue formation and matrix formation/remodelling which overlap in time (Clark, 1995) (Figure 1.7).

Abnormalities in the wound healing process can cause hypertrophic scar formation and wound contracture as a result of injury, most commonly those more severe in either surface area or depth (Scott et al., 2007). Several studies have shown increased numbers of nerve axons in hypertrophic scars (Zhang and Laato, 2001, Crowe et al., 1994). In addition Zhang and Laato suggested hyperinnervation could be due to abnormal wound healing or vice versa. Systemic decrease in cutaneous innervation has been identified post burn injury, while another study showed increased levels of CGRP immunoreactive nociceptive fibres in chronic burn patients (Anderson et al., 2010, Hamed et al., 2011). Abnormal wound healing can cause patient discomfort and increase psychological distress. Therefore it is an important goal of injury treatment to minimize excessive scarring and restore normal sensory innervation in the skin.
Figure 1.7a-c. Three major phases of wound healing. (a) Inflammation, which lasts up to 48 hours after injury. (b) Re-epithelialization and granulation tissue formation which lasts for 2 to 10 days after injury. (c) Matrix formation and tissue remodelling which can last up to 1 year or more. (a) Illustrates a wound within 24 to 48 hours after injury causing platelet aggregation forming a fibrin clot and infiltrating neutrophils to fight against bacteria. (b) Illustrates a wound between 5-10 days after injury. Re-epithelialization under the eschar and vascularization can be observed. (c) Illustrates a cutaneous wound between 1-12 months. A hypertrophic scar with disorganised collagen without skin appendages can be observed (Source: Gurtner et al. 2008).

1.5.2 Inflammation

Extravasation of blood after tissue injury causes blood coagulation and platelet aggregation, causing the formation of a fibrin rich clot. Activated platelets secrete cytokines and growth factors (Martin, 1997). Coagulation pathways activate complement pathway and injured or activated parenchymal cells also secrete vasoactive mediators and chemotactic factors. As a consequence of these factors, inflammatory...
leukocytes infiltrate the wounded site. Infiltrating neutrophils cleanse the injured area of foreign particles (Clark, 1995). Infiltrating peripheral blood monocytes are activated and become macrophages, releasing growth factors, which lead to granulation tissue formation and wound debridement (Singer and Clark, 1999, Brown, 1995). Adherence of monocytes and macrophages to the extracellular matrix induces the expression of colony-stimulating factor 1, tumour necrosis factor alpha (TNF-α), TGF-α, TGF-β and PDGF (Singer and Clark, 1999). These growth factors act as chemo attractants for many cells (PDGF, TGF-β), angiogenic stimulators (TGF-α), keratinocyte activity stimulators (IL-1, TGF-α) and stimulators of collagen synthesis and collagenase secretion (PDGF, TGF-β, TGF-α, IL-1) (Linares, 1996). Macrophages play a role in the transition between inflammation and the later stages of wound repair, providing the link between inflammation and the induction of granulation tissue formation and re-epithelialisation (Clark, 1995).

1.5.2.1 Possible role of Eph receptors and Ephrin ligands in inflammation

Decreased adhesion between endothelial cells in the vascular endothelium and increased adhesion of circulatory leukocytes to the vascular endothelium leads to leukocyte extravasation during inflammation. Possible roles of Eph receptors and Ephrin ligands have been proposed in this inflammatory process (Ivanov and Romanovsky, 2006). In the presence of the proinflammatory cytokine TNF-α, EphrinA1 (formerly known as B61 protein) was expressed in umbilical vein endothelium (Holzman et al., 1990). Immune modulatory cytokines and growth factors such as Interleukin-1β (IL-1β), basic fibroblast growth factor, Interleukin-2 (IL-2), epidermal growth factor, and transforming growth factor-β modulate EphA2 and Ephrin-A1 expression in the gut epithelium. Furthermore addition of Ephrin-A1 into injured intestinal epithelial monolayers promotes wound healing (Rosenberg et al., 1997). Therefore Eph receptors and Ephrin ligands may have an important role in modulating epithelial barriers in response to pro-inflammatory cytokines during inflammation.

1.5.3 Re-epithelialisation (Re-establishing a cutaneous cover)

Reepithelialisation begins within hours of injury. Keratinocytes of the stratified epidermal sheet and hair follicle move one over the other in a leapfrog fashion to recover the injured site (Clark, 1995). Migrating epithelial cells undergo many key
phenotypic changes including altered keratin expression, reorganization of keratin filaments, dissolution of intercellular desmosomes, development of peripheral cytoplasmic actin filaments and changes in gap junction mediated intercellular communication (GJIC) via differential expression of connexins (Goliger and Paul, 1995, Gabbiani et al., 1978, Paladini et al., 1996). Loss of tenacious binding between the epidermis and dermis as well as dissolution of hemidesmosomal links between the epidermis and the basement membrane results in lateral mobility of the epidermal cells and therefore enhanced ability to cover the wound (Singer and Clark, 1999). In addition, differential expression of integrins in keratinocytes during re-epithelialization lead these migratory epithelial cells to attach and crawl over the provisional wound matrix (Clark et al., 1996, Martin, 1997).

Epithelial cell proliferation starts 24 to 48 hours after injury behind the migrating epidermal cells. It is suggested that the absence of neighbour cells at the wound margins signals to increase epithelial cell migration and subsequent proliferation of the cells at the margin (Singer and Clark, 1999). The local release of growth factors such as transforming growth factor α, heparin binding epidermal growth factor (HB-EGF), keratinocyte growth factor (KGF), which act on epidermal cells through paracrine pathways or in an autocrine or juxtacrine manner is also important in promoting re-epithelialisation (Werner et al., 1994, Higashiyama et al., 1991, Clark, 1995). Re-epithelialisation requires a matrix for cells to migrate and proliferate across in order to restore the epidermal barrier between the organism and the environment. Therefore, occurring at the same time as the expansion and migration of epithelial cells, there is enhanced dermal matrix deposition within the wound. This granulation tissue deposition is a key part of this second phase of wound repair.

1.5.3.1 Effects of Ephrin ligands and Eph receptors on epithelial tissue

EphA1 and Ephrin-A3 have been shown to be expressed in pregastrulation embryos during the epithelialisation of epiblasts and increased expression of EphA1 has been observed after the onset of gastrulation (Duffy et al., 2006). Null mutations of the Eph receptor VAB-1 (variable abnormal) in C.elegans embryos resulted in defective epidermal cell migration, defective ventral closure of the epidermis and defective neuroblast cell movement during ventral gastrulation cleft closure. Since VAB-1 is not expressed in epidermal cells and expressed in neuronal cells, it was suggested that
epidermal cells might be receiving guidance cues from neuronal cells (George et al., 1998, Miao and Wang, 2009).

Many studies have reported the role of Eph receptors and Ephrin ligands in regulating epithelial integrity via interactions with cell junction molecules. Tight junctions present in the epithelial cells are comprised of occludins, claudins and junctional adhesion molecules (Miao and Wang, 2009). Cell-cell contact dependent tyrosine phosphorylation of EphrinB1 caused by physical interaction of Ephrin-B1 and claudins (1 or 4) present on the same cell surface of epithelial tissues attenuated tight cell-cell adhesion (Tanaka et al., 2005b). On the other hand, interaction of activated EphA4 with claudin 4 on the same cell resulted in tyrosine phosphorylation of claudin 4, which in turn enhanced paracellular permeability of tight junctions (Tanaka et al., 2005a).

E-cadherin is a calcium dependent adhesion molecule of epithelial cells and present in adherens junctions. E-cadherin null mutant embryonic stem cells expressed increased levels of Ephrin-A1, Ephrin-A2, Ephrin-B1, Ephrin-B2, Ephrin-B3, EphB4 and decreased levels of EphA2 compared to wild type cells, suggesting a possible role for E-cadherin in regulating the expression of Eph and Ephrins (Orsulic and Kemler, 2000). Another study reported that tyrosine phosphorylation of EphA2 and localization of Eph receptors to the cell-cell contact site was dependent on E-cadherin expression (Zantek et al., 1999).

Addition of human Fc-conjugated Ephrin-A ligands on to keratinocyte cell cultures showed that all the Ephrin-A ligands inhibit epidermal keratinocyte migration. However Ephrin-A ligands did not seem to have an effect on epidermal keratinocyte attachment to the substratum (Walsh and Blumenberg, 2011). Microarray studies revealed that genes regulated by Ephrin-A ligands promote epidermal keratinocyte differentiation, which was confirmed by the expression of epidermal keratinocyte markers such as keratin 1, keratin 10 and the presence of cornified envelopes in epidermal keratinocyte cell cultures treated with Ephrin-A ligands (Walsh and Blumenberg, 2011).

There is substantial indirect and some direct evidence that Ephrin signalling is important in the maintenance of the epidermis and potentially in wound repair. Further studies to better understand the roles of individual Ephrins and how Ephrin signalling influences the epidermis and wound healing have the potential to shed more light on these important processes and how Ephrins are integrated with other important pathways in the skin.
1.5.4 Granulation tissue formation/neovascularization and Ephrins

1.5.4.1 Granulation tissue formation

Granulation tissue formation and the invasion of fibroblasts, macrophages and blood vessels into the wound space occur within days of the injury (Singer and Clark, 1999). Provisional matrix comprised of fibrin and fibronectin acts as a scaffold for the epithelial cells to migrate over to form the barrier while also providing the template for the granulation tissue (Clark et al., 1982a, Singer and Clark, 1999). Extracellular matrix synthesis, deposition and remodelling are carried out by fibroblasts. Migrated fibroblasts in the wound synthesize extracellular matrix and the provisional extracellular matrix is gradually replaced with a collagen rich granulation tissue. Fibroblasts reduce the production of collagen once the granulation tissue is rich in collagen in response to TGF-β (Clark et al., 1995, Singer and Clark, 1999).

1.5.4.2 Possible role of Ephrins in granulation tissue formation

A study carried out on the microvasculature network in tissue-engineered constructs showed extracellular matrix proteins such as collagen IV and laminin production responded to a gradient of poly ethylene glycol conjugated Ephrin-A1 (Saik et al., 2011).

It has also been reported that Ephrin-B2 and EphB4 are expressed in normal dermal fibroblasts (Avouac et al., 2011). However, to date there is limited information regarding a role for specific Ephrins in replacement of the dermal matrix during skin wound repair.
1.5.4.3 Neovascularization

Microvascular endothelial cells migrate into the granulation tissue and proliferate to form new blood vessels via modulating extracellular matrix components and soluble factors (Madri et al., 1996). Many factors such as TGFβ, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and thombospondin regulate angiogenesis (Folkman and D’Amore, 1996, Iruela-Arispe and Dvorak, 1997). It has been reported that angiogenesis is initially stimulated by bFGF, while VEGF stimulates angiogenesis later during the formation of granulation tissue (Nicholas et al., 1998). Perivascular deposition of fibronectin by proliferating microvascular endothelial cells and the increased expression of fibronectin receptors on endothelial cells have been suggested to have a role in the movement of endothelial cells into the wound during neovascularisation (Clark et al., 1982b, Brooks et al., 1994, Singer and Clark, 1999).

Tissue destruction and hypoxia following injury cause release of bFGF from macrophages and VEGF from endothelial cells. Proteolytic enzymes degrade extracellular matrix proteins into protein fragments that recruit peripheral blood monocytes to the wounded site, which in turn become activated macrophages. Activated macrophages release bFGF and stimulate endothelial cells to release plasminogen activator and procollagenase giving rise to plasmin and collagenase from their precursors. These two proteases degrade basement membrane and allow endothelial cells to move and form new blood vessels (Singer and Clark, 1999).

1.5.4.4 Effects of Eph receptors and Ephrin ligands in vascular development

Ephrin-B ligands induce capillary sprouting in vitro, which suggests the importance of Ephrins in vasculogenesis (Adams et al., 1999). Bidirectional signalling of Ephrin-B2 and EphB4 is essential in angiogenesis, while Ephrin-B2 knockout mice displayed a defective embryonic vascular system (Gerety et al., 1999, Adams et al., 1999). Furthermore it was identified that EphB4 is expressed on venous endothelial cells and Ephrin-B2 is expressed on artery endothelial cells from the onset of angiogenesis. Ephrin-B2 deficient yolk sacks and heads of Ephrin-B2 deficient embryos displayed defects in angiogenesis that led to a block at the primary capillary plexus stage (Wang et al., 1998). Possible roles of Ephrin-B2 in the arterial muscle wall formation during development and expression of Ephrin-B2 at the sites of adult physiological angiogenesis such as in the female reproductive system have been reported (Gale et al.,
2001) Consistent with previous studies, Ephrin-B2 was expressed in a subset of small blood vessels of a wounded region during cutaneous wound healing as well as in a subset of ingrowing blood vessels in a corneal micropocket assay suggesting a role of Ephrins in adult neovascularization (Shin et al., 2001).

EphrinB1 induced assembly of human renal micro vascular endothelial cells (HRMEC) into capillary like structures in vitro, while EphrinA1 promoted human umbilical vein endothelial cell (HUVEC) to assemble into capillary like structures in vitro (Daniel et al., 1996). Consistent with these findings, Ephrin-A1 also mediated TNF-α induced angiogenesis in rat corneas (Pandey et al., 1995).

EphA2 receptor is necessary for Ephrin-A1 mediated vascular assembly and endothelial migration in vitro. In vivo diminished angiogenic response to EphrinA1 was observed in EphA2 deficient endothelial cells, suggesting the importance of EphA2 in angiogenesis (Brantley-Sieders et al., 2004).

Therefore all these studies depict that Ephrin ligands and Eph receptors play an important role in vascular development and might be essential in neovascularisation during wound healing.

1.5.5 Wound contraction and extracellular-matrix reorganization

Mechanical stress, TGF β and cellular fibronectin influence wound fibroblasts to transform into a myofibroblast phenotype, which contains F-actin microfilaments and fibronectin receptors causing wound contraction (Welch et al., 1990, Gabbiani, 2003). Furthermore it has been reported that α2β1 integrin expression on fibroblasts and telopeptide sites in collagen play a major role in collagen matrix contraction (Schiro et al., 1991, Woodley et al., 1991).

Transition of granulation tissue to scar during collagen remodelling is largely dependent on continuous collagen anabolism and catabolism at a low rate. Matrix metalloproteinases degrade collagens and different phases of wound repair have shown distinct expression levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases (TIMP) (Singer and Clark, 1999, Madlener et al., 1998).
There are no reports to date in the current literature that suggest possible roles of Ephrins in wound contraction and extracellular matrix reorganization, which is to be unravelled in the future.

1.6 Cutaneous innervation and wound healing

1.6.1 Contribution of innervation during wound healing

Many studies have reported the effects of cutaneous innervation on wound healing. Calcitonin gene-related peptide (CGRP) and substance P are neuropeptides released from cutaneous nerves that stimulate human dermal microvascular endothelial cells (HDMEC). In addition CGRP causes secretion of neutrophil chemotactic factor, interleukin 8 and substance P, which in turn results in expression of vascular cellular adhesion molecule 1 (VCAM-1) on HDMEC, suggesting a role of cutaneous innervation in inflammation and wound healing (Ansel et al., 1997). Furthermore the stimulatory effect of epithelial growth factor on corneal epithelial cell migration was enhanced by substance P (Nakamura et al., 1997). CGRP also promotes human umbilical vein endothelial cell proliferation, while substance P induced endothelial cell differentiation into capillary like structures, suggesting a role of these neuropeptides in the formation of new blood vessels (Haegerstrand et al., 1990, Wiedermann et al., 1996). Multiple studies have reported that denervation of the skin significantly impairs the wound healing rate in rats (Engin, 1998, Carr et al., 1993). Furthermore decreased levels of CGRP released from nerves were associated with impaired wound closure (Toda et al., 2008), while increased levels of CGRP promoted wound closure via contraction (Engin, 1998). These findings illustrate the significant impact of cutaneous innervation on wound healing.

1.6.2 Cutaneous re-innervation and sensory function in wound repair

Numerous studies in the literature have demonstrated an association between cutaneous sensory innervation and wound healing. A significantly decreased wound healing rate was observed in rats after denervation of the sensory nerves in skin (Engin, 1998, Carr et al., 1993). It has also been shown that cutaneous sensory nerves may influence myofibroblast activity in wounds and that the timing of sensory neuron regrowth after
injury is important to promote efficient wound healing (Liu et al., 1999). Reduction of sensory innervation via capsaicin treatment has been shown to impair wound healing (Maggi et al., 1987, Smith and Liu, 2002), whilst other studies have implicated neuropeptides secreted by sensory fibres in wound repair (Toda et al, 2008, Engin, 1998). A reduction in CGRP released from nerves was associated with significantly suppressed wound closure (Toda et al., 2008) whereas in contrast elevated levels of CGRP increased the rate of wound closure via elevated rates of contraction (Engin, 1998).

Other evidence has suggested a role for nerves in wound healing pathologies. Partial thickness and full-thickness wounds including burns can result in abnormal wound healing, leading to hypertrophic scar formation, wound contracture and in some individuals keloid formation (Scott et al., 2007). Hypertrophic scars contain increased numbers of nerve axons compared to normal wounds (Zhang and Laato, 2001) and nerve fibres immunoreactive to SP, CGRP, neuropeptide Y, vasoactive intestinal polypeptide and dopamine beta-hydroxylase are also increased in human hypertrophic scars (Crowe et al., 1994). Neuroinflammation has been postulated to be a driving mechanism behind hypertrophic scar formation (Scott et al., 2005). Neuropeptides derived from sensory nerves are known to have functions in the normal wound repair (Scott et al., 2007). Neuropeptides such as Substance P, CGRP are involved in inflammation during cutaneous wound injury (Barnes et al., 1986, Hosoi et al., 1993). In addition neuropeptides are also known to be involved in keratinocyte proliferation (Tanaka et al., 1988, Paus et al., 1995) and proliferation of connective tissue cells such as dermal fibroblasts (Nilsson et al., 1985). Therefore it is postulated that increased number of nerves and high levels of neuropeptides contribute to profuse inflammation in hypertrophic scars (Scott et al., 2007).

Multiple studies report differential cutaneous nerve patterning in different types of wounds, including burn wounds with poor scar outcome and sensory function problems (Nedelec et al., 2005, Anderson et al., 2010, Zhang and Laato, 2001). Cutaneous nerve regeneration beneath burn wounds was observed in rats (Ward et al., 1998). A significant increase in cutaneous nerve fibres was observed in normotrophic burn wound scars compared to hypertrophic burn wound scars suggesting the involvement of cutaneous innervation in the burn wound healing outcome (Altun et al., 2001). In contrast another study reports that the hyper innervation pattern of a superficial skin wound returns to its normal innervation pattern when the wound heals with minimal or
no scarring in rats (Aldskogius, 1987). Skin grafts of burn patients possessed increased touch, cold and warmth thresholds resulting in decreased sensory function along with decreased nerve terminal density (Nedelec et al., 2005).

These findings suggest that cutaneous re-innervation has an impact on wound healing and sensory function. Eph/Ephrin signalling is also important in skin innervation as mentioned earlier. Therefore it is important to assess the effects of Ephrin-A2 and Ephrin-A5 in skin innervation and sensory function during wound healing.

1.7 Link between hair follicle morphogenesis/cycling, cutaneous innervation and wound healing

Multiple studies show the hair cycle dependent changes in cutaneous innervation (Botchkarev et al., 1997a, Botchkarev et al., 1997b) as well as hair follicle morphogenesis dependent changes in cutaneous innervation (Peters et al., 2002). As discussed in this chapter cutaneous innervation is associated with wound healing via neuropeptide mediated wound closure (Toda et al., 2008, Engin, 1998) and formation of new blood vessels (Ansel et al., 1997) as well as re-innervation leading to sensory function deficits (Anderson et al., 2010, Nedelec et al., 2005). In addition hair follicle cycling is also known to contribute to wound healing as mentioned in this chapter (Taylor et al., 2000). Therefore hair follicle morphogenesis/cycling, cutaneous innervation and wound healing are interdependent processes in the skin.

1.8 Summary

Hair follicle cycling, cutaneous innervation and wound healing are important and interacting processes in the skin. Eph receptors and Ephrin ligands are widely expressed in the skin and have been reported to be important in this tissue. An impact of Ephrin-A2 and –A5 ligands on cutaneous innervation has recently been identified in chick embryos. However, the role of Ephrin-A2 and –A5 ligands on cutaneous development including on hair follicle morphogenesis and cycling, innervation and wound repair is not clear and would benefit from further studies. In particular, using model organisms closer to human is important to gain a better understanding of the role of these specific signalling proteins. Therefore the effects of Ephrin-A2 and –A5 ligands on cutaneous...
development and wound healing will be investigated in this thesis predominantly using a transgenic mouse model to identify the effects of these proteins.
1.9 Hypothesis

Ephrin-A2 and –A5 ligands are important modulators of;

i) Hair follicle morphogenesis

ii) Hair follicle cycling

iii) The development of cutaneous innervation

iv) Wound repair

The effects of Ephrin A2 and A5 on these processes are likely to be both direct and indirect as they are significantly interrelated and interdependent.

1.10 Aims

1.10.1 Role of Ephrin A2 and A5 in Hair follicle morphogenesis and cycling

1: To determine the expression of Ephrin-A2 and A5 ligands in the epidermis and hair follicles in wild type mice

2: To determine the effects of Ephrin-A2 and –A5 ligands in hair follicle morphogenesis using Ephrin-A2+/−, Ephrin-A5+/−, Ephrin-A2A5+/− and wild type genotypes

3: To investigate the effects of Ephrin-A2 and –A5 ligands in hair follicle cycling using Ephrin-A2+/−, Ephrin-A5+/−, Ephrin-A2A5+/− and wild type genotypes

4: To investigate the effects of Ephrin-A2 and –A5 ligands in hair follicle density using Ephrin-A2+/−, Ephrin-A5+/−, Ephrin-A2A5+/− and wild type genotypes

1.10.2 Role of Ephrin A2 and A5 in development of cutaneous innervation

1: To determine the effects of Ephrin-A2 and –A5 ligands on cutaneous innervation using Ephrin-A2+/−, Ephrin-A5+/−, Ephrin-A2A5+/− and wild type genotypes

2: To identify the impact of Ephrin-A2 and –A5 ligands on sensory function, using Ephrin-A2+/−, Ephrin-A5+/−, Ephrin-A2A5+/− and wild type genotypes
1.10.3 Role of Ephrin A2 and A5 in wound repair

1: To determine the effects of Ephrin-A2 and –A5 ligands on the migration and proliferation of dermal fibroblasts in vitro, using Ephrin-A2<sup>-/-</sup>, Ephrin-A5<sup>-/-</sup>, Ephrin-A2A5<sup>-/-</sup> and wild type genotypes

2: To determine the effects of Ephrin-A2 and –A5 ligands on keratinocyte migration in vitro using Ephrin-A2<sup>-/-</sup>, Ephrin-A2A5<sup>-/-</sup> and wild type genotypes

3: To determine the effects of Ephrin-A2 and –A5 ligands on excisional wound closure using Ephrin-A2<sup>-/-</sup>, Ephrin-A2A5<sup>-/-</sup> and wild type genotypes

1.11 Experimental design

The aim of this thesis is to further our understanding of the effects of Ephrin-A2 and –A5 ligands on cutaneous development and wound healing. This study will investigate the expression and function of Ephrin-A2 and –A5 ligands in the epidermis and hair follicles of wild type mice. In addition, the impact of Ephrin-A2 and –A5 ligands on hair follicle morphogenesis and cycling will be determined. The effects of Ephrin-A2 and A5 on cutaneous innervation and sensory function will also be investigated. Finally, the role of these ligands in wound healing will be assessed. The research will focus on the use of transgenic mice. Ephrin-A2<sup>-/-</sup>, Ephrin-A5<sup>-/-</sup>, Ephrin-A2A5<sup>-/-</sup> and C57BL/6 wild type genotypes will be used. For in vitro assessments, cells isolated from these four genotypes will be investigated and compared. The use of the transgenic animals will provide a novel method of investigation with significant advantages compared to many previous studies of Ephrins in the skin. The murine model is widely accepted as a good model for human cellular biology and the use of these specific strains will allow for better understanding of the roles of these ligands in vivo.
11.12 Significance

There is evidence in the literature that Ephrins are important in multiple aspects of skin development and function. Further knowledge of the roles of specific Ephrins will be important to improve our understanding of how functional skin is generated and maintained, as well as whether Ephrins are important for either functional or aesthetic recovery after injury. This has the potential to lead ultimately to new therapeutic approaches aimed at improving wound repair and ameliorating either the functional or aesthetic deficits associated with scar.
Chapter 2
Chapter 2: Role of Ephrin-A2 and Ephrin-A5 in hair follicle morphogenesis and hair follicle cycling

2.1 Introduction

Hair follicles are important to many functions of the skin including angiogenesis, immunity and sensation, with many of these functions co-regulated by the processes involved in hair follicle cycling (Mecklenburg et al., 2000, Paus et al., 1998).

Hair follicles form from the primitive epidermis as a consequence of neuroectodermal – mesodermal interactions (Schmidt-Ullrich and Paus, 2005). The initial formation of the whole hair follicle from the primitive epidermis is called hair follicle morphogenesis. Nine stages (stage 0 to stage 8) have been described during morphogenesis (Stenn and Paus, 2001, Paus et al., 1999). Immediately after they form, hair follicles undergo a highly organised cycling process. Hair follicle cycles are divided into a growth phase (anagen), a regression phase (catagen) and a quiescence phase (telogen). The anagen phase is divided into six stages and the length of anagen VI determines the length of the hair follicle at different regions of the body (Stenn and Paus, 2001, Muller-Rover et al., 2001, Trotter, 1924). The anagen phase is followed by the catagen phase, which ceases hair follicle growth. The catagen phase, is divided into eight stages and follows a series of events including the cessation of cell growth and pigmentation, release of papilla from the hair bulb, disappearance of the differentiated layers of the lower follicle and extracellular matrix remodelling. At the end of catagen, the hair follicle resides in the upper dermis, where it remains during telogen and attains its shortest length (Muller-Rover et al., 2001, Stenn and Paus, 2001). In mice, hair follicle morphogenesis and the first postnatal hair follicle cycling is synchronised and follows a precise time course (Muller-Rover et al., 2001), which makes it ideal to study the mechanisms involved in these processes. This cycling process continues throughout the life of the adult but cycles become desynchronised making investigation more complex at later stages.

As mentioned earlier many biological functions in the skin are affected by the phase of the hair follicle cycle. For example, during hair follicle cycling, vascular patterns around hair follicles change (Ellis and Moretti, 1959). In vivo studies have demonstrated that the anagen phase of hair follicle cycling is associated with angiogenesis.
(Mecklenburg et al., 2000). In addition, loss of blood vessels in the dermal papilla occurs during catagen as a secondary effect of hair growth cessation (Ellis and Moretti, 1959). A possible mechanism for these vascular changes is regulation of vascular endothelial growth factor (VEGF) expression during specific phases of hair follicle cycling (Goldman et al., 1995).

In addition to vascular changes, hair follicle cycling is associated with immune changes due to changes in the number of immune cells in hair follicle and peri-follicular skin compartments during synchronised cycling (Paus et al., 1998). There is also evidence that the phase of the follicle cycle alters dermal cell activity: during anagen IV (active hair growth), there are increased numbers of cells in S phase in the dermis compared to epidermis (Pierard and de la Brassinne, 1975) and there is increased DNA synthesis in dermal endothelial and perivascular cells during anagen (Sholley, 1976).

Several studies in the literature demonstrate a role for Ephrin-Eph signalling in hair follicle morphogenesis and cycling. For example, Ephrin-B2 is transiently expressed in hair buds and mesenchymal cells adjacent to the epidermis during embryogenesis, and Ephrin-A3 and EphA receptors are expressed in hair buds (Egawa et al., 2009, Yamada et al., 2008). In addition, the expression of Ephrin-A3 is hair cycle phase dependent and accelerates the onset of anagen (Yamada et al., 2008). Ephrin ligands and Eph receptors were also expressed in CD34 positive stem cells present in the bulge area of hair follicles undergoing telogen as well as anagen. Mice injected with Ephrin-A2-Fc or Ephrin-B2-Fc to inhibit Ephrin-Eph interactions displayed increased proliferation of telogen hair follicle keratinocytes. Even though Ephrin-Eph signalling inhibition did not induce anagen, it did negatively regulate proliferation of adult hair follicular stem cell niche (Genander et al., 2010).

To investigate whether Ephrin-A2 and A5 are important in hair follicle morphogenesis and/or cycling, we first examined Ephrin-A2 and Ephrin-A5 expression in early postnatal wild type mice to determine whether their expression patterns suggest a role in hair follicle development and cycling. Subsequently hair follicle morphogenesis and cycling in early postnatal wild type and Ephrin-A-/- mice was also examined. We found that Ephrin-A2 and –A5 ligands were expressed in the epidermis and hair follicles at P1, P19 and P25 in C57BL/6 wild type mice. In addition hair follicle morphogenesis was retarded in Ephrin-A5-/- and Ephrin-A2A5-/- genotypes compared to wild type mice at P1, although these effects did not persist at P19 and P25 where densities were normal. Furthermore hair follicle cycling was also retarded in Ephrin-A5-/- genotype at P25, with
a similar trend in the Ephrin-A2A5\textsuperscript{−−} genotype. This data suggests that Ephrin A2 and A5 are important in hair follicle morphogenesis and that Ephrin A5 remains important in the control of hair follicle cycling.
2.2 Materials and Methods

2.2.1 Animals

Gender matched C57BL/6 wild type mice, Ephrin-A2\textsuperscript{−/−} mice, Ephrin-A5\textsuperscript{−/−} mice, Ephrin-A2A5\textsuperscript{−/−} mice were maintained in standard housing with food and water provided \textit{ad libitum}. Approval was obtained by Institutional ethics committees and experiments performed in accordance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. (Ethics number: RA3/100/824)

Knockout mice were a generous gift from David Feldheim (Feldheim et al., 2000). Original Ephrin-A\textsuperscript{−/−} mice were back crossed onto C57Black/6J wild type line (Animal research centre, Murdoch University, and Animal Care Unit, UWA). Ephrin-A\textsuperscript{−/−} mice were bred from heterozygous parents (A2\textsuperscript{−/−} and A5\textsuperscript{+/−} or A2\textsuperscript{+/−} and A5\textsuperscript{−/−}), at UWA (Biomedical Research Facility, UWA) and genotyped at weaning. Animals were housed in single sex cages on 12-hour light dark cycle with food and water \textit{ad libitum} (Animal Care Unit, UWA).

2.2.1.1 Genotyping

2.2.1.1.2 DNA extraction

A piece (1-2mm) of mouse tail was received from the animal care services (UWA) at weening and incubated overnight at 55°C (Ratek, Orbital Mixer Incubator) with 750µL of NTES (H\textsubscript{2}O, 510µL; 0.5M EDTA, 150µL; Tris pH 8.0, 37.5µL; 20% SDS, 37.5µL; 5M NaCl, 15µL) and 80µL of Proteinase K (10mg/ml). Samples were shaken vigorously and placed on a shaker (eppendorf, Thermomixer comfort) for 5 minutes. Then 310µL of 5M NaCl was added and shaken for 5 minutes. The complete samples were shaken by hand and centrifuged (eppendorf, Centrifuge 5810R) at 19000 x g for 10 minutes at room temperature. The DNA phase (~800μL) was transferred to a new tube and DNA was precipitated with 500µL of isopropanol. The tube was shaken by hand and placed on shaker for 2 minutes followed by centrifugation at 19000 x g for 5 minutes at room temperature. The supernatant was removed and the pellet was washed with 70% ethanol followed by centrifugation at 19000 x g for 30 seconds. The supernatant was removed and DNA pellets left to dry for 10 minutes and resuspended in 50µL ddH\textsubscript{2}O.

2.2.1.1.2 PCR

Genotyping was performed based on methods previously published for these mice(Frisen et al., 1998, Feldheim et al., 2000). However the primer sequences were slightly modified to give more reliable amplification. Extracted DNA was diluted 1:10 in ddH20 and 1µL combined with a QIAGEN Multiplex PCR kit and primers (QIAGEN Master Mix, 5µL; Q-solution, 1µL;
0.25μL of Primers A2 geno F1, A2 geno R1, A5 P2, A5 P1, A5 P3; table X), vortexed and incubated on a thermal cycler (BIO RAD, DNAEngine Peltier Thermal Cycler; 95°C for 15mins, 35x [94°C for 30sec, 62°C for 1min30sec, 72°C for 1min30sec], 72°C for 10min). PCR samples were diluted threefold with tank buffer and 5µL of solution was loaded into a 2% agarose gel with 5µL of loading dye for electrophoresis. The gel was run for 2-3 hours or until the bands had separated sufficiently to read the genotypes. A wildtype sample was used as a control.

2.2.1.2 Study design

In mice, hair follicle morphogenesis and the first postnatal hair follicle cycling follow a precise time course (Muller-Rover et al., 2001), which makes it ideal to study the mechanisms involved in these processes. We euthanased mice at P1, P19 and P25 to obtain hair follicles at specific stages. Hair follicle morphogenesis occurs at P1, while hair follicles go through catagen phase at P19. Furthermore hair follicles are in telogen phase from P21 to P27 and enter the anagen phase at P28 in C57BL/6 female mice (Paus et al., 1999, Muller-Rover et al., 2001). However it is also reported that the time scale is dependent on the genetic background, gender (female mice have a prolong telogen phase) and environmental factors (Muller-Rover et al., 2001). At P19 one female and two male mice per genotype were used, while at P25 two male and two female mice per genotype were used.

2.2.2 Immunohistochemistry (Ephrin-A2 and Ephrin-A5 ligands)

Immunohistochemistry was carried out to identify the expression pattern of Ephrin-A2 and Ephrin-A5 ligands in hair follicles.

2.2.2.1 Ephrin-A2 and Ephrin-A5 staining

Tissue sections were deparaffinized in xylene (3x5 minutes) and rehydrated in descending order of 100%, 90%, 80% and 70% ethanol. Then tissue sections were rinsed in running water for 10 minutes and incubated in 0.1 M phosphate buffered saline (PBS) for 5 minutes. Antigen retrieval was carried out by incubating tissue sections in 1mg/ml of trypsin (Sigma-Aldrich, Missouri, USA) at 37°C for 20 minutes. Then the sections were rinsed with cold 0.1M PBS (2x2minutes) and incubated in blocking solution (10% donkey serum [Millipore, North Ryde, NSW, Australia] with 0.3% TritonX-100 [BDH Chemicals, Kilsyth, VIC, Australia] and 0.1% sodium azide [Sigma-Aldrich, Missouri, USA]) for 1 hour at room temperature. Anti Ephrin-A2 rabbit polyclonal primary antibody (SC-912, Santacruz, California, USA; 1:100 diluted
with 10% donkey serum with 0.3% TritonX-100 and 0.1% sodium azide) and anti Ephrin-A5 rabbit polyclonal primary antibody (SC-20722, Santa Cruz, California, USA; 1:100 diluted with 10% donkey serum with 0.3% TritonX-100 and 0.1% sodium azide) were added to skin tissues. All the tissue sections with anti Ephrin-A2 and anti Ephrin-A5 primary antibodies were incubated at 4°C for 48 hours in a humidified chamber. The method carried out to identify the optimal concentrations of anti Ephrin-A2 and anti Ephrin-A5 primary antibodies is explained in 2.2.2.2

Sections were rinsed in Tris phosphate buffered saline (TPBS; 3x15 minutes) and incubated in Alexa Flour 568 donkey anti-rabbit secondary antibody (Invitrogen Molecular Probes, Eugene, Oregon, USA) diluted in 1:1500 with 5% donkey serum in TPBS with 0.3% TrironX-100, for 4 hours at room temperature. Tissue sections were rinsed in TPBS (3x15 minutes). Nuclear staining was carried out with 2μg/ml Hoeschst 33258 (Invitrogen, Eugene, Oregon, USA) with an incubation period of 15 minutes followed by rinsing in TPBS (3X2minutes). Sections were mounted with ProLong Gold antifade reagent (Invitrogen Molecular Probes, Eugene, Oregon, USA) and stored at 4°C.

2.2.2.2 Establishing optimal concentrations for anti-Ephrin-A2 and Anti-Ephrin-A5 primary antibodies

To establish the optimal concentration for anti Ephrin-A2 rabbit polyclonal primary antibody (SC-912, Santacruz, California, USA), dilutions of 1:100, 1:200 and 1:300 primary antibody with 10% donkey serum with 0.3% TritonX-100 and 0.1% sodium azide, was used. Anti-Ephrin-A2 antibody dilution range was decided based on previous work carried out in different contexts such as in the visual system etc. (Rodger et al., 2001, Davenport et al., 1998, Stubbs et al., 2000). Primary antibody dilutions were added to wild-type skin tissue sections as the test sample and to Ephrin-A2A5−/− skin tissue as the negative control. The same method was followed to establish the optimal concentration of anti Ephrin-A5 rabbit polyclonal primary antibody (SC-20722, Santa Cruz, California, USA), by using dilutions of 1:75, 1:100 and 1:200 primary antibody with 10% donkey serum with 0.3% TritonX-100 and 0.1% sodium azide. Anti-Ephrin-A5 antibody dilution range was decided based on a previous publication using the antibody in granulosa cells (Buensuceso and Deroo, 2013). The optimal concentration for both anti Ephrin-A2 and anti-Ephrin-A5 antibody was 1:100 in 10% donkey serum with 0.3% TritonX-100 and 0.1% sodium azide and this was used for all data presented here.
2.2.3 Gender determination of paraffin wax embedded mouse skin samples for hair follicle cycling study

Since female mice show a prolonged telogen (Muller-Rover et al., 2001), These samples were collected prior to noting of the gender. Therefore gender determination was carried out retrospectively in order to match the gender at P19 and P25.

2.2.3.1 Paraffin wax dissolution and ethanol rehydration

The technique was slightly modified from a previously published method (Pikor et al., 2011). Paraffin wax dissolution was carried out by adding 0.5-1ml of xylene to sterile eppendorf tubes containing paraffin wax embedded skin samples. The tube was inverted 5 times and incubated for 5 minutes at room temperature. Samples were then centrifuged at 13,000 rpm for 3 minutes at room temperature. The supernatant was carefully discarded. These steps were repeated until paraffin wax was completely dissolved. Ethanol rehydration of skin tissue samples was carried out by a series of decreasing ethanol concentrations from 100% (v/v) molecular biology grade ethanol (Sigma-Aldrich®, Missouri, USA) to 70% and lastly 50%. After addition of ethanol at each step, the samples were centrifuged at 13,000 rpm for 3 minutes and supernatant was discarded prior to next rehydration step. After the last rehydration step, the mouse skin sample was air dried for 5 minutes in the fume hood.

2.2.3.2 Mouse (Deoxyribonucleic acid) DNA extraction and DNA Precipitation

The mouse skin sample was then digested in 750μl of NTES buffer containing 6.8ml of nuclease free water (Qiagen®, Hilden, Germany), 2ml of 0.5M ethylenediaminetetraacetic acid (EDTA), 500μl 1M Tris pH 8.0, 500μl 20% sodium dodecyl sulphate (SDS), 200μl 5M sodium chloride (NaCl) and 80μl of 10mg/mL proteinase K (Sigma-Aldrich, Missouri, USA). Samples were incubated at 55°C overnight with continuous shaking. The next day, tubes were shaken vigorously for 5 minutes and 310μl of 5M NaCl was added to each tube. The tubes were centrifuged at 13,000rpm for 10 minutes at room temperature and the supernatant was kept for DNA precipitation. 800μl of supernatant was transferred to a new sterile eppendorf tube. Standard ethanol precipitation was performed. In summary, 2x volume of cold 100% (v/v) molecular grade ethanol was added with 1/10 volume of 3M sodium acetate (pH 5.2). The solution was mixed and left to precipitate in -80°C overnight. The next day,
the samples were centrifuged at top speed at 4°C for 20 minutes. The supernatant was discarded and washed with 70% ethanol. The supernatant was discarded and the pellet was air-dried at room temperature inverted for 10 minutes. The dried pellet was re-suspended in 50μl of nuclease-free water (Qiagen®, Hilden, Germany).

2.2.3.3 Polymerase chain reaction (PCR) for gender determination on extracted mouse genomic DNA

Gender determination of mice skin samples was carried out by conventional PCR, using a novel pair of primers in a single reaction tube which amplifies DNA fragments from a X-chromosome-specific gene, Jarid1c and the Y-chromosome-specific gene Jarid1d (Clapcote and Roder, 2005).

The PCR protocol was carried out with Expand High Fidelity Polymerase in 25uL reaction volumes (Roche). Each reaction contained nuclease free water, 10X reaction buffer + MgCl₂, deoxyribonucleotide (dNTPs), 1μM of forward (Jarid1c) and reverse primer (Jarid1d) (Fisher Biotec, Wembley, Australia), Q-solution (Qiagen, Multiplex PCR Kit, Hilden, Germany) and 0.5 units of Taq polymerase. 1μl of genomic DNA was added from each mouse skin sample (diluted 1:10 with nuclease free water). For every PCR run, a “no template control (NTC)” as well as positive male and female samples was included.

After PCR, the samples were loaded onto a 2% agarose gel [2g of agarose in 100 mLs of 1x Tris/Borate/EDTA (TBE) buffer] containing gel red solution. Loading buffer (5μl) was added into 25μl of PCR product and 5μl of the mixture was loaded onto the gel. A molecular weight ladder was included. The gel was run at 70V for 4 to 5 hours and viewed under a UV trans illuminator (Biorad, California, USA). Gender of mice samples was determined by presence of two distinct bands corresponding to male (PCR product from X chromosome and Y chromosome, 331bp and 302bp respectively) and female (single distinct band of 331bp).

2.2.4 Tissue preparation and imaging

Formalin fixed, paraffin embedded, 5μm thick skin tissue sections from the dorsum were taken from wild-type (n=3), Ephrin-A2+/− (n=3), Ephrin-A5+/− (n=3), Ephrin-A2/A5−/− (n=3) mice at 1 day after birth (P1) and at 19 days after birth (P19). Paraffin embedded, 5μm thick skin tissue sections from the dorsum were also harvested from wild-type (n=4), Ephrin-A2−/− (n=4), Ephrin-A5−/− (n=4), Ephrin-A2A5−/− (n=4) mice at
25 days after birth (P25). All the sections were stained with haematoxylin and eosin (H&E) followed by mounting in Entellan® new rapid mounting medium for microscopy (Merk Millipore, Australia) and 0.17mm thick coverslips. High resolution, transmitted-light images of the whole 5μm thick sections were produced using 20X /0.75 Plan Apo objective with an aperio scanscope digital slide scanner (Aperio®, Vista, California). Images were visualized using aperio imagescope software at 100X magnification (Aperio®, Vista, California).

2.2.5 Histological analysis of hair follicle morphogenesis

Distinct stages of hair follicle morphogenesis were categorized using the basic criteria for classification of hair follicle morphogenesis (Paus et al., 1999). The percentages of hair follicles assigned to each stage of hair follicle morphogenesis were calculated (derived from three mice per genotype at P1). At least 50 longitudinal hair follicles per genotype/animal were assessed.

2.2.6 Histological analysis of hair follicle cycling

Basic criteria for classification of hair follicle cycling was used to identify the distinct stages of hair follicle cycling (Muller-Rover et al., 2001). A minimum of 75 dorsal skin hair follicles per genotype at P19 derived from 3 mice and a minimum of 100 hair follicles per genotype at P25 derived from 4 mice were assessed. The percentage of hair follicles in each defined hair cycle stage was obtained. Hair cycle score (HCS) was calculated to compare catagen phase at P19 and anagen phase at P25 between the genotypes as previously described (Muller-Rover et al., 2001, Maurer et al., 1997). Briefly, each stage was assigned a factor in ascending numerical order (eg: anagen I= factor 1, anagen II= factor 2 etc. and catagen I= factor 1, catagen II= factor 2 etc.). The number of hair follicles in each stage of each mouse was multiplied by the corresponding factor and the results were added and divided by the total number of hair follicles in the respective catagen or anagen phase per mouse. As a result anagen phase gives rise to 1-6 HCS and catagen phase gives rise to 1-8 HCS.

2.2.7 Hair follicle density

The number of hair follicles with clear hair bulbs per 1mm of skin was counted at P19 and P25 in order to identify hair follicle density per genotype (Yamada et al., 2008). Kruskal-Wallis test was carried out to assess the possible differences in hair follicle
density at P19 and P25 across the four genotypes and the level of significance was set at p<0.05.

2.2.8 Statistical analyses

All results were analysed using SPSS version 19. The non-parametric Kruskal-Wallis test was carried out to compare the hair follicle cycling and hair follicle density between the four genotypes at P19 and P25 (α=0.05), where there were 3 or 4 animals per genotype. This test makes no assumption about the distribution of the data. The Kruskal-Wallis test was also used to compare hair follicle percentage between the genotypes at each stage of morphogenesis (α=0.05). Mann-Whitney pairwise test (α=0.05) was conducted if necessary.
2.3 Results

2.3.1 Expression of Ephrin-A2 ligand in hair follicles

Ephrin-A2 and Ephrin-A5 ligands are expressed in the epidermis, hair bulb, dermal papilla and outer root sheath at P1 in wild type hair follicles (Figure 2.1:a, d). The epidermis and the germ capsule express Ephrin-A2 ligand and Ephrin-A5 ligand at P19 (Figure 2.1:b, e). Both the ligands are expressed in the epidermis, strand of keratinocytes, bulb and dermal papilla at P25 (Figure 2.1:c, f).

The normal expression pattern of Ephrin-A2 ligand in hair follicles did not change in the absence of Ephrin-A5 ligand at P1, P19 and P25 (Figure 2.1:g-i). Similarly normal expression of Ephrin-A5 ligand in hair follicles did not change in the absence of Ephrin-A2 ligand at P1, P19 and P25 (Figure 2.1: j-l). Furthermore Ephrin-A2 and –A5 ligands were not expressed in Ephrin-A2A5−/− mice, confirming the specificity of the antibodies, which were, used (Supplementary figure 1).
Figure 2.1a-l Expression of Ephrin-A2 and Ephrin-A5 ligands in hair follicles. a-c: Ephrin-A2 ligand expression in wild type hair follicles at P1, P19 and P25. d-f: Ephrin-A5 ligand expression in wild type hair follicles at P1, P19 and P25. g-i: Ephrin-A2 ligand expression in Ephrin-A5<sup>−/−</sup> hair follicles at P1, P19 and P25. j-l: Ephrin-A5 ligand expression in Ephrin-A2<sup>−/−</sup> hair follicles at P1, P19 and P25. DP, dermal papilla; Hb, hair bulb; GC, germ capsule; ORS, outer root sheath. Scale bar=50 μm.
2.3.2 Hair follicle morphogenesis is retarded in Ephrin-A5\textsuperscript{-/-} and Ephrin-A2A5\textsuperscript{-/-} mice at P1

Kruskal Wallis nonparametric statistical analysis revealed statistically significant differences (p<0.05) between the four genotypes at all stages of postnatal hair follicle morphogenesis except stage 7(Figure 2.2). Ephrin-A2A5\textsuperscript{-/-} mice showed significantly increased percentages (p<0.05) of hair follicles compared to wild type mice at early stages (stages 2,3,4) as well as at stage 5 of morphogenesis. However the percentage of hair follicles in Ephrin-A2A5\textsuperscript{-/-} mice was significantly reduced (p<0.05) compared to wild type at stage 6. This indicates a slight retardation of hair follicle morphogenesis in Ephrin-A2A5\textsuperscript{-/-} mice compared to wild type (Figure 2.3). Ephrin-A5\textsuperscript{-/-} mice showed significantly high percentages (p<0.05) of hair follicles compared to wild type at early stages (stages 1,2,3,4). But the percentage of hair follicles in Ephrin-A5\textsuperscript{-/-} mice was significantly reduced (p<0.05) compared to wild type at stage 8 suggesting a retardation of hair follicle morphogenesis in these mice compared to wild type mice. Ephrin-A2\textsuperscript{-/-} mice showed significantly increased percentage of hair follicles at stage 6 (p<0.05) compared to wild type with no hair follicles at stage 8. Furthermore wild type and Ephrin-A2\textsuperscript{-/-} mice did not contain any hair follicles at stage 1 of morphogenesis suggesting hair follicle induction is completed before P1 in wild type and in Ephrin-A2\textsuperscript{-/-} mice. Presence of hair follicles in Ephrin-A5\textsuperscript{-/-} mice and Ephrin-A2A5\textsuperscript{-/-} mice at stage 1 of morphogenesis at P1 suggests that hair follicle induction is delayed in these two genotypes.
Figure 2.2 Percentage of hair follicles at each stage of hair follicle morphogenesis in wild type, Ephrin-A2\textsuperscript{+/-}, Ephrin-A5\textsuperscript{+/-}, Ephrin-A2A5\textsuperscript{+/-} genotypes. There is a significant increase in the percentage of hair follicles at early stages in Ephrin-A5\textsuperscript{+/-}, Ephrin-A2A5\textsuperscript{+/-} genotypes compared to wild type mice. n=3 animals per genotype. Data are represented as mean +/- SEM. *, p < 0.05.
Figure 2.3 H&E sections of hair follicle morphogenesis in wild type, Ephrin-A2\(^{-/-}\), Ephrin-A5\(^{-/-}\), Ephrin-A2A5\(^{-/-}\) genotypes.

Hair follicle morphogenesis is retarded in Ephrin-A5\(^{-/-}\) and Ephrin-A2A5\(^{-/-}\) mice at P1. Scale bar= 500\(\mu m\)
2.3.3 Hair follicle cycling is retarded in Ephrin-A5⁻/⁻ mice at P25 and Ephrin-A2A5⁻/⁻ mice show a strong tendency to decrease hair follicle cycling at P25

Hair follicle cycling score analysis (Kruskal Wallis test) at P25 showed a significant difference between the four genotypes (p= 0.038) (Table 2.1:b, Figure 2.4:b). Mann-Whitney pairwise comparison indicated a significant retardation of hair follicle cycling in Ephrin-A5⁻/⁻ mice (p=0.029) (Figure 2.6), and a strong tendency to slower hair follicle cycling in Ephrin-A2A5⁻/⁻ mice compared to wild type (p=0.083). However Ephrin-A2⁻/⁻ mice did not show a significant difference in hair follicle cycling at P25 compared to wild type (p=0.767). Furthermore Ephrin-A5⁻/⁻ mice contained hair follicles in catagen phase and other three genotypes did not contain hair follicles in catagen phase confirming the hair follicle retardation of Ephrin-A5⁻/⁻ mice at P25 (Figure 2.5:b) Hair follicle cycle score analysis (Kruskal Wallis test) at P19 did not show a statistically significant difference (p= 0.186) (Table 2.1a, Figure 2.4:a). However Ephrin-A5⁻/⁻ mice and Ephrin-A2A5⁻/⁻ mice did contain hair follicles in mid catagen at P19, whereas wild type and Ephrin-A2⁻/⁻ mice did not contain hair follicles in mid catagen at P19 (Figure 2.5:a). This suggests that the lack of statistical significance could be a false negative result due to the limited number of samples of each genotype (n=3) in the study.

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<td>Ephrin-A2A5⁻/⁻ (n=3)</td>
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**Table 2.1 Hair cycle score per animal.** (a) Catagen hair cycle scores of wild type, Ephrin-A2⁻/⁻, Ephrin-A5⁻/⁻ and Ephrin-A2A5⁻/⁻ genotypes at P19. (b) Anagen hair cycle scores of wild type, Ephrin-A2⁻/⁻, Ephrin-A5⁻/⁻ and Ephrin-A2A5⁻/⁻ genotypes at P25.
2.4 Hair cycle scores of wild type and Ephrin-A\textsuperscript{A/-} mice.

(a) at P19, and (b) at P25. Ephrin-A\textsuperscript{A5/-} mice showed a significant retardation of hair follicle cycling compared to wild type mice at P25. \(n=3-4\) animals per genotype. Data are represented as mean +/- SEM. *, \(p < 0.05\).

2.3.4 Hair follicle density is not changed in Ephrin-A\textsuperscript{2/-}, Ephrin-A\textsuperscript{5/-} and Ephrin-A\textsuperscript{2A5/-} compared to wild type mice

The number of hair follicles per mm was not significantly different at P19 (\(p=0.950\)) or at P25 (\(p=0.298\)) between the four genotypes (Figure 2.5: c, d).
Figure 2.5 a-d The percentage of hair follicles at each stage of hair follicle cycling and the hair follicle density of Ephrin-A^{-/-} and wild type mice. (a, c) at P19. (b, d) at P25. n=3 animals per genotype. Data are represented as mean +/- SEM.
Figure 2.6 H & E sections of Ephrin-$$A^{-/-}$$ and wild type mice. Hair follicle cycling is retarded in Ephrin-$$A^{-/-}$$ mice compared to wild type mice at P25.
2.4 Discussion

We identified the expression of both Ephrin-A2 and Ephrin-A5 ligands in the epidermis and hair follicles at P1, P19 and P25 in wild type mice. Hair follicle morphogenesis was retarded in Ephrin-A5−/− and Ephrin-A2A5−/− genotypes at P1. However this effect did not have a lasting impact on hair follicle density in older Ephrin-A−/− mice, which have normal hair follicle density at P19 and at P25. Importantly, hair follicle cycling was retarded in Ephrin-A5−/− with a similar trend in Ephrin-A2A5−/− at P25. In summary the results suggest the importance of Ephrin–A5 ligand in hair follicle morphogenesis and cycling.

2.4.1 Ephrins and hair follicle morphogenesis

Our results showing expression of Ephrin-A2 ligand in wild type hair follicles at P1 is consistent with a previous study showing mRNA expression of Ephrin-A1, Ephrin-A2 and Ephrin-A3 ligands in the skin (Yamada et al., 2008). Our study adds that Ephrin-A5 ligands are also expressed and largely overlap with Ephrin-A2 ligands in hair follicles at similar stages of morphogenesis. Importantly, lack of Ephrin-A2 or Ephrin-A5 ligands in knockout mice does not appear to result in compensatory changes in expression of the other ligand in the skin: expression of Ephrin-A2 ligand in hair follicles and epidermis was not affected by the absence of Ephrin-A5 ligand and vice versa. By contrast a previous study has reported that Ephrin-A5 knockout mice up regulated Ephrin-A2 ligand expression in cortical layers including a region where Ephrin-A2 ligand is not expressed in C57BL/6 wild type animals (Peuckert et al., 2008). Therefore the stability of each Ephrin expression in knockout skin is confirmed in the current study. This suggests that that there are no compensatory differences in the expression pattern of ligands in the skin, unlike in the brain.

In addition, our data on hair follicles at P1 suggest that Ephrin-A5 may be involved in the very early stages of follicle morphogenesis. Truncal hairs of the type we examined include both tylotrich (guard) and non-tylotrich (awl, zig zag, auchene) hair follicles (Sundberg et al., 2005). Induction of both types of hair follicles occurs during slightly different periods of embryonic development, but for both is complete at P0.5 (Shimomura, 2008). Our data in wild type and Ephrin-A2−/− mice match those previously reported, suggesting that hair follicle induction is complete before P1 and Ephrin-A2 is
not involved in this process. By contrast Ephrin-A5−/− and Ephrin-A2A5−/− mice at P1 possessed hair follicles at the very earliest immature stage 1, suggesting a delay in hair follicle induction in these mice. It is also possible that induction was normal, but development was delayed at one or more early stages of morphogenesis, resulting in a higher prevalence of hair follicles at the earliest 4-5 stages. However preliminary results show that Ephrin-A−/− mice have no impact on the percentage of tylotrich and nontylotrich hair follicles (Supplementary figure 2). Identification of TGF-β receptor type II positive pregerm in stage 0 of hair follicle morphogenesis at P1 will enable to identify the precise hair follicle induction in knockout mice (Paus et al., 1999). This would determine whether the hair follicle induction is delayed or the development is delayed in Ephrin-A5−/− and Ephrin-A2A5−/− mice compared to wild type mice at P1.

Hair follicle formation occurs as a consequence of mesodermal and epithelial interaction during embryogenesis (Oro and Scott, 1998). It has been suggested that three key signalling pathways namely Wnt/β-catenin/ Lef-1, Eda/Eda-R and TGF-β2/TGF-β-RII are associated with hair placode formation (Botchkarev and Paus, 2003). Molecular mechanisms associated with the induction of the hair placode, down growth of the hair placode to the dermis and differentiation of cells in the hair follicle during morphogenesis (Botchkarev and Paus, 2003) have been reported and discussed previously (Danilenko et al., 1996). Our data now add that Ephrin-Eph signalling, and in particular Ephrin-A5 is likely to be involved in these important processes.

Despite the retardation of hair follicle development in Ephrin-A5−/− mice, adult mice had normal hair follicle densities. The number of hair follicles is determined during embryogenesis and is known to be consistent throughout life (Yamada et al., 2008). However, as suggested by Yamada et al. (2008), hair germs at P0.5 could disappear without developing into full hair follicles under normal conditions. The opposite can also occur if hair germs are over stimulated: overexpression of Ephrin-A3 promoted the development of supernumerary hair germs into complete hair follicles, leading to increased number of hair follicles in the adult (Yamada et al., 2008). The varying effects of individual Ephrin-A ligands emphasizes that despite the renowned redundancy and promiscuity of these proteins and their receptors (O'Leary and Wilkinson, 1999), individual ligands can exert highly specific effects.
2.4.2 Ephrins and hair follicle cycling

Growth factors and cytokines associated with hair follicle cycling and molecules that regulate transition between hair cycle stages have been discussed in detail (Danilenko et al., 1996, Botchkarev and Paus, 2003), but little is known about Ephrin/Eph signalling in hair follicle cycling. Similar to our data for hair follicle morphogenesis, the presence of Ephrin-A2 ligand in wild type hair follicles at P19 and P25 is consistent with previous work showing the expression of Ephrin-A1, Ephrin-A2 and Ephrin-A3 mRNA that is coordinated with the hair cycle (Yamada et al., 2008). The current study shows Ephrin-A5 ligand expression in hair follicles at P19 and P25, which largely overlaps with the expression pattern of Ephrin-A2 ligand at similar phases of hair follicle cycling. A previous study carried out using immunohistochemical localization of Eph receptors and Ephrin ligands reported that both Ephrin ligands and Eph receptors are expressed in telogen and anagen hair follicles, where both the ligands and receptors are present in the bulge region. Ephrin-A ligands were also identified in the outer root sheath of anagen hair follicles which is consistent with our findings (Genander et al., 2010). Also Genander et al reported that hair matrix of anagen hair follicles is devoid of EphA receptors, while we found expression of Ephrin-A2 and Ephrin-A5 ligands in the matrix. Consistent with their findings Ephrin-A2 and Ephrin-A5 ligands were also present in the epidermis and sebaceous glands. Ephrin-A2 and Ephrin-A5 ligands were expressed in anagen hair follicles at P25 in the dermal papilla of wild type mice.

However, despite the similar expression patterns of Ephrin-A2 and Ephrin-A5, our hair follicle cycling results suggest that these ligands have distinct roles in hair follicle cycling. We have shown that hair follicle cycling is normal in Ephrin-A2<sup>−/−</sup> mice, but delayed in Ephrin-A5<sup>−/−</sup> mice and to a lesser extent, in Ephrin-A2A5<sup>−/−</sup> mice. It is known that cells in the dermal papilla activate slow cycling stem cells in the bulge area during early anagen (Cotsarelis et al., 1990) and bulge cells proliferate at the onset of anagen to produce the new follicle during hair follicle cycling (Lyle et al., 1998). It is possible that Ephrin-A5 ligands present in the dermal papilla interact with EphA4 expressing bulge stem cells (Tumbar et al., 2004) in order to regulate stem cell proliferation. Therefore future studies should be carried out to identify the role of Ephrin-A5 in stem cell proliferation of hair follicles.
2.5 Conclusion

Our results suggest the importance of A5 ligands in hair follicle morphogenesis and cycling, and together with data published by others, emphasize the specificity of signalling by different Ephrin ligands. In order to use these proteins as therapeutic targets, for example for preventing hair loss, it will be important to identify the receptors that they interact with, as well as their downstream signalling pathways. Here, the results suggest that Ephrin-A5 ligand plays a dominant role in hair follicle morphogenesis and cycling whereas the Ephrin-A2 ligand had very limited effects.
Chapter 3
3.1 Introduction

Cutaneous innervation and sensory function play an important role in normal skin homeostasis, while altered cutaneous innervation and sensory function problems have been identified post injury and in disease conditions (Albrecht et al., 2006, Hamed et al., 2011, Anderson et al., 2010). Therefore it is important to understand how cutaneous innervation is initially established as well as how it can be restored after injury.

Cutaneous innervation is functionally divided into afferent somatosensory innervation and efferent autonomic innervation (Hendrix et al., 2008). Peripheral information from the skin is transmitted to the central nervous system via afferent sensory nerve fibres of two types. Peripheral Aδ and C nerve fibres terminate as free nerve endings and large myelinated Aα and Aβ axons terminate with encapsulated non-neural structures. Aα and Aβ axons with encapsulated terminals mediate somatic modalities of touch and proprioception. Meissner’s corpuscles mediate light touch, Merkel disk receptors mediate pressure, Pacinian corpuscles mediate vibration and ruffini endings identify skin stretch. The smaller myelinated Aδ nerve fibres and the unmyelinated C fibres are receptive for painful (nociception) or thermal sensation (Gardner, 2000).

All of these sensory nerves terminate at very specific locations within the dermis and epidermis. Among the large myelinated Aβ axons with specialized proneural structures, rapidly adapting type 1- Meissner’s corpuscles are located in the papillary ridges of the skin, while slowly adapting type 1- Merkel cells are present at the tip of the epidermal ridges. By contrast, rapidly adapting type 2- Pacinian corpuscles are present in the subcutaneous tissue and slowly adapting type 2-Ruffini endings terminate in the dermis (Vallbo and Johansson, 1984, Ward et al., 2004). The smaller myelinated Aδ nerve fibres and the unmyelinated C fibres also terminate at different levels of the dermis and epidermis (Ward et al., 2004). However, there is little information about the mechanisms and molecules that are important in establishing these different nerves to their specific locations within the skin.

Developing sensory nerves have specialised structures at their tips called growth cones. These cones are involved in sampling the environment and responding to molecular...
guidance cues in the local environment that direct the axon to its appropriate location. Growth cone guidance is regulated by different mechanisms such as contact repulsion, contact attraction, chemo attraction and chemo repulsion (Tessier-Lavigne, 1996).

During fetal development neuronal axons extend from somatic sensory ganglia to the skin and into the central nervous system. Directed growth of these neurons is known to depend primarily on multiple target derived neurotrophic molecules such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Oaklander and Siegel, 2005, Guthrie, 2008). NGF is known to establish peripheral sensory and sympathetic innervation levels (Albers, 1994), and BDNF promotes sensory innervation (LeMaster et al., 1999). Many other studies have reported the role of target-derived neurotrophic factors in promoting axon growth and guidance during development (Guthrie, 2008). It has been reported that the innervation of epidermis, upper dermis and the upper portion of the hair follicle are regulated by neurotrophic factors and their receptors, the receptor tyrosine kinases (trkA, trkB, trkC) signalling (Rice et al., 1998). However, the neurotrophins are diffusible proteins and give very limited spatial information to guide developing axons.

Another family of axon guidance cues that may play a role in guiding innervation of the skin is the Ephrin ligand family. Ephrins are cell surface bound molecules that bind to and signal via Eph receptors. Ephrin ligand binding to Eph receptors can cause either contact repulsion or attraction of neuronal growth cones in both the central and peripheral nervous system (Poliakov et al., 2004). Most studies to date have focussed on the role of Ephrins in the brain, where Ephrin-A2 and Ephrin-A5 are crucial in the formation of neuronal maps, particularly in the visual (Haustead et al., 2008, Feldheim et al., 1998, Wilks et al., 2010) and olfactory systems (Knoll et al., 2001, Smith and Bhatnagar, 2000). Furthermore, there is evidence in the visual system that Ephrin ligands and Eph receptors interact with neurotrophin receptors to guide axons to their target (Poopalasundaram et al., 2011, Fitzgerald et al., 2008). Ephrin-A5 and trkB interaction increases retinal ganglion cell branching (Marler et al., 2008) while Ephrin-A6 interaction with neurotrophin receptor p75 guides retinal ganglion cell axons (Poopalasundaram et al., 2011). EphA receptor and TrkB receptor interaction is also known to regulate retinal ganglion cell response during development (Fitzgerald et al., 2008). In addition Ephrin-A5 ligand activation has been shown to suppress BDNF evoked neuronal motility (Meier et al., 2011).
Taken together, the role of Ephrins in guiding axons to specific targets, in particular in the CNS, as well as their interaction with neurotrophin signalling pathways, makes them compelling candidates for regulating innervation of the skin. This hypothesis is supported by studies using in vitro labelling of live sensory neurons with a recombinant Ephrin-A5 protein (Ephrin-A5–Fc), which binds to all the Eph-A class receptors, to show that nearly all sensory growth cones express Eph-A class receptors (Muñoz et al., 2005). Furthermore, ectopic expression of Ephrin-A5 in ovo in chicks inhibited cutaneous innervation (Muñoz et al., 2005), and defects in epaxial sensory neuronal projections were identified in Ephrin-A2A5−/− chick embryos (Wang et al., 2011). This strongly suggests a functional role of Ephrin-A signalling in the development of cutaneous sensory axonal growth and/or patterning.

Therefore in this study the effects of Ephrin-A2 and Ephrin-A5 on cutaneous innervation and sensory function during development and in adults have been further assessed using the transgenic mouse models previously described. Protein gene product 9.5 (PGP 9.5) was used for immunohistochemistry as a neuronal marker to quantify epidermal and dermal nerve fibres in the mouse skin (Wang et al., 1990, Navarro et al., 1995) using a protocol previously developed in our laboratory (Anderson et al., 2010). Ephrin-A2 and –A5 ligands were both found to have an effect on cutaneous innervation during development and on cutaneous innervation in adult mice. However, only the Ephrin-A5 ligand appeared to have a functional impact with an effect on measured light touch sensory function.
3.2 Materials and methods

3.2.1 Animals

C57BL/6 wild type mice, Ephrin-A2−/− mice, Ephrin-A5−/− mice, Ephrin-A2A5−/− mice were maintained in standard housing with food and water provided *ad libitum*. Approval was obtained by Institutional ethics committees and experiments performed in accordance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. (Ethics numbers: RA3/100/824, RA3/100/1162).

3.2.2 Tissue preparation

Wild type (n=5), Ephrin-A2−/−(n=5), Ephrin-A5−/− (n=5), Ephrin-A2A5−/− (n=5) mice were euthanized at 1 day after birth (P1) and at 3-6 months after birth (adults). Wild type (n=4), Ephrin-A2−/− (n=4), Ephrin-A5−/− (n=4), Ephrin-A2A5−/− (n=6) mice were euthanized at 19 days after birth (P19). A piece of skin tissue (1cm²) from the dorsum was harvested and fixed in 4% paraformaldehyde from euthanized mouse. Tissue was processed, paraffin embedded and serially sectioned at 5μm. Three 5μm thick tissue sections were taken within a distance of 500μm skin tissue for protein gene product (PGP) 9.5 immunohistochemistry.

3.2.3 PGP 9.5 immunohistochemistry

Tissue sections were deparaffinized in xylene (3x5 minutes) and rehydrated in descending order of 100%, 90%, 80% and 70% ethanol for 2 minutes at each concentration. Then the tissue sections were rinsed in running water for 10 minutes and incubated in 0.1 M phosphate buffer (PB) for 5 minutes. Antigen retrieval was carried out by incubating tissue sections in 1mg/ml of trypsin (Sigma-Aldrich, Missouri, USA) at 37°C for 15 minutes. Then the sections were rinsed with 0.1M PBS (2x2minutes) and incubated in blocking solution (10% donkey serum [Millipore, North Ryde, NSW, Australia] with 0.3% TritonX-100 [BDH Chemicals, Kilsyth, VIC, Australia] and 0.1% sodium azide [Sigma-Aldrich, Missouri, USA]) for 1 hour at room temperature. Primary, polyclonal, anti-rabbit PGP 9.5 antibody (AbD Serotec, Oxford, UK,) in 1:1,500 dilution, with 10% donkey serum with 0.3% TritonX-100 and 0.1% sodium
azide, was added to tissue sections and incubated at 4°C for 48 hours in a humidified chamber. Sections were rinsed in Tris phosphate buffered saline (TPBS; 3x15 minutes) and incubated in Alexa Flour 568 donkey anti-rabbit secondary antibody (Invitrogen Molecular Probes, Eugene, Oregon, USA) diluted in 1:1500 with 5% donkey serum in TPBS with 0.3% TrironX-100, for 4 hours at room temperature. Tissue sections were rinsed in TPBS (3x15 minutes), mounted with ProLong Gold antifade reagent (Invitrogen Molecular Probes, Eugene, Oregon, USA) and stored at 4°C.

3.2.4 Imaging and cutaneous nerve quantification

Epidermal and dermal innervation was quantified separately for each animal. Initially five tissue sections within a range of 500μm and six fields from each tissue section of skin were assessed to quantify dermal innervation. Subsequently, due to the observed uniformity of innervation between fields across the sample, three tissue sections within a range of 500μm skin tissue and one field from each tissue section were used to quantify dermal innervation. Dermal innervation was visualized using a Nikon Eclipse 90i upright microscope (Nikon Corporation, Tokyo, Japan; 20x objective) with a 0.75 emission filter at 594 nm and photographed with a Coolsnap Pro digital camera (Media Cybernetics, Houston, TX). Epidermal innervation was quantified by studying three fields from one 5μm thick skin tissue section. Epidermal innervation was visualized and images were taken using a Leica TCS SP2 A OBS Multiphoton Confocal microscope (Leica Microsystems, Wetzlar, Germany; 20X oil objective) at an emission wavelength of 594nm. Images were taken in 1μm optical z- series sections and the average projection of the z-series sections was produced using Leica imaging software.

The area of the epidermal and dermal innervation was outlined and quantified using Image J software (Rasband). The analysis was confined to the interfollicular regions and single nerve fibres as previously described (Morellini et al., 2008, Anderson et al., 2010). Non-specific background fluorescence was excluded in order to achieve a clear image of nerve fibres by manually adjusting the lower threshold, which was derived from the brightness of the PGP 9.5. The upper threshold was set consistently at 255. All the structures in the selected area with the brightness equal to or higher than the pre-set threshold were considered as immuno-positive and the innervation was quantified as the percentage of the area covered by nerves in the selected region (as previously described (Morellini et al., 2008, Anderson et al., 2010)).
3.2.5 Tactile sensory function testing using Semmes-Weinstein monofilaments

C57BL/6 wild type (n=10), Ephrin-A2<sup>+/−</sup> (n=10), Ephrin-A5<sup>+/−</sup> (n=10) and Ephrin-A2A5<sup>+/−</sup> (n=10) mice were used to identify the effects of Ephrin-A2 and –A5 ligands on tactile sensory function. Mice were placed in red coloured plastic cylinders on a wire mesh for 15 minutes prior to testing in order to relax the animal and reduce stress caused by handling (Richner et al., 2011). Semmes-Weinstein monofilaments were applied on the mid plantar surface of the left hind foot of the mouse in an ascending order of force (0.008g-4g; filaments beyond 4g lift the paw (Figure 3.1). Each filament was applied 10 times. Each application of the filament was spaced 2-3 s apart. The number of withdrawal reflexes to each filament was recorded in spread sheets. Sudden paw withdrawal was considered as a positive response (Chaplan et al., 1994). Any detectable positive response was scored as 1 and the absence of a response was scored as 0. The number of positive responses was recorded as a percentage. The whole experiment was carried out three times on separate days (Chaplan et al., 1994).

![Figure 3.1a-b. Tactile sensory function in adult wild type, Ephrin-A2<sup>+/−</sup>, Ephrin-A5<sup>+/−</sup> and Ephrin-A2A5<sup>+/−</sup> genotypes were identified by applying Semmes-Weinstein monofilaments (a), on the mid plantar surface of the left hind foot of the mouse (b).](image-url)
3.2.6 Statistical analyses

All results were analysed using International Business Machines Corporation- Statistical Product and Service Solutions (IBM-SPSS) statistics version 19. Epidermal and dermal nerve density results were compared between Ephrin-A2<sup>−/−</sup>, Ephrin-A5<sup>−/−</sup>, Ephrin-A2A5<sup>−/−</sup> and wild type mice at each time point (P1, P19 and adults) using one-way Analysis of variance (ANOVA; α=0.05) and Bonferroni post hoc test (α=0.05 as significant) was carried out if necessary. Epidermal and dermal nerve density results were compared between P1, P19 and adults per genotype using one-way ANOVA (α=0.05) and Bonferroni post hoc test (α=0.05) was carried out if necessary. Separately tactile sensory function test results for 50% and 80% sensitivity threshold were also compared between adult, Ephrin-A2<sup>−/−</sup>, Ephrin-A5<sup>−/−</sup>, Ephrin-A2A5<sup>−/−</sup> and wild type mice using one-way ANOVA (α=0.05) and Bonferroni post hoc test (α=0.05).
3.3 Results

Different innervation density patterns were detected in Ephrin-A2<sup>−/−</sup>, Ephin-A5<sup>−/−</sup>, Ephrin-A2A5<sup>−/−</sup> and wild type mice during different stages of development P1, P19 and in adult phase (3 to 6 months old).

3.3.1 Preliminary results of dermal nerve density of one wild type mouse

Dermal nerve density results of one wild type mouse sample across 500μm skin tissue were not highly variable (Table 3.1, Figure 3.2 and Supplementary figure 3). Hence as mentioned in 3.24, three tissue sections within a range of 500μm skin tissue and one field from each tissue section were used to quantify dermal innervation density. The mean ranged from 0.92-1.06 % area and SD ranged from 0.09-0.2. The coefficient of variation was 0.15.
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Table 3.1 Interfollicular dermal nerve density results of one wild type mouse sample across 500μm skin tissue. Sections were cut at a thickness of 5μm and sections were taken at 5μm, 100μm, 200μm, 300μm, 400μm and 500μm. Six fields from each section were tested.
Interfollicular dermal nerve density was not significantly different in Ephrin-A2-/-, Ephrin-A5-/-, Ephrin-A2A5-/- and wild type mice at either P1 or adult mice (one way ANOVA; p>0.05)(Figure 3.3a, c). However, the interfollicular dermal nerve densities of the four genotypes were significantly different (one way ANOVA; p=0.000) at P19. Dermal innervation density of Ephrin-A2-/- animals was significantly reduced compared to wild type (Bonferroni; p=0.028) Ephrin-A5-/- (Bonferroni; p=0.001) and Ephrin-A2A5-/- (Bonferroni; p=0.000) mice at P19 (Figure 3.3b). Dermal nerve density of Ephrin-A2A5-/- mice was also significantly reduced compared to wild type at P19 (Bonferroni; p=0.041) (Figure 3.3b).

Across P1, P19 and adult wild type mice a significant difference in dermal nerve density was not detected. Ephrin-A2-/- mice were significantly different between different age groups (one way ANOVA; p=0.05) with a tendency to increase the nerve density from P19 to adult phase. Dermal innervation density of Ephrin-A5-/- animals was significantly
different across the age groups (one way ANOVA; p=0.038), where it showed a significant increase in dermal nerve density at P19 compared to P1 (Bonferroni; P=0.037). Dermal nerve density of Ephrin-A2A5−/− mice showed a significant difference across the age groups (one way ANOVA; p=0.003) with a significant increase of the dermal nerve density at P19 compared to P1 and adult mice (Bonferroni; p=0.004 and p=0.016) (Figure 3.4 a-d).

Looking over time i.e from P1 to adult stage, suggests no significant change in dermal innervation of each genotype. This suggests dermal innervation is complete at P1 and no further changes occur after this timepoint.
Figure 3.3 Interfollicular dermal nerve densities of wild type and Ephrin-A⁻/⁻ mice. (a) at P1. (b) at P19 (c) in adults. d:g Cutaneous dermal nerves labelled with PGP9.5 in, adult wild type (d), adult Ephrin-A2⁻/⁻ (e), adult Ephrin-A5⁻/⁻ (f), adult Ephrin-A2A5⁻/⁻ (g). n=4 to 6. Data are represented as mean +/- SEM. Scale bar= 50μm. * is < 0.05 and ** is < 0.01
Figure 3.4. Interfollicular dermal nerve densities across P1, P19 and adults in different genotypes. a-d: Interfollicular dermal nerve densities of wild type (a), Ephrin-A2^-/- (b), Ephrin-A5^-/- (c), Ephrin-A2A5^-/- (d), genotypes across P1, P19 and adult stages. n= 4-6. Data are represented as mean +/- SEM. * is < 0.05 and ** is < 0.01.

3.3.3 Epidermal nerve density

Epidermal nerve density of the four genotypes was significantly different at P1 (one way ANOVA; p=0.01), P19 (one way ANOVA; p=0.000) and in adult mice (one way ANOVA; p=0.01). Ephrin-A5^-/- epidermal innervation was significantly reduced compared to Ephrin-A2^-/- animals (Bonferroni; p=0.038) at P1 (Figure 3.5a). Adult Ephrin-A2A5^-/- mice showed a significant reduction of epidermal innervation compared to wild type (Bonferroni; p= 0.045), Ephrin-A2^-/- (Bonferroni; p=0.034) and Ephrin-A5^-/- mice (Bonferroni; p= 0.018)(Figure 3.5c,d,g). In contrast epidermal innervation of Ephrin-A2^-/- animals was significantly reduced compared to wild type (Bonferroni; p=0.017) as well as to Ephrin-A5^-/- mice (Bonferroni; p=0.000) at P19 (Figure 3.5b).
Ephrin-A5−/− mice showed a significant increase in epidermal innervation compared to Ephrin-A2A5−/− mice at P19 (Bonferroni; p=0.000). (Figure 3.5 b).

Wild type, Ephrin-A2−/− and Ephrin-A5−/− mice showed a significant difference in the epidermal innervation densities across time i.e. from P1 to adult phase (one way ANOVA; p=0.035, p=0.003 and p=0.000). Epidermal nerve density of adult wild type mice were significantly reduced compared to P19 wild type mice (Bonferroni; p=0.037). Epidermal nerve density of Ephrin-A2−/− animals were significantly reduced at P19 (Bonferroni; p=0.004) and in adult mice (Bonferroni; p=0.002) compared to mice at P1. Ephrin-A5−/− mice at P19 showed significant increase in epidermal nerve density compared to P1 (Bonferroni; p=0.000) and adult Ephrin-A5−/− (Bonferroni; p=0.000) mice (Figure 3.6 a-d).
Figure 3.5 Interfollicular epidermal nerve densities of wild type and Ephrin-A<sup>−/−</sup> mice. (a) at P1. (b) at P19 (c) in adults. d: g Cutaneous epidermal nerves labelled with PGP9.5 in, adult wild type (d), adult Ephrin-A2<sup>−/−</sup> (e), adult Ephrin-A5<sup>−/−</sup> (f), adult Ephrin-A2A5<sup>−/−</sup> (g). n=4 to 6. Data are represented as mean +/- SEM. Scale bar= 100μm. * is < 0.05 and ** is < 0.01
Figure 3.6. Interfollicular epidermal nerve densities across P1, P19 and adults in different genotypes. a-d: Interfollicular epidermal nerve densities of wild type (a), Ephrin-A2−/− (b), Ephrin-A5−/− (c), Ephrin-A2A5−/− (d), genotypes across P1, P19 and adult stages. n= 4-6. Data are represented as mean +/- SEM. * is < 0.05 and ** is < 0.01.
3.3.4 Sensory function test using Semmes-Weinstein monofilaments

Tactile sensory function tests carried out on adult mice (3-6 months) showed a statistically significant difference (one way ANOVA; P=0.001) in 50% paw withdrawal response between the four genotypes (Figure 3.7). Bonferroni post hoc tests confirmed that the 50% paw withdrawal response of Ephrin-A5−/− mice were significantly different compared to wild type (P=0.002), Ephrin-A2−/− (p=0.003) and Ephrin-A2A5−/− (p=0.024) mice.

Median values of 50% paw withdrawal threshold (Chaplan et al., 1994) for wild type and Ephrin-A2−/− mice were 0.07g (ranging from 0.008g- 4g), while Ephrin-A2A5−/− mice possessed a median value of 0.16g. Ephrin-A5−/− mice had the highest median value for 50% paw withdrawal threshold, which was 0.5g. Hence sensory function is reduced in Ephrin-A5−/− mice. There is a trend for reduced touch sensory function in Ephrin-A2A5−/− mice, which suggests that the lack of Ephrin-A5 has some effect on sensory function. However, the simultaneous loss of Ephrin-A2 may be opposing the effect of Ephrin-A5 loss and hence leading to no evidence of decrease in sensory function.

![Figure 3.7 Tactile sensory function of adult wild type, Ephrin-A2−/−, Ephrin-A5−/−, Ephrin-A2A5−/− genotypes at 50% touch sensitivity threshold.](image)

Figure 3.7 Tactile sensory function of adult wild type, Ephrin-A2−/−, Ephrin-A5−/−, Ephrin-A2A5−/− genotypes at 50% touch sensitivity threshold. The touch sensitivity of Ephrin-A5−/− mice were significantly increased for 50% touch sensitivity threshold. n=10 animals per genotype. * is < 0.05 and ** is < 0.01
3.4 Discussion

The results show that light touch sensory function of adult Ephrin-A5<sup>-/-</sup> mice is impaired. However, the functional impairment does not correlate with a reduced density of total cutaneous innervation as estimated by PGP9.5 immunolabelling, implying that Ephrin ligands may affect only specific nerve fibre subtypes. Dermal nerve density was similar in the four genotypes at the timepoints studied (P1 and adult), suggesting that Ephrin-A2 and –A5 ligands do not have a significant effect on dermal innervation. By contrast, epidermal innervation was significantly different in Ephrin-A<sup>-/-</sup> mice at different times during development, suggesting that Ephrin-A2 and –A5 ligands have a significant effect on specific aspects of the development of epidermal innervation. A previous study showed the role of Ephrin ligands in retinal ganglion cell axonal branching at their precise terminal zones (Yates et al., 2001). Furthermore branching of nerve fibres occur as nerves go from dermal to epidermal layer in the normal skin (Kennedy et al., 1996). Therefore the effects of Ephrins found on the epidermal innervation could be due to their impact on axonal branching in the epidermis.

3.4.1 Cutaneous sensory function

Semmes Weinstein filaments were used to assess the light touch sensation of the plantar surface of the skin and show for the first time that mice lacking Ephrin-A5 ligand have reduced light touch sensitivity in the skin (Figure 3.7). These findings suggest that the Ephrin-A5 ligand is involved in some aspect of development and/or function of the nerves associated with light touch sensation. A large percentage of nerve fibres in the dorsal root ganglion which conduct non-nociceptive sensation are Aα/β nerve fibres (Fang et al., 2005). Tactile units of the glabrous skin in the hand are comprised of Aα nerve fibres, whereas C nerve fibres and Aδ nerve fibres are associated with nociceptive and thermo sensitive units (Vallbo and Johansson, 1984). Furthermore Aα/β nerve fibre terminals encapsulated by Meissner’s corpuscles are receptive for light touch in the glabrous skin (Gardner, 2000). Therefore, it is likely that Ephrin-A5 ligands are required for the development and/or function of Aα/β nerve fibres in the glabrous skin. However, light touch sensory function of the hairy skin is detected by C fibre low-threshold mechanoreceptor (LTMR) lanceolate endings, Aδ LTMR and Aβ rapidly adapting LTMR innervating different types of hair follicles (Roudaut et al., 2012). The antibody PGP 9.5 which is a pan neuronal marker was used to identify interfollicular cutaneous nerve densities in this study. Therefore this may in part underlie the disconnect between the interfollicular dermal and epidermal nerve density and the light
touch sensory function found in this study. In addition touch domes are structures in hairy skin containing Merkel cells receptive for light touch (Doucet et al., 2013, Maricich et al., 2009). Therefore it is possible that the loss of Ephrin-A5 expression leads to a reduction in the number of these touch domes in the hairy skin and subsequent loss of sensory function. Further analysis using antibodies to identify these structures (including Krt8 (Doucet et al., 2013)) will be important to determine if this underlies the changes in sensory function observed. Different neuronal markers including substance P, CGRP, and calretinin have all been used previously to quantify specific nerve fibre subtypes. (Pare et al., 2001, Schulze et al., 1997). Neurofilament 200 (NF200) is a marker for myelinated A fibres (Ma, 2002) and transient receptor potential vanilloid 1(TRPV1) is specific to nociceptive C fibres responding to heat (Lawson et al., 2008). Therefore the use of these more specific markers will be beneficial to more closely relate the neuroanatomical findings with the assessments of sensory function.

Further sensory function tests could also be conducted to identify mechanical pain sensation and thermal thresholds for innocuous cold sensation and heat pain (Selim et al., 2010) in different genotypes. In addition to the more specific fibre subtype labelling experiments, this may contribute to a better understanding of the role of Ephrin-A2 and –A5 ligands in the development and function of cutaneous sensory innervation.

3.4.2 Ephrin-A2 and Ephrin-A5 have different effects on dermal and epidermal nerve density

In order to determine whether the reduction in sensory function might be due to a developmental delay or other abnormality in sensory innervation, we studied the density of innervation in the dermis and epidermis of Ephrin-A-/- mice. The density of dermal innervation in wild type mice was stable between P1 and adulthood (Figure 3.4a). These results are consistent with previous reports showing no significant change in dermal innervation in wild type mice between birth and P17 (Peters et al., 2002). In contrast, in Ephrin-A2A5-/- and Ephrin-A5-/- mice, dermal nerve density was significantly altered at P19 compared to wild type (Figure 3.3b). Our data suggest that Ephrin-A2 enhances dermal nerve density acting as a promoter of innervation, while Ephrin-A5 appears to act as an inhibitor of dermal innervation. Our results are consistent with a previous study showing inhibitory effects of the Ephrin-A5 ligand on embryonic sensory innervation (Muñoz et al., 2005) and add that different Ephrin-A ligands can have opposing effects on dermal innervation.
Similar to the dermal nerve density results, the data shows that Ephrin-A2 and –A5 ligands have distinct effects on epidermal nerve density, which may suggest that they act at different developmental time points. Wild type epidermal innervation peaks at P19 followed by a significant reduction in epidermal innervation in the adult (Figure 3.5b). This is consistent with previous studies showing that the density of CGRP and SP immunoreactive epidermal sensory nerves progressively increases from birth and peaks at P21. Density of both nerve types then decreases to reach mature adult levels (Schotzinger and Landis, 1990). By contrast, the present study found a strong trend for epidermal nerve density of Ephrin-A5<sup>−/−</sup> and Ephrin-A2A5<sup>−/−</sup> mice to reduce at P1 compared to wild type (Figure 3.5a). In chapter 2 we identified that hair follicle morphogenesis is retarded in Ephrin-A5<sup>−/−</sup> and Ephrin-A2A5<sup>−/−</sup> mice at P1. In addition a previous study has shown that epidermal innervation increases towards the later stages of hair follicle morphogenesis (Peters et al., 2002). Therefore we hypothesise that the reduction of epidermal innervation in Ephrin-A5<sup>−/−</sup> and Ephrin-A2A5<sup>−/−</sup> mice at P1 may be associated with the observed retarded hair follicle morphogenesis.

At P19, Ephrin-A2<sup>−/−</sup> mice have decreased epidermal innervation (Figure 3.2e), which has returned to normal in adults (Figure 3.5c), suggesting that the Ephrin-A2 ligand acts as a promoter of innervation at that specific time point. By contrast, innervation of Ephrin-A5<sup>−/−</sup> mice epidermis is not significantly different from normal at P19 (Figure 3.5b), suggesting that innervation levels have recovered from their developmental delay at P1. However, Ephrin-A2A5<sup>−/−</sup> retain significantly lower levels of innervation at P19 (Figure 3.5b) and through to adulthood (Figure 3.5c), suggesting that the loss of both Ephrin-A ligands has a severe and irrevocable impact on epidermal innervation that cannot be compensated by other ligands in the family. These data suggest that Ephrin-A2 and Ephrin-A5 act sequentially in epidermal innervation. Firstly, Ephrin-A5 is required for a very early aspect of innervation at P1 and this may be linked to aspects of hair follicle morphogenesis. Subsequently at P19, Ephrin-A2 promotes some aspect of epidermal innervation at P19, but this process can be compensated by other mechanisms, resulting in normal albeit delayed innervation density in the adult.

In addition light touch sensory function results suggest that Ephrin-A5<sup>−/−</sup> genotype impairs Aα/β nerve fibres with Meissner’s corpuscles. NF200 is a neuronal marker for Aα/β nerve fibres (Ma, 2002). Previously Meissner’s-like corpuscles were identified with antibodies to S100 protein, p75LNGFR protein and vimentin (Albuerne et al., 2000), while CGRP and SP nerve fibres were identified in close relation to the
epidermis in the glabrous skin (Ishida-Yamamoto et al., 1988). Nonetheless, as mentioned earlier in this chapter the light touch sensory function in the dorsal skin is associated with innervation around different types of hair follicles in the hairy skin (Roudaut et al., 2012). Detecting Aβ nerve fibres innervating hair follicles with neuronal marker NF200 (Ma, 2002) in the mouse dorsal skin at P1, P19 and adult stage might enable us to identify the time point where Ephrin-A5 ligand has an impact on the nerve subtype.

This combined immunohistochemical and functional data from the transgenic Ephrin mice suggests that Ephrin-A2 and –A5 ligands have different effects on specific and different nerve subtypes in the skin. This data suggests both Ephrin A2 and A5 are important in the development of cutaneous innervation and that there are specific functions of each ligand in the skin that are not compensated for by other family members (O'Leary and Wilkinson, 1999).

3.5 Conclusion

The data shows that Ephrin-A2 and –A5 ligands are important in cutaneous nerve development and sensory function. Ephrin-A5 ligand is important for normal light touch sensory function associated with Aαβ nerve fibres, Meissner’s corpuscles and Merkel cells in the skin. The absence of the A5 ligand led to significantly reduced sensitivity. In contrast, no effect was observed in mice lacking the Ephrin-A2 ligand, suggesting this ligand is either not important for light touch sensation in the skin or that its role can be compensated for by other Ephrin proteins. Ephrin-A2 and –A5 ligands have opposing effects at P19 on dermal innervation, where Ephrin-A2 acts as a promoter and Ephrin-A5 acts as an inhibitor. However, dermal nerve density was similar in the four genotypes at the time-points studied (P1 and adult), suggesting that Ephrin-A2 and –A5 ligands do not have a significant effect on dermal innervation. Ephrin-A2 and –A5 ligands have different effects on epidermal innervation, where Ephrin-A5 ligand promotes innervation at P1 and Ephrin-A2 acts as a promoter at P19. Furthermore, reduced epidermal innervation observed during development was not regained in the adult Ephrin-A2A5−/− genotype mice, suggesting the loss during development is irreversible.

This study emphasises the importance of both Ephrin-A2 and –A5 ligands in epidermal innervation. The functions of these Ephrins cannot be compensated by other ligands in the Ephrin family, at least not in the skin.
In summary Ephrin-A2 and –A5 ligands are important in cutaneous nerve development and sensory function. Furthermore, effects of Ephrin ligands are likely to be specific to nerve fibre subtypes at different time points of development. Future studies could be carried out to identify the effects of these Ephrins on different nerve fibre subtypes using different neuronal markers. In addition different sensory function tests could be conducted in the Ephrin-A−/− genotypes to identify the impact of these Ephrins on specific sensory functions.
Chapter 4
Chapter 4: Effects of Ephrin-A2 & -A5 on wound healing

4.1 Introduction

Wound healing is a complex process with three major phases: early and late inflammation, re-epithelialization and granulation tissue formation and matrix formation and remodelling. Keratinocytes participate in the re-epithelialization process, while dermal fibroblasts are the dominant cell type involved in the formation, deposition and remodelling of the extracellular matrix (Singer and Clark, 1999). In this study the effects of Ephrin-A2 and –A5 on the phenotype of these two major skin cell types and on wound closure in vivo were investigated.

Re-epithelialization after wounding consists of formation of a provisional wound bed matrix, keratinocyte migration from the wound edge, proliferation, stratification and differentiation of newly formed epithelium. The effects of different molecules including basement membrane components such as type IV collagen and laminin I, provisional matrix components such as fibronectin and vitronectin and type I collagen in the dermis on keratinocyte migration and re-epithelialisation have been extensively investigated as have the roles of many growth factors and cytokines secreted during the inflammatory phase (Gurtner et al., 2008, Singer and Clark, 1999, O'Toole, 2001)

Epidermal keratinocytes migrate via protrusion and contraction, mediated by insulin like growth factor 1 (IGF-1) signalling (which induces protrusion) and epidermal growth factor (EGF) signalling (which can induce contraction) (Haase et al., 2003). Contraction of keratinocytes by EGF is mediated through the mitogen activated protein kinase (MAPK) pathway whilst IGF-1 mediates protrusion through the phosphatidylinositol-3 kinase pathway (Haase et al., 2003). Fibroblast growth factor -2 (FGF2) mediated Ephrin-A1 stimulation has been shown to cause stimulation of the MAPK pathway in differentiated P19 cells (Yokote et al., 2005). In addition, phosphatidylinositol-3 kinase pathway dependent Ephrin-B reverse signalling occurred in fusing palate epithelium (San Miguel et al., 2011). This suggests overlap and possible interactions between Ephrin signalling pathways and those involved in keratinocyte migration. However, the role of Ephrin-A2 and –A5 ligands in these pathways and their effects on keratinocyte migration are as yet unknown.
Previous studies of gene expression showed that Ephrin-A1, -A3 and -A4 were expressed in normal human epidermis whilst no expression of Ephrin-A2 and –A5 was detected (Walsh and Blumenberg, 2011). Despite normal keratinocytes in human epidermis not apparently expressing Ephrin A2 or A5, it is possible that under stress or injury expression of these ligands may be induced as part of the repair process. Furthermore, the addition of Ephrin ligands i.e. Ephrin-A1 to –A5 to keratinocyte cultures was shown to induce synthesis of differentiation markers and prevent keratinocyte proliferation (Walsh and Blumenberg, 2011).

Hemidesmosomes attach basal epithelial cells to the basement membrane, with α6β4 integrin an important component of these structures (Stepp et al., 1990, Jones et al., 1991). Antibodies against α6β4 integrin block assembly of hemidesmosomes resulting in cell detachment (Kurpakus et al., 1991, Larjava et al., 1996), which might promote keratinocyte migration and therefore be important in wound repair. Keratinocytes treated with human Fc-conjugated Ephrin-A ligands, including Ephrin-A2 and –A5, suppressed the expression of the important cell adhesion genes such as integrin β6 and integrin β4, suggesting the Ephrin signalling pathway could influence migration and healing through reduced cell attachment and increased motility (Walsh and Blumenberg, 2011). Furthermore β1 integrin play a crucial role in wound repair (Grose et al., 2002), while Eph-A3 mediated activation of Ephrin-A2 or Ephrin-A5, resulted in a beta 1-integrin-dependent increased adhesion of Ephrin-A-expressing Human Embryonic Kidney 293 cells to laminin (Huai and Drescher, 2001). Therefore further investigations of the role of Ephrin A2 and A5 on keratinocytes may elicit a better understanding of the role of this pathway and these specific Ephrins in wound repair.

Fibroblasts play a major role in wound healing via migration, proliferation and extracellular matrix synthesis. Many factors are known to enhance fibroblast migration and proliferation in vitro. Basic fibroblast growth factor and TGF-β both promote fibroblast migration in vitro (Schreier et al., 1993), while platelet derived growth factor-BB promotes type I collagen attached dermal fibroblast migration in vitro (Li et al., 2004a). Platelet derived growth factor-AA, platelet derived growth factor-BB, platelet derived growth factor-AB, epidermal growth factor and basic fibroblast growth factor all increase proliferation of fibroblasts in vitro (Narine et al., 2006). There has been extensive research into the activation and control of fibroblast activity in wound repair, given its central role in matrix deposition and therefore in scar appearance, texture and pliability. However, there have been no investigations of a possible role for Ephrin
signalling in these cells. Given the wide ranging functions of Ephrin signalling, further investigation of the role of Ephrins in this cell type may also be important in better understanding the control of wound repair and scar formation.

Here, the effects of Ephrin-A2 and –A5 on migration and proliferation of dermal fibroblasts as well as the effects of these Ephrins on the migration of keratinocytes in vitro has been investigated. The impact of these ligands on wound closure has also been investigated in vivo.

There was no significant effect of Ephrin-A2 and –A5 ligands on fibroblast phenotype in vitro. A tendency for these ligands to have an effect on keratinocyte migration was observed. Ephrin-A2 and –A5 ligands did not appear to have a significant impact on the rate of wound closure. However qualitative changes in the healed wound were observed, most likely due to an impact on contraction during healing.
4.2 Materials and methods

4.2.1 Animals

C57BL/6 wild type mice, Ephrin-A2−/− mice, Ephrin-A5−/− mice and Ephrin-A2A5−/− mice were maintained in standard housing with food and water provided ad libitum. Approval was obtained by Institutional ethics committees and experiments performed in accordance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. (RA/3/100/1162).

4.2.2 Dermal fibroblast isolation and culture

Hair was removed from the mouse skin using depilatory cream and a scalpel blade. The skin is rinsed in phosphate buffered saline (PBS) and soaked in povidone-iodine solution (Pfizer, Perth pty limited) for 5 min at room temperature. The skin is then washed in PBS and a 1cm² piece of skin is placed in 1ml of digestion solution containing 2mg/ml Collagenase/Dispase (Roche Applied Science, Penzberg, Germany) in Dulbecco’s modified eagle medium nutrient mixture F-12 (DMEM/F12, GIBCO®, Carlsbad, California, USA). The piece of skin was incubated in the digestion solution at 37°C for 3 hours in a water bath. During digestion the mixture was agitated periodically. The resulting cell suspension was passed through a cell strainer and centrifuged at 300g for 10min at 4°C. The cells were washed in PBS (pH7.4) and centrifuged twice at 1500rpm for 3min at 4°C. Cells were re-suspended in DMEM/F12 glutamax+10% fetal bovine serum (FBS, Invitro technologies, Noble Park North, Australia), 0.5μg/ml fungizone (Gibco®, Invitrogen™, Carlsbad, California, USA), 100μg/ml kanamycin (Gibco®, Invitrogen™, Carlsbad, California, USA), 1% penicillin/streptomycin (GIBCO® by life technologies, Carlsbad, California, USA) and incubated in a T25 flask at 37°C in 5% CO₂. After another 24 hours media was removed and cells washed with PBS. Finally cell culture media was added without fungizone and kanamycin and cells were maintained in DMEM/F12 glutamax+10% fetal bovine serum at 37°C in 5% CO₂.
4.2.3 *In vitro* wound healing assay (Scratch assay)

Dermal fibroblasts from Wild-type (n=5), Ephrin-A2<sup>−/−</sup> (n=5), Ephrin-A5<sup>−/−</sup> (n=5) and Ephrin-A2A5<sup>−/−</sup> (n=5) mice, isolated as described above, were plated in 6 well tissue culture plates. The seeding density of dermal fibroblasts was 2x10<sup>5</sup> cells per ml of Dulbecco’s modified eagle medium nutrient mixture F-12 (DMEM/F12, GIBCO<sup>®</sup> Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS, Invitro technologies, Noble Park North, Australia) and 1% penicillin/streptomycin (GIBCO<sup>®</sup> by life technologies, Carlsbad, California, USA) into each well. Each experimental group contained 3-6 replicate wells (dependent on cell number isolated prior to the experiment). Plates were incubated at 37°C in 5% CO<sub>2</sub> for the cells to reach full confluence over two days. A microscope slide was placed across the side of a well as a guide and a straight scratch was created down the middle of each well with a 200 μl yellow pipette tip (approximately 0.8mm in diameter). The scratch was made with adequate pressure to remove all the cells in the scratch zone. After making the scratch the wells were rinsed gently with media. Wells were then washed twice with phosphate buffered saline, pipetting the solution around the well to wash away all of the scraped cells. After washing, 2ml of DMEM/F12 + 10%FBS was added into each well. Photographs of the scratches were taken at 0 hours, 6 hours, 24 hours, 30 hours and 48 hours until cells completely covered the scratch area created. Photographs were taken using a light microscope (Olympus 1x51, Japan) at a final objective 10X, numerical aperture 0.13. The total photographed area and the area of the scratch was measured using the ImageJ software program (NIH, Bethesda, MD). At 0 hours the percentage of the scratch surface area was 100%. The percentage closure was calculated by subtracting the percentage of the scratch area remaining at each time point from the percentage of the scratch at 0 hours (100%) to obtain the percentage closure (% surface area of the scratch that was covered by cells). Cell migration was represented as the percentage closure of the scratch over time.
4.2.4 Primary dermal fibroblast MTS [3-(4, 5-dimethylthiazol-2-yl)- 5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium] proliferation assay

Cell proliferation was quantified using a colorimetric assay based on the measurement of bio reduced MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt). Dermal fibroblasts from wild-type (n=5), Ephrin-A2 knockout (n=5), Ephrin-A5 knockout (n=5) and Ephrin-A2/A5 knockout mice (n=5), were seeded at 1.5x10³ cells per well in 96 well microplates in 100μl of DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin. Each sample contained 3-5 replicate wells. Control wells contained 100μl of DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated at 37°C with 5% CO₂. Separate plates were used for 24, 48, 72 and 96 hours incubation times after seeding dermal fibroblasts. At each time-point, 20μl of CellTiter 96® AQueous Non-Radioactive cell solution (Promega cooperation, Madison, USA) was added into each well including the control wells and incubated for 3 hours. Absorbance was read at 492nm using a Multiskan Transmit RC program (Labsystems, Frankland, Massachusetts, U.S.A). Mean absorbance of the test wells of each sample of each genotype were subtracted by the absorbance of the control well to calculate the dermal fibroblast proliferation of each sample at each incubation time. The mean absorbance of all the samples in each genotype at each time point was calculated and plotted to provide a measure of proliferation/viability of each cell type over time.

4.2.5 Keratinocyte isolation and culture

Fur was removed from the dorsal mouse skin and cleaned with povidone-iodine solution (Pfizer, Perth pty limited) and 70% ethyl alcohol using autoclaved cotton wool. Dorsal skin was collected in 1ml of Epigrow™ human epidermal keratinocyte complete media (Merck, Millipore, Massachusetts, USA). Skin was taken to the laminar flow hood and fatty tissue beneath the dermis removed using a sterile surgical blade (No.23) and forceps. Skin tissue was cut into pieces of 3-5mm². The pieces of skin were collected in 5ml of Epigrow™ human epidermal keratinocyte complete media with 20μl of fungizone (Gibco®, Invotrogen™, Carlsbad, California, USA) and 100μl of kanamycin (Gibco®, Invotrogen™, Carlsbad, California, USA), 0.0127g of Dispase (Life technologies™, Carlsbad, California, USA) per 5ml (2.4U/ml)
was added. This solution was incubated for 24 hours at 4°C. This method is a modified version of a protocol published by the CELLNTEC company (CellNTEC, 2011)

After 24 hours, the epidermis was separated from the dermis using sterile fine tip forceps and the pieces of epidermis transferred into 500 μl of Tryple Select (Gibco®, Invitogen™, Carlsbad, California, USA) placed in a sterile petri dish. The epidermal tissue pieces were spread out flat on the surface of Tryple Select and to prevent evaporation of the solution the petri dish was covered with a lid. After an incubation period of 20 to 30 minutes at room temperature the petri dish was tilted at a 30° angle and 2 ml of human epidermal keratinocyte complete media was added. With the help of sterile forceps epidermal tissue pieces were rubbed gently against the base of the petri dish in order to generate a single cell suspension. The resulting cell suspension was collected in a 15 ml centrifuge tube and the previous step repeated to increase the yield of keratinocytes. The second preparation of a single cell suspension was then added to the same 15 ml centrifuge tube. The combined cell preparation was then centrifuged at 200 g for 3 minutes. The cell pellet was resuspended in 5 ml of human epidermal keratinocyte complete media. Cells were cultured on T25 tissue culture flasks (Sarstedt, Germany) coated with 1 ml of 20 μg/ml type IV collagen in phosphate buffered saline (type IV from human placenta, Sigma-Aldrich, Missouri, USA).

4.2.6 Type IV Collagen coating

Stock solution of type IV collagen with a concentration of 1 mg/ml in 10 mM acetic acid (Chem supply, Gillman, Australia) was made. Then the working solution of type IV collagen with a concentration of 20 μg/ml in phosphate buffered saline was made from the stock solution. Tissue culture flasks (T25) were coated with 1 ml of type IV collagen working solution and incubated for 3 hours at 37°C or at 4°C overnight (Redvers and Kaur, 2005).

4.2.7 Optimization of Dispase concentration

A series of Dispase concentrations in CnT-07 medium (Cellntec, Bern, Switzerland) were tested to identify the optimum concentration of Dispase in order to separate the epidermis from the dermis. Initially 5 mg/ml, 7.5 mg/ml, 10 mg/ml, 12.5 mg/ml, 15 mg/ml, 17.5 mg/ml, 20 mg/ml and 22.5 mg/ml of Dispase in CnT-07 medium (Cellntec) were tested by incubation the series of solutions with skin tissue pieces of 0.3 mm X 0.3 mm at 4°C overnight. The epidermis was easily separated from the dermis at 5 mg/ml, 7.5 mg/ml, 10 mg/ml and 12.5 mg/ml of Dispase/ CnT-07 solutions. However a lot of cell
debris was observed. The skin tissue pieces were partially digested in 17.5mg/ml, 20mg/ml and 22.5mg/ml of Dispase/ CnT-07 solutions. Therefore 2.5532mg/ml (2.4U/ml) of Dispase in CnT-07 medium (Cellntec) was tested to identify the optimum Dispase concentration using the same procedure and ultimately confirmed as the optimum concentration with reduced cell debris and high levels of cell separation.

4.2.8 Keratinocyte migration assay (Time lapse microscopy)

A glass bottom dish with a diameter of 35mm and an inner glass diameter of 10mm (Mat Tek co-operation, Massachusetts, USA) was coated with 20μg/ml of collagen IV in PBS (Redvers and Kaur, 2005). Keratinocytes were seeded at a density of 1X10^5 cells/cm² and incubated for 24 hours at 37°C with 5% CO₂ (Siemes et al., 2006). Keratinocytes were visualised at a magnification of 10X and a numerical aperture of 0.55 using differential interference contrast (DIC) and an Olympus IX81 inverted microscope (Olympus, Tokyo, Japan). Images were taken at an interval of 1 minute for 1 hour with a resolution of 1376X1032 pixels by a U-TV1X-2 camera (Olympus, Tokyo, Japan). The distance of the keratinocyte nuclear migration was measured using the Fiji software package (Schindelin et al., 2012).

4.2.9 Full thickness surgical excisional wound injury

Mice aged 8 weeks were received in the pre-clinical facility (PCF) and were given 7 days to acclimatize to animal handling and the PCF environment. On the day of surgery, mice aged 9 weeks were given general anaesthesia using isofluorane administered by anaesthetic chamber and maintained by facemask. The surgery was conducted in a surgery room in PCF zone B. Sterile gloves were worn throughout and the equipment used was all sterile single use equipment except the clippers used to shave the animals which were stored and washed in 70% ethanol. While the animal was under general anaesthesia the area to be wounded was shaved and cleaned with povidone-iodine solution (Pfizer, Perth pty limited). A sterile 12mm punch biopsy (Acuderm inc., Fort Lauderdale, USA) was used to create an incision on the back of the mouse. Using sterile surgical scissors the tissue was removed following the outline made by the punch biopsy incision, creating a 12mm diameter full-thickness excisional wound. The wound was created away from all limbs and on one side of the back away from the spine to prevent impacting on movement and activity of the mouse. The wound was covered with tegaderm dressing using tissue adhesive to maintain the dressing in place. All animals were given buprenorphine for analgesia (0.1mg/kg intramuscular) immediately
prior to surgery while under general anaesthesia. Oral paracetamol in drinking water (1mg/ml) was given for 5 days post injury. Animals were recovered in individual cages and monitored until they were fully recovered from anaesthesia. Once recovered the animals were group housed and allowed food and water ad libitum. All the mice were monitored for their behaviour, grooming and lack of activity. During the acute post injury phase i.e. 5 days post injury, animals were monitored twice per day for any signs of infection and hydration in the wound site, weight of the mice, social behaviour, vocalization, posture, facial grimace and grooming. After 5 days post-injury animals were monitored once per day until 14 days post injury when the wound was completely healed and animals had recovered from the injury. For the period of 5-14 days, wound site and animal behaviour were monitored.

4.2.10 Imaging

A Canon Powershot S100 digital camera was used to photograph wounds daily for the first seven days post-injury and then at days 9,11,13,14 and 16. The total wound area and the scar (circularity and the size) were measured using the ImageJ image processing and analysis program (NIH, Bethesda, MD) (Poutahidis et al., 2013).

4.2.11 Sample collection

Mice were sacrificed 28 days post injury and scar tissue collected and fixed in Zamboni’s fixative and tissue samples embedded in paraffin for further analysis.

4.2.12 Assessment of collagen orientation

H & E staining was carried out post excisional (day 28) scar tissue pieces obtained from wild type (n=4), Ephrin-A2−/− (n=4) and Ephrin-A2A5−/− (n=4) mice. Three sections were used from each animal. Collagen fibres were visualized using Leica TCS SP2 A OBS Multiphoton Confocal microscope (Leica Microsystems, Wetzlar, Germany; 20X oil objective) at an excitation wavelength of 561nm as previously described (Vanderhaeghen et al., 2000). Orientation of collagen was assessed using orientation J of image J software (Rezakhanliha et al., 2012).

4.2.13 Statistical analyses

All results were analysed using SPSS version 19. Dermal fibroblast proliferation and migration were compared between Ephrin-A2−/−, Ephin-A5−/−, Ephrin-A2A5−/− and wild type mice using one-way ANOVA (α=0.05). In these experiments there were 5 animals per genotype and it was assumed that the dermal fibroblast migration and proliferation
rates have a normal distribution and the values in each group have homogeneous variances. Keratinocyte migration was compared between Ephrin-A2<sup>−/−</sup>, Ephrin-A2A5<sup>−/−</sup> and wild type mice using the non-parametric Kruskal-Wallis test (α=0.05). This was used because the group size was only 3 animals per genotype, and this test makes no assumptions about the distribution of the data. One-way ANOVA (α=0.05) was conducted between Ephrin-A2<sup>−/−</sup>, Ephrin-A2A5<sup>−/−</sup> and wild type mice in order to identify differences in wound closure at each time point, scar circularity and the scar size. Mean coherency of collagen orientation ranging from 0 (perfectly random) to 1 perfectly parallel) was compared between wild type (n=4), Ephrin-A2<sup>−/−</sup> (n=4) and Ephrin-A2A5<sup>−/−</sup> (n=4) mice using one-way ANOVA (α=0.05) and Bonferroni post hoc test (α=0.05).
4.3 Results

4.3.1 Ephrin-A2 & -A5 ligands have no significant effect on dermal fibroblast migration \textit{in vitro}

The migration rate of wild type, Ephrin-A2$^{-/-}$, Ephrin-A5$^{-/-}$ and Ephrin-A2A5$^{-/-}$ dermal fibroblasts was assessed following scratch injury (Figure 4.1a-l). The scratch injury was completely closed by 96 hours in all four genotypes. There was no statistically significant difference in the percentage closure of the scratch injury at any time point i.e. 6, 24, 30, 48, 54, 72 and 78 hours between the four genotypes (One way ANOVA; p>0.05) (Figure 4.1m). Therefore the absence of either the Ephrin-A2 or A5 ligands does not have a significant effect on dermal fibroblast migration \textit{in vitro}. 
The migration rate of Ephrin-A$^{-/-}$ and wild type dermal fibroblasts.

4.3.2 Ephrin-A2 & -A5 ligands have no significant effect on dermal fibroblast proliferation in vitro

The proliferation rate of wild type, Ephrin-A2$^{-/-}$, Ephrin-A5$^{-/-}$ and Ephrin-A2A5$^{-/-}$ dermal fibroblasts was assessed using the MTS colorimetric assay. The analysis of proliferation was carried out between 24 hours post incubation to 96 hours post incubation (Figure 4.2). There was no significant difference in the average proliferation rate of dermal fibroblasts between 24 to 96 hours post incubation (One way ANOVA; p>0.05) between the four genotypes. Also no significant difference was found in the proliferation rate of dermal fibroblasts at each time point i.e. 24, 48, 72 and 96 hours.
(One way ANOVA; p>0.05) between the four genotypes. Therefore the absence of expression of both Ephrin-A2 & A5 ligands does not have a significant effect on dermal fibroblast proliferation in vitro.

Figure 4.2. The proliferation rate of wild type and Ephrin-A^-/- dermal fibroblasts. The proliferation rates were not significantly different (p>0.05) among the four genotypes namely wild type, Ephrin-A2^-/-, Ephrin-A5^-/- and Ephrin-A2A5^-/- mice. n=5 animals per genotype. Data are represented as mean +/- SEM.

4.3.3 Ephrin-A2 & -A5 ligands might have an effect on keratinocyte migration in vitro

The migration rate of wild type, Ephrin-A2^-/-, Ephrin-A5^-/-, and Ephrin-A2A5^-/- keratinocytes was assessed using time lapse imaging (Figure 4.3a-c). Kruskal-Wallis analysis of keratinocyte migration rate among the four genotypes showed a p value of 0.09 (Figure 4.3d). Further investigation with additional samples is required to determine whether the lack of significance is due to small sample number (ie this is a false negative) or whether the Ephrin-A2 and -A5 do not affect keratinocyte migration.
Figure 4.3a-d. The migration rate of Ephrin-A2<sup>-/-</sup>, Ephrin-A2A5<sup>-/-</sup> and wild type keratinocytes. a-c: Time lapse images taken to identify the keratinocyte migration per hour in wild type (a), Ephrin-A2<sup>-/-</sup> (b), Ephrin-A5<sup>-/-</sup> (c), and Ephrin-A2A5<sup>-/-</sup> (d) mice. e: Keratinocyte migration rate was not significantly different (p>0.05) between wild type, Ephrin-A2<sup>-/-</sup>, Ephrin-A5<sup>-/-</sup> and Ephrin-A2A5<sup>-/-</sup> mice. n=3 animals per genotype. Data are represented as mean +/- SEM.
4.3.4 Ephrin-A2 & -A5 ligands have no effect on the rate of excisional wound closure in vivo

Excisional wound closure of wild type, Ephrin-A2−/− and Ephrin-A2A5−/− was measured to identify whether Ephrin-A2 and/or Ephrin-A5 ligands have an effect on the rate of wound closure (Figure 4.4a-i). Wound closure, which is defined as a stable covering (Drew et al., 2001) was completed by day 16 post-injury in all four genotypes. The Ephrin-A2 and A5 ligands do not appear to have an effect on the rate of wound closure (Figure 4.4j)
Figure 4.4a-j. The rate of excisional wound closure in Ephrin A2<sup>−/−</sup>, A2A5<sup>−/−</sup> and wild type mice. a-i: Excisional wound closure of wild type mice. (a) 1 day post injury, (b) 7 days post injury, (c) 16 days post injury. d-f: Excisional wound closure of Ephrin-A2<sup>−/−</sup> mice. (d) 1 day post injury, (e) 7 days post injury, (f) 16 days post injury. g-i: Excisional wound closure of Ephrin-A2A5<sup>−/−</sup> mice. (g) 1 day post injury, (h) 7 days post injury, (i) 16 days post injury. j: There was no significant difference (p>0.05) in wound closure rates between the genotypes. n=7 animals per genotype. Data are represented as mean +/- SEM. Scale bar: 1mm.
4.3.5 Ephrin-A2 & -A5 ligands have an effect on wound contracture in vivo

Wound healing and contracture of injuries in wild type mice occurs in all directions leading to a circular scar similar in shape to the original injury but significantly reduced in size (primarily due to contraction of the wound). In Ephrin-A2/− and Ephrin-A2A5/− mice healing appeared to be impacted as the resulting scar (observed at day 16 post-injury), although of similar surface area (one-way ANOVA; p>0.05) (Figure 4.6:a), was observed to be more linear compared to wild type mice with a longer, but more narrow scar in the anterior/posterior direction (Figure 4.5a-c). The circularity of the scars was significantly different between the three genotypes (one-way ANOVA; p<0.01) (Figure 4.6:b). Shape of the scar in wild type mice was significantly different compared to Ephrin-A2/− (Bonferroni; p<0.01) and Ephrin-A2A5/− (Bonferroni; p<0.01) mice (Figure 4.6:b).

Coherency of collagen orientation between wild type, Ephrin-A2/− and Ephrin-A2A5/− mice were significantly different (one-way ANOVA p<0.05), while the coherency of collagen orientation of Ephrin-A2/− mice was significantly different from wild type mice (Bonferroni; p<0.05) day 28, post excisional wounding (Figure 4.7). Furthermore Ephrin-A2/− mice showed more randomized collagen orientation compared to wild type mice. Therefore contracture of the wound may be affected in these mice.

Figure 4.5a-c. Scarring at 16 days post-injury in Ephrin transgenic mice is impaired. Wound scar outcome of wild type (a), Ephrin-A2/− (b), Ephrin-A2A5/− (c) at 16 days post injury. Wild type mice show a circular scar, while Ephrin-A2/− and Ephrin-A2A5/− show an elongated scar. Scale bar: 1mm.
Figure 4.6 Size and the circularity of the scars at 16 days post injury in Ephrin-A^-/- and wild type mice. Size of the scars (a), Circularity of the scars (b). n=7 animals per genotype. Data are represented as mean +/- SEM. ** p<0.01.
Figure 4.7 Coherency of collagen orientation of the scars at 28 days post injury in Ephrin-A$^{-/-}$ and wild type mice. Ephrin-A2$^{-/-}$ mice show a significant difference in the coherency of collagen orientation compared to wild type mice. n=4 animals per genotype. Data are represented as mean +/- SEM. * p<0.05.
4.4 Discussion

This study shows that there is no significant effect of either the Ephrin-A2 or Ephrin-A5 ligands on dermal fibroblast migration or proliferation *in vitro*. Ephrin-A2 and Ephrin-A5 may have an effect on keratinocyte migration, which will require further investigation. Finally, *in vivo* wound healing analysis shows that these two ligands do not appear to affect the rate of wound closure. However, both ligands appear to have an effect on scar outcome, potentially through an impact on contraction of the wound. Other possible effects of the ligands on healing also need to be investigated further, including impacts on sensory function restoration and reinnervation after injury.

4.4.1 Effects of Ephrin-A2 and –A5 on dermal fibroblast and keratinocyte phenotype *in vitro*

Scratch assays have previously been used to identify the effects of cell-cell interactions on cell migration (Liang et al., 2007), which is important for cell contact dependent Ephrin/Eph signalling. The scratch assay is also a well characterized *in vitro* wound healing assay that has become a routine assay for investigating the effects of molecules on wound healing (Walsh and Blumenberg, 2011, Morellini et al., 2008). Therefore scratch assays were performed to assess whether there were any effects of Ephrin-A2 and –A5 on cell migration. Previous studies have shown an impact of Ephrin-B/ EphB signalling on melanoblast and neural crest cell migration (Santiago and Erickson, 2002). However, in this study no significant effect of Ephrin-A2 or Ephrin-A5 on cell migration was observed. This could be due to several reasons. Firstly, as described in previous chapters, the Eph/ Ephrin family has extensive functional redundancy and other ligands may have compensated for the loss of Ephrin-A2 and Ephrin-A5. Alternatively, the Ephs and Ephrins may be expressed on different cell types *in vivo*, leading to an impact on migration *in vivo* that is not observed in single cell type isolated culture models such as the scratch assay used here. Finally, it is well known that extracellular matrix and soluble growth factors directly regulate cutaneous cell motility (Li et al., 2004b) *in vivo*. Therefore the effects of Eph /Ephrin signalling may involve factors during *in vivo* wound healing that are not present in this *in vitro* system, masking the effect of these ligands. Nevertheless, despite these other possible explanations the data here strongly suggest that neither Ephrin A2 nor Ephrin A5 are important in fibroblast migration.
Dermal fibroblasts from Ephrin-A<sup>−/−</sup> mice also showed a normal rate of proliferation in vitro when compared to wild type cells. By contrast, stimulation of endogenous EphA with Ephrin-A1 inhibited the proliferation of several cell types, but not mouse embryonic fibroblasts, via negatively regulating the Ras/MAPK pathway (Miao et al., 2001). However the data presented here strongly suggest it is likely that Ephrin-A2 and A5 ligands are not critical to either the normal proliferation or activity of fibroblast cells.

Even though gene expression studies of human skin have reported the absence of Ephrin-A2 and –A5 ligands in human epidermis (Walsh and Blumenberg, 2011), we found expression of Ephrin-A2 and -A5 ligands in the mouse epidermis (chapter 3). However it is important to detect the impact of these ligands as differential expression of Eph receptors and Ephrin ligands has been identified in skin lesions such as psoriasis and skin ulcers (Kulski et al., 2005, Piruzian et al., 2010, Hafner et al., 2006). Furthermore, addition of Ephrin-A ligands into human keratinocyte scratch assays inhibited keratinocyte migration in vitro (Walsh and Blumenberg, 2011). The current study does not show any impact of Ephrin-A2 and –A5 ligands on the migration rate of keratinocytes in vitro. However, this would need to be validated using additional samples as insufficient numbers of isolated cell populations were obtained for this experiment to reach the threshold for statistical significance, and it is therefore not possible to be sure if this was a false negative due to limited numbers or due to there being no effect of these Ephrins.

We were unable to detect differences in the proliferation rates between the four genotypes using the MTS colorimetric assay (data not shown). Mouse keratinocytes possess a low proliferative index (2-4%) (Taylor et al., 2000, Potten et al., 1982) making it difficult to assess the changes. Thus due to difficulties in isolating mouse keratinocytes, previous studies have found it necessary to use time lapse microscopy and low cell densities (Siemes et al., 2006) to identify keratinocyte migration rate. In addition a previous study showed that intravenous injection of Ephrin-A2-Fc and Ephrin-B2-Fc in mice negatively regulates keratinocyte proliferation (BrdU) via blocking Ephrin-Eph interactions (Genander et al., 2010). The in vivo BrdU labelling technique used in Genander et al. 2003, is more sensitive to changes in the rate of proliferation than our MTS assay and allowed counting of single cells, suggesting that effects on keratinocyte proliferation are small and might have been missed in our study due to technical limitations.
4.4.2 Effects of Ephrin-A2 and –A5 on wound closure and contracture in vivo

The wound healing process in full thickness excisional wound models begins from the margins and the base of the wound by the formation of granulation tissue and migration of epidermal tissue. Wound contraction is important in the closure of the wound as it involves the rapid shrinkage of the surrounding skin, reducing the wound dimensions. This is particularly important in rodent healing, as rodents rely more extensively on this process of contraction to heal wounds in contrast to humans where wounds contract, but to a lesser extent (Davidson, 1998). Wound contraction is mediated in part by fibroblasts and myofibroblast cells generating force across the wound matrix and ‘pulling’ the edges of the wound together (Shin and Minn, 2004). Wound contraction is important and beneficial to wound repair, but can, if unregulated or excessive, lead to wound contracture, with excessive or an extended period of contractile force generated in the granulation tissue and scar tissue leading to hypertrophic scarring and contracture (Davidson, 1998, Shin and Minn, 2004). No significant effects of Ephrin-A2 and –A5 ligands on initial wound contraction or the rate of wound closure were observed (Figure 4.4).

After the initial contraction of the wound, fibroblasts differentiate into myofibroblasts. F- actin filaments of myofibroblasts are aligned across the long axis of cross section of the wound and F- actin filaments aligned parallel to the en face plane (Petroll et al., 1993). This causes contracture of the wound in all directions. However, we observed a lack of wound contracture along the anterior posterior axis in Ephrin-A-/- mice. During somitogenesis, presumptive somites are patterned along the anterior posterior axis prior to boundary formation due to anterior posterior expression patterns of Ephrin ligands and Eph receptors in the presomitic mesoderm (Durbin et al., 1998). Somitogenesis gives rise to the vertebrae, skeletal muscles and the dermis (Retnoaji et al., 2014). Therefore impaired Ephrin/Eph signalling along the anterior posterior axis in the absence of Ephrin-A2 and –A5 ligand expression may reflect the developmental role of Ephrins in the establishment of the anterior-posterior segmental boundaries in the dermis. To further investigate what underlies the differences observed in the scars formed in Ephrin A-/- mice, the orientation of F-actin filaments in cross section as well as in the en face plane in wild type and knockout animals could be detected using phalloidin-FITC (Petroll et al., 1993). This would show whether there was an increase/decrease in the filament arrangements in each axis, which may explain the
altered scar formation. The number of fibroblasts within the wounds may also be important in producing the observed effects. This could be assessed using propidium iodide nuclear staining (Petroll et al., 1993) and would enable us to understand the impact of Ephrin-A2 and –A5 on F-actin filament orientation as well as the number of fibroblasts in the lateral/medial direction and anterior/posterior direction of the wound. Furthermore the number of myofibroblasts in the wound site could be assessed by the detection of α-smooth muscle actin expression in myofibroblasts (Skalli et al., 1986, Darby et al., 1990) as well as potentially assessing the force production of individual myofibroblasts if it was suspected that Ephrins were involved in this aspect of contraction.

Combined contribution of fibroblasts and myofibroblasts to defective extracellular matrix production can lead to excessive scar formation and wound contracture (Nedelec et al., 2000). In addition Ephrin-A1 induced extra cellular protein genes, specifically fibrilla collagen (Walsh and Blumenberg, 2011). Previous studies have shown that myofibroblasts are more responsible for wound contracture (Shin and Minn, 2004). In addition Fibroblast locomotion has been associated with collagen reorientation and contraction in vitro (Ehrlich and Rajaratnam, 1990). Current study shows that collagen orientation of Ephrin-A2−/− mice is more randomized than the wild type mice. Myofibroblasts and fibroblasts could be isolated from Ephrin-A−/− and wild type scar tissue using magnetic activating cell-sorting column. Furthermore measuring collagen gel contraction by isolated myofibroblasts and fibroblasts could be used to identify the differences in wound contracture between the genotypes (Shin and Minn, 2004). This will enable us to identify the effects of Ephrin-A2 and –A5 ligands on wound contracture. However the differences in collagen orientation in the current study confirms that Ephrin-A2 ligand has an effect on wound contracture.

The assembly of F-actin filaments in fibroblasts and fibronectin matrix formation during wound healing has been shown to occur synchronously with fibroblast integrin α5β1 receptor expression in the periphery, causing wound contraction (Welch et al., 1990). Short term Ephrin-A5 activation by its cognate Eph receptor has also been shown to cause increased adhesion of an immortalized mouse fibroblast cell line (NIH 3T3) to various components of extracellular matrix via modulating integrin affinity (Davy and Robbins, 2000). Finally, Ephrins have been shown to affect integrin expression (Walsh and Blumenberg, 2011). Therefore the impact of Ephrins on
$\alpha_5\beta_1$ integrin during wound contraction could also be investigated to try to understand the observed differences in wound contraction and scar.

4.5 Conclusion

The absence of Ephrin-A2 and –A5 ligands does not have a significant effect on dermal fibroblast migration and proliferation \textit{in vitro}. This could be due to the functional redundancy of Ephrin ligands and Eph receptors or the use of a single cell type in the assay, preventing cross-talk between skin cell types. However, Ephrin-A2 and –A5 ligands had no tendency to influence keratinocyte migration. Finally, although Ephrin-A2 and –A5 ligands did not have an impact on the rate of wound closure, there were clear qualitative differences between the healed scar in transgenic and wild type mice. This is the first time Ephrin expression has been implicated \textit{in vivo} in scar formation. This interesting result requires further investigation to understand the mechanism by which these Ephrins impact on scar formation and their normal physiological role. This could ultimately lead to novel ways of intervention in wound repair to ameliorate scar formation and improve patient outcomes. Further research on the impact on reinnervation and sensory function will also be essential as these are critical to regain tissue with normal sensory function post injury. The evidence presented in previous chapters strongly suggests that Ephrin-A2 and A5 are important in cutaneous innervation and this is likely to extend to reinnervation after injury. Using samples already collected in this study preliminary data can be obtained on the impact of the absence of these ligands on both innervation and wound contraction. This will be a critical future experiment.
Chapter 5
Chapter 5: General discussion

5.1 Summary of preceding chapters

The importance of Ephrin-A2 and –A5 ligands is well established in the case of axonal guidance of some central and peripheral nerves during development and regeneration. However, very little work has been done to identify the role of these ligands in the development of cutaneous innervation and its restoration during wound healing. The current study addresses this knowledge gap of expression and function of Ephrin-A2 and –A5 ligands in cutaneous development and wound healing.

The role of Ephrin-A2 and –A5 ligands in skin and their expression was investigated in the epidermis and hair follicles at P1, P19 and P25. Hair follicle morphogenesis was negatively regulated in Ephrin-A5+/− and Ephrin-A2A5+/− mice at P1. In addition, hair follicle cycling was negatively regulated in Ephrin-A5+/− mice with a strong tendency to decrease the rate of hair follicle cycling in Ephrin-A2A5+/− at P25. However Ephrin-A2 and -A5 ligands do not seem to affect final hair follicle density (Chapter 2).

Furthermore the study of epidermal and dermal nerve development and sensory function, reports that Ephrin-A2 and –A5 ligands have opposing effects on epidermal and dermal nerve densities at P19 where Ephrin-A2 ligand acts as a promoter and –A5 ligand acts as an inhibitor of innervation. In addition diminished light touch sensory function in Ephrin-A5+/− adult mouse skin suggests that Ephrin-A5 ligands are required for the development and/or function of Aα/β nerve fibre subtypes receptive for light touch sensory function (Chapter 3).

Finally, since both hair follicles and sensory innervation are known to be important in the response to skin injury, Ephrin-A−/− mice were used to examine wound healing in the skin. Even though Ephrin-A2 and –A5 do not seem to have any effect on dermal fibroblast phenotype in vitro, and keratinocyte migration in vitro. Furthermore, an excisional wound healing study showed that the two ligands have an impact on wound contracture and the scar outcome although they had no significant effects on the rate of wound closure (Chapter 4).

In summary, the thesis provides evidence that these two ligands are important for normal cutaneous development and wound repair.
5.2 Advantages and disadvantages of using Ephrin-A−/− mice over other techniques

As mentioned earlier in chapter 1, Ephrin/Eph signalling sends a forward signal to Eph receptor expressing cells and a simultaneous reverse signal to the ligand expressing cell (Arvanitis and Davy, 2008). Use of Ephrin-A−/− mice prohibits all of the signalling effects of the ligands. This includes both forward and reverse signalling, as well as signalling in trans (to other cells) and in cis (on the same cell expressing both ligand and receptor). Inconsistent differences in the effects found on keratinocyte migration by the addition of Fc-conjugated Ephrin-As onto keratinocyte cell cultures are relatively non-specific (Walsh and Blumenberg, 2011), due to the promiscuous binding of Ephrin/Eph (O'Leary and Wilkinson, 1999) and the concentrations of conjugated Ephrins being used potentially being substantially higher than the normal physiological level. Addition of specific Fc-conjugated Ephrin-As might override the naturally occurring interactions of other Ephrin-A ligands with the Eph receptors that would mask true Ephrin/Eph interactions. The use of the transgenic model described here allows the effects observed to be specifically attributed to the role of the individual (or combined) Ephrins on the system being tested, providing a more accurate representation of the in vivo and true physiological effects of the proteins compared to the many cell culture models used to date (Lin et al., 2010, Walsh and Blumenberg, 2011).

However, there are disadvantages in using Ephrin-A−/− mice to identify the effects of Ephrin ligands on wound healing and skin development. For example, anterior posterior expression patterns of Ephrins and Eph receptors in the presomitic mesoderm are known to play a role in presumptive somite patterning along the anterior posterior axis during somitogenesis (Durbin et al., 1998). Furthermore, somitogenesis gives rise to the dermis (Retnoaji et al., 2014). Therefore the lack of anterior-posterior wound contracture (reported in chapter 4) could be due to the lack of Ephrin A2 or A5 expression during development of the animal and a consequent change to the underlying programming of cells as to their position on the body’s anterior-posterior axis. Therefore it is possible that there is no functional role related to these Ephrins during the healing process. However, this is very unlikely as an underlying change in anterior posterior axis programming result in severe defects, which was not apparent in Ephrin-A−/− mice. In addition the current studies showed that Ephrin-A2 and –A5 ligands have an impact on hair follicle morphogenesis, cycling and cutaneous innervation during cutaneous development. Therefore the impact of these ligands on wound healing in adults could be
partly due to the effects of Ephrins on these developmental processes rather than a direct effect at the time of injury.

To overcome these disadvantages in the future it may be possible to use inducible and/or conditional promoter systems to produce transgenic mice in order to better examine the effects of these ligands on specific tissues and at specific time points (Hakamata and Kobayashi, 2010). For example, tetracycline-regulated gene expression in a transgenic mouse that is specific to the epidermis has been reported (Romano and Sinha, 2010). This approach could be modified for controlling the expression of Ephrins, either in the epidermis or other important cell types, or alternatively restricting expression to an area of interest (for example to the injury site) through topical application of the inducing agent. This would provide a system whereby the potential developmental effects of the Ephrins would be bypassed, as normal expression would be maintained until the administration of the gene inducing agent. This approach would provide better evidence than that from the mice used here for the role of these Ephrins in wound repair.

Another disadvantage of using mouse models is differences identified in the cutaneous Ephrin/Eph ligand expression patterns compared to human skin (Lin et al., 2012). For instance EphA1 is expressed in all viable cell layers of the human epidermis (Hafner et al., 2006), whereas EphA1 expression is confined to the basal layer of mouse epidermis (Coulthard et al., 2001). Furthermore gene expression analysis revealed that Ephrin-A2 and –A5 ligands are not expressed in the human epidermis (Walsh and Blumenberg, 2011), whereas we showed the expression of Ephrin-A2 and –A5 ligands in the mouse epidermis (Chapter 2). However differential expression of Eph receptors and Ephrin ligands in skin lesions such as psoriasis and skin ulcers (Kulski et al., 2005, Piruzian et al., 2010, Hafner et al., 2006), warrants to identify the role of these ligands in the skin.

Finally possible redundancy of the Ephrins (Walsh and Blumenberg, 2011), can mask effects of single knockout genotypes. This is less likely in the double knockout genotype, as demonstrated by the observed decrease in epidermal innervation in adult mice, which was not identified in single knockout genotypes (Chapter 3).
5.3 Linking hair follicle cycling and cutaneous innervation

The impact of Ephrin-A2 and –A5 ligands on hair follicle cycling and cutaneous innervation has been discussed previously in chapter 2 and 3. Here the potential links between hair follicle cycling, cutaneous innervation and Ephrins are discussed.

It is generally agreed that hair follicle morphogenesis/cycling and cutaneous innervation are closely associated and interacting processes. However, in our study of Ephrin-A2−/−, A5−/− and A2A5−/− mice, there was no observed association between these processes. This may be due to experimental limitations or alternatively due to ‘uncoupling’ of these linked processes due to the lack of Ephrin-A2 or A5 expression.

5.3.1 Hair follicles – cutaneous innervation

Previous studies show interactions between hair follicle cycling and cutaneous innervation (Botchkarev et al., 1997a, Botchkarev et al., 1997b). For example, the density of PGP9.5 immunoreactive dermal and epidermal nerves was shown to be significantly increased during the transition from telogen to anagen II stages of hair follicle cycling, with the increased level sustained to anagen IV (Botchkarev et al., 1997a). It was also shown that during the subsequent transition from anagen IV to anagen VI there was a significant decrease in cutaneous innervation (Botchkarev et al., 1997a). The number of nerve fibres in the skin that were immunoreactive for CGRP and SP has significantly changed at different hair follicle cycling stages (Botchkarev et al., 1997b). In our study, we identified a delay in hair follicle cycling at P25 in Ephrin-A5−/− mice (Chapter 2). However, we were unable to directly compare this with the density of cutaneous innervation data as cutaneous innervation wasn’t assessed at P25 due to time constraints. Skin tissue samples were collected at P1, P19, P25 and from adult mice as the hair follicle morphogenesis and first postnatal hair follicle cycling follows a precise time scale (Muller-Rover et al., 2001). Hair follicle morphogenesis can be observed at P1, while 100% of pelage hair enter into stage 8 of morphogenesis by P9. The first morphologic signs of synchronized catagen development could be observed at P17-18 (Paus et al., 1999). Therefore hair follicles go through catagen phase at P19, where as hair follicles are in telogen phase from P21 to P27 and enter the anagen phase at P28 in C57BL/6 female mice (Muller-Rover et al., 2001). However it is also reported that the time scale is dependent on the genetic background, gender (female mice have a
prolonged telogen phase) and environmental factors (Muller-Rover et al., 2001). In future studies skin tissue from all these animals should be assessed using PGP9.5 to better determine the relationship between the two processes.

Examination of the expression of the neurotrophic factors NT-4, BDNF and their receptor TrkB showed expression was variable during the hair follicle cycle and peaked in catagen hair follicles. This suggests the importance of these neurotrophic factors and their receptor signalling in catagen development (Botchkarev et al., 1999). It is also well known that these same (and other) neurotrophic factors are important to establish and promote peripheral sensory innervation (Albers, 1994, LeMaster et al., 1999). Therefore the correlations previously observed between hair follicle cycling and cutaneous innervation could be due to neurotrophic factor expression. Furthermore multiple studies have reported interactions between neurotrophic receptors and Ephrins as well as Eph receptors during retinal ganglion cell branching and guidance (Marler et al., 2008, Poopalasundaram et al., 2011). The neurotrophic factors and their receptor expression could be determined using immunohistochemistry in Ephrin-A<sup>-/-</sup> and wild type skin tissues collected. This would enable to identify the impact of Ephrins on neurotrophic factors and their receptor expression in the skin.

In addition to the close relationship between hair follicle cycling and innervation a relationship between hair follicle morphogenesis and cutaneous innervation has also been reported (Peters et al., 2002). In normal wild type mice, all tylotrich hair follicles enter stage 5 or 6 of hair follicle morphogenesis at P1. In addition stage 1-4 nontylotrich hair follicles are also present in the mouse back skin at P1 (Peters et al., 2002). In the current study similar findings were obtained, with numerous hair follicles in stage 6 of morphogenesis identified at P1 in wild type as well as in Ephrin-A2<sup>-/-</sup> mice. In stark contrast, Ephrin-A2A5<sup>-/-</sup> mice showed a significantly reduced percentage of hair follicles compared to wild type at stage 6 with a similar trend in hair follicle percentage reduction in Ephrin-A5<sup>-/-</sup> mice. This finding further confirms the retardation in hair follicle morphogenesis at P1 in Ephrin-A5<sup>-/-</sup> and Ephrin-A2A5<sup>-/-</sup> mice (Chapter 2). Peters et al. (2002) reports that epidermal innervation detected with PGP9.5 peaks at P5, when the hair follicles enter stage 7 or 8 of hair follicle morphogenesis and decreases by P17 when the hair follicles enter catagen phase of the first hair cycle. The results from Peters et al. suggest that epidermal innervation increases as hair follicle morphogenesis progresses to the later stages (stages 7/8). However, in Ephrin-A5<sup>-/-</sup> and Ephrin-A2A5<sup>-/-</sup> mice there was a tendency to observed reduced levels of epidermal
innervation compared to wild type mice. This did not reach statistical significance but the study was limited due to the small number of transgenic mice from each strain obtained at this time-point and for this study. From the findings presented in this thesis, showing expression of Ephrin-A2 and A5 ligands in wild type hair follicles at P1 (Chapter 2) as well as the impact of these ligands on hair follicle morphogenesis and cycling, together with the studies of others showing mRNA expression of Ephrin-A2 ligands in the skin at P1 (Yamada et al., 2008) suggests that there maybe direct communication between the hair follicles and cutaneous nerves that is mediated by Ephrins. Therefore in the Ephrin knockout mice, these interactions are impaired and the correlation between innervation and hair follicle morphogenesis is altered. Essentially, the absence of the Ephrin expression has led to ‘uncoupling’ of the process of hair follicle morphogenesis and cycling to cutaneous nerve growth into the dermis and epidermis. Hair follicle morphogenesis and cycling could be assessed in Ephrin-A-/- genotype post surgical cutaneous denervation (Engin, 1998), and compare it with hair follicle data in the current study (Chapter 2). This would enable to identify whether there is a direct communication between the hair follicles and cutaneous nerves that is mediated by Ephrins.

Despite providing evidence that Ephrin-A2 and A5 are involved in hair follicle development and cutaneous innervation, this study suggests that the Ephrin-mediated effects occur independently in each process and does not show evidence of clear interactions between these processes. Interestingly the functional study (Chapter 3) examining sensory function in these transgenic mice revealed that Ephrin-A5 ligands impaired light touch sensory function associated with Aβ nerve fibres and Meissner’s corpuscle endings. Therefore retardation in hair follicle induction and/or morphogenesis at P1 in Ephrin-A5-/- might be associated with altered densities of different nerve subtypes. Further studies could be conducted to examine Ephrin expression in specific hair follicle cycling stages and the types of cutaneous innervation using different immunoreactive markers such as substance P, CGRP, NF200 etc. (Schotzinger and Landis, 1990, Ma, 2002). Neurofilament 200 (NF200) is a marker for myelinated A fibres (Ma, 2002), while TRPV1 is expressed by nociceptive C fibres responding to heat (Lawson et al., 2008, Cavanaugh et al., 2009). Unmyelinated C fibres are identified by CGRP, while substance P is a marker for unmyelinated C fibres and nociceptive fibres (Anderson et al., 2011). This would further probe the relationship between hair follicle
cycling and innervation, as well as elucidate the role of Ephrin-A5 in cutaneous innervation.

5.4 Do the effects of Ephrin-A2 and –A5 ligands on cutaneous innervation and hair follicle cycling have an impact on wound contracture and/or sensory function during wound healing?

An important recent study clearly depicts the interdependence of hair follicle cycling, cutaneous innervation and wound healing (Martinez-Martinez et al., 2012). Bulge stem cells possess receptors to neuropeptides produced by sensory neurons and mice treated with capsaicin, which reduces sensory neuronal terminals have reduced migration of hair follicle bulge stem cell progeny to the epidermis post wounding compared to control (Martinez-Martinez et al., 2012). Another study showed Sonic Hedgehog from neurons signal to a population of bulge stem cells, which contributes to wound healing (Brownell et al., 2011). However, the complex molecular mechanisms underlying these links are yet to be determined.

5.4.1 Hair follicles – wound healing

Many studies have identified the importance of hair follicles during wound healing. As described in the introduction, hair follicles are a source of stem cells that become long-term repopulating epidermal cells in the wound epidermis (Levy et al., 2007). It is possible that EphA4 expressing bulge stem cells (Tumbar et al., 2004) interact with Ephrin-A5 ligand expressing dermal papilla (chapter 2) during wound healing. Another interesting and very important study has been carried out to identify the impact of hair follicle cycling on wound healing (Ansell et al., 2011). This study identified a significant acceleration of the wound healing process when mice were wounded during late anagen compared to catagen and telogen hair cycle stages. Ansell et al. (2011) also reported a significant increase in re-epithelialization, decreased immune cell infiltration, increased angiogenesis and extracellular matrix deposition during the anagen phase of hair follicle cycling. In the current study a significant retardation of hair follicle cycling in Ephrin-A5−/− mice and a strong tendency to reduce hair follicle cycling in Ephrin-A2A5−/− mice at P25 have been identified. In addition Ansell et al. (2011) observed a strong correlation between the up-regulated genes in the anagen phase and during normal wound healing. Therefore gene expression analysis of anagen hair follicles of Ephrin-A−/− genotypes could be useful to identify differences in their gene expression
profile compared to normal hair follicles and therefore to better understand their impact on wound healing.

5.4.2 Sensory innervation - wound healing

Recent studies have shown that in addition to the well-characterised changes in sensory function in scar, altered cutaneous innervation identified post injury appears to be related to scar pathology, including the incidence of chronic pain (Albrecht et al., 2006, Hamed et al., 2011, Anderson et al., 2010). All of this work points to an important interdependent and reciprocal relationship between cutaneous innervation and wound repair. Using the transgenic mouse model, the potential for Ephrin A2 and A5 to be part of the mechanistic link between these processes was investigated. Cutaneous epidermal innervation was reduced in Ephrin-A2A5−/− adult mice, but this was not associated with an impaired rate of wound closure in adult animals (Chapter 4). As mentioned earlier neuropeptide substance P is involved in keratinocyte proliferation (Paus et al., 1995, Tanaka et al., 1988). It is possible that Ephrin-A2 and –A5 ligands do not impair nerve fibres releasing substance P, which promote keratinocyte proliferation in vivo. This could be identified using immunoreactive markers for substance P (Pare et al., 2001). Alternatively in the present study we identified that Ephrins might have an effect on keratinocyte migration. Therefore the impact of Ephrins on keratinocyte phenotype might counter the loss of nerves during re-epithelialisation. However, clear differences in the qualitative scar appearance were observed in transgenic animals, implicating a role for these Ephrins in wound repair. Studies examining cutaneous reinnervation during wound healing could not be carried out within the timeframe of the thesis, but will be important to understand the role of Ephrins in the skin following injury. This can be identified using immunoreactive markers for SP, CGRP (Pare et al., 2001) as well as pan-neuronal marker PGP9.5 (Wang et al., 1990, Navarro et al., 1995, Schulze et al., 1997) in the skin tissue sections as described in this thesis.

5.4.3 Ephrins may be involved in independent processes that are specific to hair follicle development and cycling, cutaneous innervation and wound healing

From our study we know that cutaneous innervation in normal skin is altered by Ephrin-A2 and Ephrin-A5 ligands. Also, in vivo experiments showed the effects of these two ligands on qualitative wound scar outcome in mice. This could be due to effects of these ligands on myofibroblast function. Previously, Liu et al. (1999) has demonstrated an effect of and potential link between cutaneous innervation and myofibroblasts. In
addition to CGRP positive nociceptive fibres and myofibroblasts detected in close proximity to wound tissue, increased levels of myofibroblasts and transient sensory hyperinnervation were observed in neonatal rats (Liu et al., 1999). It has also been reported that increased levels of nerve growth factor in myofibroblasts observed in neonatal rats promote nerve sprouting into the wound (Hasan et al., 2000). Therefore it is possible that the different appearance of scar observed in the transgenic mice is due to the direct effects of the Ephrin ligands on myofibroblast activity, which could also impact on the innervation. Further investigation of this possibility is important and would include analysing the numbers and activity of myofibroblasts in transgenic mice (including by the detection of α-smooth muscle actin expression (Skalli et al., 1986, Darby et al., 1990)), and assessment of the extra cellular matrix contraction by the dermal cells using collagen gel contraction assays *in vitro* (Rouabhia et al., 2013).

Sensory function problems such as loss of sensory function, pain and itching have been identified post wound healing (Waris et al., 1989, Niessen et al., 1999, Ward et al., 2004, Nedelec et al., 2005). Substance P immune-positive neurons, which account for a small proportion of total cutaneous nerves, were observed to be significantly increased post excisional wounding, potentially a cause of pruritis and pain (Henderson et al., 2006). It has also been reported that CGRP is a sensory mediator during wound healing as its contribution to the total innervation is increased whilst non-SP and non–CGRP nerve fibres were highly reduced during wound reinnervation (Henderson et al., 2006). However Henderson *et al.* also reported that the overall innervation pattern detected with PGP9.5 was not altered in excisional wounds, suggesting that changes in these subtypes of fibre are offset by reciprocal changes in other fibre types that were not investigated. In the current study, in addition to the altered total cutaneous nerve densities identified with the use of PGP9.5 in Ephrin-A2−/−, Ephrin-A5−/−, Ephrin-A2A5−/− transgenic mice, the absence of the Ephrin-A5 ligand reduced light touch sensitivity in normal mouse skin. Therefore it is likely that these ligands do also impact on the process of reinnervation after injury (in addition to the observed impact on innervation of normal skin) and that this will be observed in analysis of the histology of the wound samples collected using immunohistochemistry for nerve fibres. Further work will be critical to examining the level and types of innervation after injury in order to determine whether it is the effects of Ephrin ligands on innervation that lead to the observed changes in scar or whether there are other direct or indirect effects of the Ephrins on the healing process.
5.5 Possible mechanisms behind Ephrin/EphA regulated hair follicle cycling and cutaneous innervation

The current study shows that hair follicle cycling is retarded in Ephrin-A5−/− mice at P25 with lower or no percentages of hair follicles in anagen II and anagen III. In contrast Ephrins are known as negative regulators of hair follicle and epidermal stem cell proliferation (Genander et al., 2010). Ephrin-A1-Fc mediated activation of EphA2 receptor interfered with PC3 cell growth with its inhibitory effects on basal MAPK signalling through p120RasGAP binding (Parri et al., 2005) (Figure 5.1). However ligand activation of EphA2 did not have an effect on EGF induced HN5 cell proliferation (Larsen et al., 2007). It is possible that Ephrin-A5/EphA signalling play an opposing role to Ephrin-A1-Fc mediated activation of EphA receptor signalling.

Ephrin-A2 and –A5 ligands are expressed in the epidermis (Chapter 2), and nearly all EphA receptors are expressed in cutaneous sensory growth cones (Muñoz et al., 2005). Therefore growth cone attraction and repulsion occur via Ephrin-A/EphA signalling in order to innervate the skin.
Figure 5.1 Mechanism behind Ephrin-A mediated EphA receptor activation in keratinocyte proliferation

5.6 Redundancy & opposing effects of Ephrin ligands

A study carried out on chick embryos showed defective epaxial sensory neuronal projections in the absence of Ephrin-A2 and –A5 ligands on sensory neurons. However the sensory neuronal projections were not completely lost, suggesting that other Ephrin-A family members compensated for their loss (Wang et al., 2011). Keratinocytes treated with different Ephrin ligands expressed a considerable number of similar and overlapping gene sets, strongly suggesting a high level of redundancy across the Ephrin family (Walsh and Blumenberg, 2011). Similarly, in the current study, as discussed in chapter 4 despite the use of the transgenic model to specifically target the effects of individual ligands, that other ligands in the Ephrin family might be compensating for the loss of Ephrin-A2 & -A5 ligands both in vivo and during in vitro dermal fibroblast migration, and proliferation and in vivo wound closure. This would effectively mask the true activity of these individual ligands, leading to false negative results in the studies presented here. However, the presence of observed changes in many of the studies shows clearly that in vivo these ligands cannot be completely compensated for and have at least some specific and unique functions in the skin.
There is also evidence that Ephrin-As can have contrasting signalling effects depending on the cells they are expressed in, the receptors they activate and even the concentration of the receptors. Ephrin-As are primarily repulsive, as evidenced by studies of Ephrin-A5 induced in astrocytes after a stroke, showing a reduction of sprouting from cortical neurons and blockade of axonal sprouting in premotor-prefrontal motor circuits (Overman et al., 2012). However, another study showed that concentration gradients of Ephrin-A ligands can cause attraction or repulsion of retinal axon projections depending on the concentration of the protein: at low concentrations, Ephrin-A2 ligands promote axonal growth, but at high concentrations they inhibit axonal growth (Hansen et al., 2004). Similarly the opposing effects of Ephrin-A2 and –A5 ligands were identified in chapter 3, where Ephrin-A2 ligand acting as a promoter and Ephrin-A5 ligand acting as an inhibitor during dermal innervation at P19. The current study and previous studies show the redundancy among the ligands of the Ephrin family and opposing effects of Ephrin-A2 and –A5 in different contexts. Our data add that these effects may also be specific to the time of development, where opposing effects were observed at P19 of dermal cutaneous innervation and not in adult animals as reported in chapter 3. This could be due to interactions of Ephrin-A2 and –A5 ligands with different Eph receptors, which occur in a time dependent manner.

5.7 Clinical importance

Because we found effects of Ephrin-A2 and –A5 ligands on hair follicle cycling, innervation and wound contracture, modulating interactions of these ligands with their receptors might be useful to achieve a better wound healing process with reduced scars and improved sensory function. For example, increased CGRP immunoreactive nociceptive fibres have been identified in chronic burn patients (Hamed et al., 2011), while another study showed systemic decrease in cutaneous innervation post burn injury (Anderson et al., 2010). Furthermore, loss of light touch sensation has been identified over grafted areas in patients with burns (Ward et al., 1989). The present study demonstrates a reduction in light touch sensory function in normal skin of Ephrin-A5+/– mice. Therefore Ephrin-A5 ligands may be useful for enhancing light touch sensory function. Inhibition of forward signalling caused by the interactions of Ephrin-A5 ligands with the cognate Eph receptors in Ephrin-A5+/– mice can be achieved by using peptides and small molecules which block EphA receptor forward signalling, [reviewed in detail in (Noberini et al., 2012)]. Since Ephrin-A5 ligand also interacts with the EphB2 receptor (Himanen et al., 2004), it will be important to identify whether EphB2
receptor forward signalling is also involved in enhancing light touch sensory function. Furthermore specific antagonistic peptides designed for EphA4, EphA5, EphA7 (Murai et al., 2003) and an antibody specific to EphB2 (Mao et al., 2004) have been reported. These antagonistic peptides and antibodies that bind to Eph receptors, block Ephrin-Eph interactions and inhibit Eph forward signalling. These molecules could be used to determine the role of Eph receptors in light touch sensory function. These peptides and small molecules could eventually be used to develop pharmaceuticals to enhance light touch sensory function as a therapeutic intervention for burn patients with loss of light touch sensory function.

Wound contracture, which occurs post burn injury can lead to patient discomfort (Kraemer et al., 1988, Yi et al., 2013). The current study identified differences in wound contracture in Ephrin-A<sup>-/-</sup> mice compared to wild type mice. F-actin filaments of myofibroblasts are known to cause contracture (Petroll et al., 1993). Future studies should be carried out to identify the role of Ephrins in myofibroblast mediated wound contracture in order to identify targets for therapeutic interventions.

Modulating Ephrin /Eph interactions as suggested above already forms the basis of promising anticancer therapies and this strategy has been recently reviewed (Xi et al., 2012, Mosch et al., 2010). For example, soluble EphA2-Fc has been used to block interactions between EphA receptors and ligands and inhibits EphA receptor forward signalling, and results in inhibition of carcinogenesis (Cheng et al., 2003). However, in these studies it has been suggested that using soluble EphA2-Fc might not efficiently inhibit Ephrin ligand reverse signalling (Brantley et al., 2002, Cheng et al., 2003, Dobrzanski et al., 2004).

In order to proceed with these techniques for therapeutic interventions in skin it will be important to investigate the signalling pathways activated by Ephrin-As. Nonetheless, the independent roles of Ephrin-A2 and –A5 in skin biology described above may provide highly specific and therefore useful therapeutic targets.

5.8 Future work

The current study identified the retardation of hair follicle cycling in Ephrin-A5<sup>-/-</sup> and –A2A5<sup>-/-</sup> mice at P25. As mentioned earlier in this chapter, gene expression profiling of anagen hair follicles in Ephrin-A<sup>-/-</sup> mice could be carried out in the future. This would
enable identification of the differences in gene expression patterns compared to anagen hair follicles in the normal skin as well as during wound healing (Ansell et al., 2011).

Future studies could be carried out in order to identify the impact of Ephrin-A2 and –A5 ligands on cutaneous re-innervation and sensory function during wound healing. Pan neuronal marker PGP 9.5 could be used to assess innervation density in skin samples at different time points post excisional wounding. Furthermore assessment of nociceptive function using Semmes-Weinstein (Morellini et al., 2012), warm or cold sensory function (Yarnitsky and Sprecher, 1994) of the small diameter myelinated/unmyelinated fibres in wounded skin could be carried out to identify the effects of these ligands on sensory function post excisional wounding. Furthermore specific subtype nerve markers such as transient receptor potential cation channel subfamily V member 1 (TRPV1) mediating thermal hyperalgesia (Huang et al., 2006), tyrosine-receptor kinase A (TrkA) mediating mechanical hyperalgesia (Summer et al., 2006) could be used to identify the effects of Ephrin ligands on cutaneous nerve subtypes.

Furthermore the role of neuronal peptides in cutaneous inflammation (Rossi and Johansson, 1998), and their association in hypertrophic scarring have been reported (Scott et al., 2005). Cytokines play a significant role in inflammation and, are used as biomarkers to detect inflammatory response (Gu et al., 2009). Therefore cytokine assays could be performed on serum samples collected from Ephrin-A-/- and wild type mice, following excisional wound injury in order to identify the role of Ephrins in cutaneous inflammation during wound healing.

Blood vessel formation occurs during wound healing (Singer and Clark, 1999), and many studies have reported the role of Ephrins and Eph receptors in vascular development in different contexts (Brantley-Sieders et al., 2004, Daniel et al., 1996, Pandey et al., 1995). Therefore vascularisation could be quantified in post excisional wound skin tissue sections of Ephrin-A-/- and wild type mice, using CD31 stain (Sahin et al., 2014), to determine the role of Ephrins.

As mentioned in chapter 4 of the thesis, differences in the orientation of F-actin filaments in the wounds among the four genotypes i.e wild type, Ephrin-A2/-, EphrinA5/- and Ephrin-A2A5/- could be assessed in order to identify the effects of the ligands on wound contracture.
Future work mentioned here will answer the questions that have arisen from the current study.

5.9 Conclusion

Throughout the thesis we have demonstrated the important role played by Ephrin-A2 & -A5 ligands in hair follicle morphogenesis, hair follicle cycling, cutaneous innervation, sensory function and wound healing. Ephrin-A2 and –A5 ligands were detected in the epidermis and hair follicles at P1, P19 and P25. Ephrin-A5\(^/-\), Ephrin-A2A5\(^/-\) showed a retardation of hair follicle morphogenesis at P1 compared to wild type, although these effects did not persist at P19 and P25 where hair follicle densities were normal. Ephrin-A5\(^/-\) showed a retardation in hair follicle cycling at P25 with a similar trend in Ephrin-A2A5\(^{-/-}\) mice. We identified that Ephrin-A2 and –A5 ligands have opposing effects on dermal innervation at P19, where Ephrin-A2 ligand acts as a promoter and Ephrin-A5 ligand acts as an inhibitor. Light touch sensory function of normal glabrous skin is enhanced by Ephrin-A5 ligand. Ephrin-A2 and –A5 ligands do not seem to have an effect on dermal fibroblast phenotype \textit{in vitro} and keratinocyte migration. Even though Ephrin-A2 and –A5 ligands do not seem to affect wound closure, these ligands seem to have an impact on wound contracture changing the wound scar outcome. Hence modulating the interactions of Ephrin-A2 and –A5 ligands with the cognate receptors may prove useful to achieve a better wound healing process with reduced scars and sensory function problems.
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Supplementary Material
Figure S1 Ephrin-A2 and Ephrin-A5 ligand expression in Ephrin-A2A5<sup>−/−</sup> mouse skin. (a) Ephrin-A2 ligand was not expressed in Ephrin-A2A5<sup>−/−</sup> mouse skin. (b) Ephrin-A5 ligand was not expressed in Ephrin-A2A5<sup>−/−</sup> mouse skin.
Figure S2 Percentage of guard, awl and zig zag hair follicles in wild type and Ephrin-A⁻ /⁻ mice.
Figure S3 Dermal nerve density results of one wild type mouse sample across 500μm skin tissue. Dermal nerves are marked with arrows and the hair follicles are marked with arrow heads.
Appendix
Ephrin-A2 and Ephrin-A5 Are Important for the Functional Development of Cutaneous Innervation in a Mouse Model

Letter to the Editor

Eph receptor/Ephrin ligand interactions are important in neuronal mapping and topography in central and peripheral nerves (Palmer and Klein, 2003). All the Eph receptors and Ephrin ligands are expressed in normal human skin (Hafner et al., 2006) and Ephrin-A ligand signaling in hair follicles and keratinocytes has been identified (Yamada et al., 2008). In animal models, sensory neurons express Eph-A receptors, whereas Ephrin-A2 and -A5 have been shown to be important in sensory axonal growth patterning (Muñoz et al., 2005; Walsh and Blumenberg, 2011). Here, we have investigated the role of Ephrin-A2 and -A5 ligands on cutaneous innervation and sensory function. We hypothesized that the loss of either Ephrin-A2 and/or A5 would modify cutaneous innervation and negatively impact sensory function.

All animal studies were approved by the University of Western Australia Animal Ethics Committee. C57BL/6 wild-type, Ephrin-A2−/−, Ephrin-A5−/−, and Ephrin-A2A5−/− mice were euthanized at day 1 (P1), day 19 (P19), and 3–6 months after birth (adult, n=5 per genotype per time point). 1 cm² of dorsal skin was harvested and fixed. Nerves were identified by protein gene product (PGP) 9.5 immunohistochemistry and innervation density quantitated (Supplementary Methods; Anderson et al., 2010; Morellini et al., 2012). All analysis used one-way analysis of variance and Bonferroni correction for multiple testing.

Both Ephrin-A2 and -A5 ligands are expressed in normal mouse skin epidermis and hair follicles at all time points tested (P1, P19, adult, Supplementary Figure S2 online). Dermal innervation density was not significantly different in any genotype at P1 or adults (Figure 1a and c). Dermal innervation density of Ephrin-A2−/− animals was significantly reduced compared with wild type (P<0.05), whereas Ephrin-A2A5−/− mice showed a significant increase in dermal innervation density compared with wild type (P<0.05) at P19. Dermal nerve density of Ephrin-A2−/− animals was also significantly decreased compared with Ephrin-A5−/− (P=0.001) and Ephrin-A2A5−/− (P<0.001) mice at P19 (Figure 1b). Across the age groups wild-type mice did not show a significant difference in dermal nerve density (Figure 2a). Ephrin-A2−/− mice showed a significant increase in nerve density from P19 to adult (P=0.05; Figure 2b). Nerve density of Ephrin-A5−/− animals was significantly increased from P1 to P19 (P<0.05; Figure 2c). Dermal nerve density of Ephrin-A2A5−/− mice showed a significant increase at P19 compared with P1 (P<0.01) and adult mice (P<0.05; Figure 2d).

Epidermal innervation of Ephrin-A−/− animals was not significantly different compared with wild-type mice (P>0.05) at P1. Epidermal innervation in Ephrin-A5−/− mice was significantly reduced compared with Ephrin-A2−/− animals (P<0.05) at P1 (Figure 1d and g–j). Adult Ephrin-A2A5−/− mice also showed a significant reduction compared with wild-type (P<0.05), Ephrin-A2−/− (P<0.05), and Ephrin-A5−/− mice (P<0.05; Figure 1f and o–r). In contrast, epidermal innervation of Ephrin-A2−/− animals was significantly reduced compared with wild-type (P<0.05) and Ephrin-A5−/− mice (P<0.001) at P19 (Figure 1e). Ephrin-A5−/− mice showed a significant increase in epidermal innervation compared with Ephrin-A2A5−/− mice at P19 (P<0.001; Figure 1e and k–n).

Epidermal nerve density of adult wild-type mice was significantly reduced compared with P19 wild-type mice (P<0.05). Epidermal nerve density of Ephrin-A2−/− animals at P1 was significantly increased compared with P19 (P<0.01) and adult mice (P<0.05). Epidermal nerve density of Ephrin-A5−/− mice at P19 was significantly increased compared with P1 (P<0.001) and adult Ephrin-A5−/− mice (P<0.001; Figure 2e–h).

Semmes-Weinstein monofilaments were used to measure sensory function (Supplementary Methods; Chaplan et al., 1994). Ephrin-A5−/− mice were significantly less sensitive compared with wild-type (P<0.01), Ephrin-A2−/− (P<0.01), and Ephrin-A2A5−/− (P=0.05) mice (Figure 2i). Mean values of 50% paw withdrawal threshold were wild type 0.135 g, Ephrin-A2−/− 0.148 g, Ephrin-A2A5−/− 0.216 g, and Ephrin-A5−/− 0.5 g. There was a trend toward loss of sensory function in A2A5−/− mice. This does not correlate directly with the density of the cutaneous innervation. However, the simultaneous loss of Ephrin-A2 may have an opposing effect to Ephrin-A5 loss and hence a trend but not significant decrease in sensory function in the double knockouts. Aβ nerve fibers predominantly conduct non-nociceptive sensation (Fang et al., 2005), suggesting Ephrin-A5 is important in the development of Aβ fibers. However, light touch sensory function of hairy skin is also detected by C-fiber low-threshold mechanoreceptor lanceolate endings (Roudaut et al., 2012). The neuronal staining focused only on interfollicular density in hairy skin, whereas the sen-
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Role of Ephrin-A2 and -A5 in Cutaneous Nerves

![Graphs and images showing nerve density](image-url)
sory function was tested on glabrous skin. This may in part underlie the limited correlation between nerve density and sensory function.

Wild-type dermal innervation density was stable between P1 and adulthood, consistent with previous reports (Peters et al., 2002), in contrast to Ephrin-A2/C0 mice. Dermal nerve density of Ephrin-A2/C0 mice was significantly lower compared with wild type at P19. Ephrin-A2A5/C0 mice showed a significant increase in dermal nerve density compared with wild-type mice at P19. Both Ephrin-A2A5/C0 and Ephrin-A5/C0 mice showed a significant increase in the dermal nerve density compared with Ephrin-A2/C0 mice at P19. These data suggest that the Ephrin-A5 ligand acts as an inhibitor of dermal innervation, consistent with previous findings (Murioz et al., 2005) and that A2 and A5 have opposing effects on the development of cutaneous nerves, similar to their effects in the auditory brain stem (Yates et al., 2014). No significant differences were observed at P19 in hair follicle cycling or density (data not shown), suggesting that the changes observed are not linked to changes in hair follicles at this time point.

The changes observed in innervation after P1 suggest that the loss of Ephrin expression changes the timing of nerve development, which is usually established by P1 (Peters et al., 2002). Nevertheless, no significant difference in dermal nerve density in adult mice

Figure 1. Interfollicular cutaneous innervation of Ephrin-A2/C0 and wild-type mice. (a–c) Interfollicular dermal nerve density in wild type, Ephrin-A2/C0, and Ephrin-A2A5/C0 genotypes at P1 (a), P19 (b), and in adults (c). (d–f) Interfollicular epidermal nerve density in wild type, Ephrin-A2/C0, Ephrin-A5/C0, Ephrin-A2A5/C0 genotypes at P1, P19, and adult stages. *P<0.05, **P<0.01, and ***P<0.001. Hair follicles are marked with arrowheads. Scale bar = 50 μm.

Figure 2. Interfollicular dermal and epidermal nerve density over time and sensory function in Ephrin-A2/C0 and wild-type mice. (a–d) Interfollicular dermal nerve densities of wild type (a), Ephrin-A2/C0 (b), Ephrin-A5/C0 (c), Ephrin-A2A5/C0 (d), genotypes across P1, P19, and adult stages. (e–h) Interfollicular epidermal nerve densities of wild type (e), Ephrin-A2/C0 (f), Ephrin-A5/C0 (g), Ephrin-A2A5/C0 (h), genotypes across P1, P19, and adult stages. *P<0.05, **P<0.01, and ***P<0.001. The touch sensitivity of Ephrin-A5/C0 mice was significantly increased for 50% touch sensitivity threshold. *P<0.05 and **P<0.01. Ephrin-A5/C0 mice were significantly less sensitive compared with wild-type (P=0.002), Ephrin-A2/C0 (P=0.003), and Ephrin-A2A5/C0 (P=0.024) mice.

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was observed, most likely reflecting functional redundancy (Klein, 2001).

In the epidermis, wild-type innervation peaks at P19 followed by a significant reduction in epidermal innervation in the adult, similar to previous studies (Schotzinger and Landis, 1990). However, we found decreased epidermal innervation in Ephrin-A2/−/− mice at P19, which returned to normal in adults. In contrast, Ephrin-A5/−/− mice showed significantly reduced innervation at P1, which returned to wild-type levels by P19. This suggests that the two Ephrins act at different time points, with the effects overcome by adulthood, most likely due to redundancy. Finally, Ephrin-A2A5/−/− mice retain significantly lower levels of innervation from P19 through to adulthood, suggesting that the loss of both Ephrin ligands has an impact on epidermal innervation that cannot be compensated.

These data strongly suggest that Ephrin-A2 and -A5 ligands are important for the development of cutaneous nerves and have different effects on innervation and function.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary Material is linked to the online version of the paper at http://www.nature.com/jid

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