Investigating the epidermal Notch activation during wound healing and the consequences of prolonged Notch activity during skin wound healing

SOULMAZ BOROUMAND

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Soulmaz Boroumand

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Declaration

I declare that this thesis is my own work and that I have correctly acknowledged the work of others. This submission is in accordance with University and school on good academic conduct.

I certify that no part of the material offered has been previously submitted by me for a degree or other qualification in this or any other university.

Soulmaz Boroumand

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List of abbreviation

4OHT	4-hydroxytamoxifen
AKN	Ankyrin repeat
BM	Basement membrane
cDC	Conventional dendritic cell
CR	Cysteine rich domain
DC	Dendritic cell
DETC	Dendritic epidermal T cells
DSL	Delta-Serrate-Lag2 region
ECD	Extracellular domain
ECM	Extracellular matrix
EGF	Epidermal growth factor
GSI	γ-secretase inhibitor
Hh	Hedgehog
Hes	Hairy/enhancer of split
HF	Hair follicles
ICDs	Intracellular domains
IFE	Interfollicular epidermis
IFN	Interferon
IL	Interleukin
ILCs	Innate lymphoid cells
IRS	Inner rooter sheath
K14	Keratin 14
KGF	Keratinocyte growth factor
LNRs	Lin12-Notch repeats
MAML	Mastermind-like
MIP	Macrophage inflammatory protein
MMPs	Matrix metalloproteases
NEXT	Notch EXternacellular Truncation
NICD	Notch intracellular domain
NK	Natural killer cells

NLS	Nuclear localization signals
ORS	Outer rooter sheath
PDGF	Platelet-derived growth factors
PEST	Domain rich in proline, aspartic acid, serine and threonine residues
RAM	RBP-Jκ associated molecule
RBP-Jκ	Recombination signal sequence binding protein Jκ
SG	Sebaceous gland
TA	Transit amplifying cells
TGFβ	Transforming growth factor β
Th	T helper cells
TMIC	Trasmembrane and Intercellular domain
TNFα	Tumor necrosis factor-α

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Abstract

The Notch signaling pathway is critically involved in cell fate decisions during skin development and homeostasis. In the present study an *in vivo* model (seven-week-old male, C57BL/6) was used to elucidate the role of epidermal Notch activation in the healing of full-thickness dermal wound. Immunolocalization of activated Notch1, Notch2 and Jagged1 was performed during skin wound healing *in vivo* in mice. The expression level of Notch1, Notch 2 and Jagged1 in the C57BL/6 skin was examined by quantitative PCR (qPCR). This study shows that skin injury rapidly actives Notch signaling in the epidermis. Epidermal forced Notch1 activation results in a Jagged1 dependent Notch2 activation in epidermis. Data presented in this thesis also demonstrate that the prolonged epidermal Notch activation via a 4-hydroxy-tamoxifen-inducible transgene before and after wounding caused an over-activation of Notch during early stages of the healing of full-thickness dermal wounds. The expression level of Notch1, Notch2 and the inflammatory Notch related genes, in the transgenic wounded skin was examined by quantitative PCR (qPCR). The phenotypes and morphology of the transgenic skin were compared with that of wild type (WT) controls. The skin response to wound healing was studied by H&E staining at the microscopic level at 2, 5 and 8 days post injury. These data show that the prolonged epidermal Notch activity may do more harm than good in terms of an increased inflammatory response at the wound site. These data suggest that Notch plays an important role in the early stages of the skin wound healing process, a finding that has implications in wound inflammatory responses. This thesis also examines skin wound healing in different anatomical locations on seven-week-old male, C57BL/6 mice model. 4mm full thickness dermal wounds were made at different anatomic regions; upper posterior, middle to posterior and posterior-most (caudal). These data showed that lower body wounds healed significantly better than those in the upper posterior, or middle to posterior of the mouse back skin. The data presented here show that anatomical location is important in wound healing responses, as reflected by differences in keratinocyte proliferation and differentiation.

1 Introduction

1.1 The Skin

The skin is the largest organ of the human body; it weighs approximately 15% of an adult's body weight. As well, the skin is in a direct contact with the outside environment, and helps to maintain four essential physical functions. These functions are retention of moisture and prevention of loss of other molecules, regulation of body temperature, sensation and protection of the body from microbes and harmful external threats. During the interaction between the organism and the environment, the skin is at risk of exposure to injury from a variety of agents. In addition to injury through trauma or disease processes, skin may be wounded as a necessary part of surgical and other invasive medical procedures. Fortunately, the skin has a great capacity for healing (Matoltsy and Odland, 1955, Slater and Goldsmith, 1993, Tobin, 2006). Therefore, a basic understanding of cutaneous biology and functions of normal skin is essential for studying the wound healing response in the skin.

1.2 Skin Structure

The skin is a dynamic organ composed of three primary layers, an outer epidermis and an inner dermis, with an underlying layer known as the hypodermis. All functional properties of the skin are a reflection of the structural features of the skin (Matoltsy and Odland, 1955, Slater and Goldsmith, 1993, Tobin, 2006). The diagram in Figure 1.1 provides an overview of the basic anatomic features of the skin.

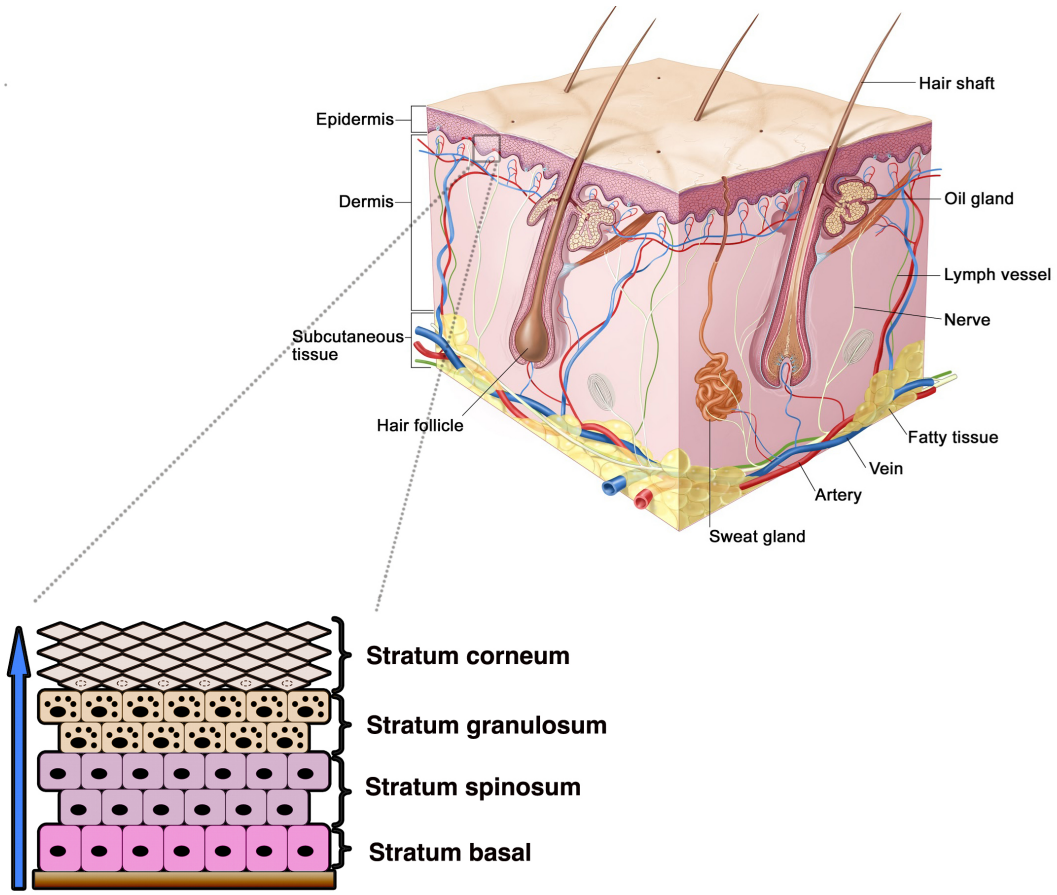


Figure 1.1 Diagram of human skin structure.

Representing the two main layers of skin epidermis and dermis. The epidermal barrier layer is attached to the underlying dermis by basement membrane zone Image adapted, from MacNeil, 2007.

1.3 Epidermis

The epidermis is the outer most layer of the skin; the predominant cell type in the epidermis is the keratinocyte that is committed to a program of terminal differentiation. Figure 1.2 illustrates the epidermal layers, which, from the bottom to the top are basal, spinous, granular and cornified layers. A continuous cell transition from the basal layer of the epidermis, entering the spinous layer first and then the granular layer and finally forming the uppermost keratinized layer of the epidermis is principle to the renewal process of the epidermis that takes 3 to 6 weeks (Grove and Kligman, 1983, Slater and Goldsmith, 1993, Blanpain and Fuchs, 2006).Keratinocytes proliferate and divide in the epidermal basal layer and traverse to the upper layers; during terminal differentiation, the keratinocyte produces intercellular keratins and lipid-filled granules, although its cell nucleus becomes inactive and condensed. The restoration of damaged epidermis is brought about by regeneration. The cells of the basal layer multiply and migrate from undamaged areas to replace damaged cells.

1.3.1 Basal layer

The basal layer is the innermost layer of the epidermis, consisting of basal cells including the epidermal stem cell subpopulation. Basal cells vary in shape from cubic to columnar. The basal cells continually divide, producing new cells that constantly push older ones up toward the surface of the skin, where they are eventually shed. Also, the basal layer is maintained by cell mitosis, although once a cell exits the basal layer, it is committed to the differentiation pathway and is no longer capable of cell division. The basal keratinocyte is the primary cell involved in the epidermal wound healing response (Slater and Goldsmith, 1993). Melanocytes constitute a small quantity of the basal cells that produce pigment to provide protection against ultraviolet radiation; other cells residing in the epidermis are markel cells, a neuroendocrine cell, which consists of a small proportion of the basal cell population. These cells are in an intimate engagement with cutaneous nerves and are involved in light touch sensation (Tobin, 2006) (Figure1.2).

1.3.2 Spinous layer

Reproduction, maturation and migration of the basal cells toward the outer layer of skin usually result in the formation of the spinous layer that is adjacent to the basal cells. Suprabasal cells are polygonal in the lower layer and somewhat flattened in the upper layers. They are larger than basal cells and contain a small amount of chromatin in their circular nucleus. Intercellular bridges that are made of desmosomes provide the "prickles" appearance to the cell population within this epidermal layer (Figure1.2). Other cells residing in the spinous layer are the Langerhans cells that are a type of dendritic immunologically active cell; these cells act as antigen–presenting cells in skin immune reactions (Slater and Goldsmith, 1993, Tanaka, 2012, Toebak et al., 2009).

1.3.3 Granular cell layer

Throughout the transition of the basal cells to the skin surface, the cells continue to be structurally flattened, losing their nuclei and their cytoplasm appears granular at this level (Figure1.2). The cells and nuclei in the granular cell layers are even flatter than those in the suprabasal layer (Slater and Goldsmith, 1993, Fuchs, 2008, Fuchs and Horsley, 2008).

1.3.4 Cornified layer

Non-viable cornified cells are flat hexagonal-shaped and found in the outer layer of epidermis which is the final outcome of the keratinocytes' maturation process, and their cytoplasm is filled with aggregated keratin fibers (Fuchs, 2008, McLafferty et al., 2012). These cells are systematically shed or sloughed from the surface of the skin. The dead cells of the cornified layer are composed of cell membranes, keratin proteins, and complex lipids, which form the major barrier against water loss and microbial or chemical invasion (Slater and Goldsmith, 1993) (Figure1.2).

1.3.5 Epidermal T-cells

There are several other cells residing in the epidermis which play key roles during skin wound healing and they are the dendritic epithelial T cells (DETC), a specialized

subset of T cells that express the γδ-T-cell receptor (Allison and Havran, 1991), while activated DETCs produce keratinocyte growth factors and chemokines, raising the prospect that DETCs are critical in wound repair cascade. Previous studies have shown a reduction in keratinocyte proliferation and tissue reepithelialization during the healing of experimental wounds in mouse skin lacking DETCs (Jameson et al., 2002). Another study in mice also has proposed that the recognition of expressed antigens from injured keratinocytes by DETCs induce the production of factors that directly affect healing (Born and O'Brien, 2002).

1.3.6 Epidermal appendages

Epidermal appendages including the hair follicle, sebaceous gland and sweat glands are specialized structures that arise from the epidermis during a defined stage of embryonic development by means of dermal-epidermal induction (Mackool et al., 1998). It has been reported that epidermal stem cells, located in the bulge of the hair follicle, contribute to the renewal of basal keratinocytes and follicular epithelium (Alonso and Fuchs, 2003, Bickenbach and Kulesz-Martin, 2007).

1.4 Basement membrane

The cutaneous basement or basement membrane (BM) is the boundary between the epidermis and the dermis. A specific assemblage of the basal keratinocyte, extracellular matrix (ECM), basal lamina, anchoring filaments and fibrils form the specialized structural integrity of the BM region (Figure1.2). Most of the components of this region are synthesized by epithelial cells and must be reproduced during wound healing. The lack of a linkage network results in the separation of the epidermis from the dermis (Chetty et al., 1992, Fuchs, 2008, Fuchs and Horsley, 2008).

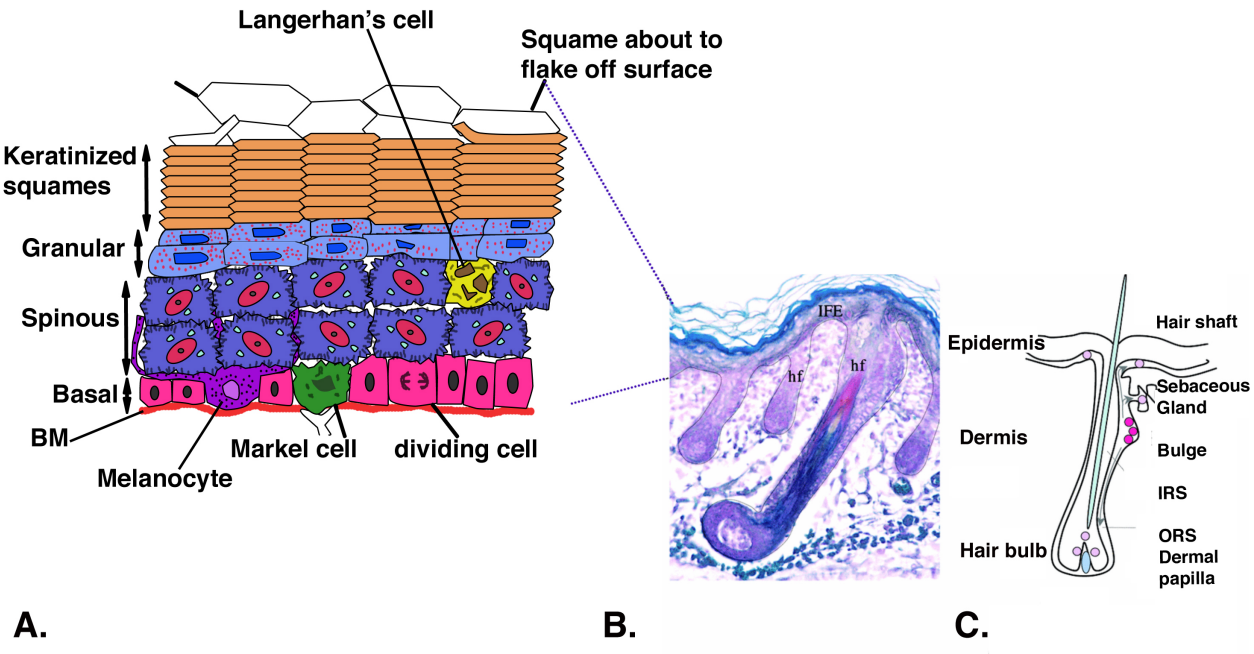


Figure 1.2 Epidermal layers and skin stem cells.

(A) , schematic representation of the stratified layers of the epidermis (basal, spinous, granular and cornified layers). The differentiated squames in the stratum corneum initiates by the differentiation of the mitotically active basal cells to spinous cells in the upper layer, which are transcriptionaly active. Subsequently the enucleated granular cells form in the granular layer. The proliferative basal layer is adjacent to the basement membrane (BM). Stem cells (SC) generate transit amplifying (TA) cells which will differentiate to form the stratified layers.(B), section of mouse skin stained to distinguish the different compartments of the hair follicle (hf).(C), schematic representation of a hair follicle and the multipotent SCs localizing to the bulge region. These cells migrate to populate the bulb region of the follicle, the sebaceous gland and the interfollicular epidermis (IFE; pink). IRS, inner root sheath; ORS, outer root sheath. Adapted from J.A.Serge, 2006.

1.5 Dermis

The dermis is the structural layer of the skin that is thicker than the epidermis and supports the epidermis. In addition, the dermis consists of different cell types including fibroblasts that are known as a source of collagen production, elastin and structural proteoglycans, mast cells and macrophages. Dermis is designed to keep its integrity under an assault of physical forces and delivers metabolic and communicative support for the epidermis. The dermal vascular network, afferent sensory nerves and nerve endings, run through the dermis and end just below the BM zone (see Figure 1.1). Epidermal appendages including sweat glands and hair follicles are also supported by dermis. Specialized smooth muscle of hair follicles, arrector pili muscle, is located within the dermal region. (McLafferty et al., 2012, Singer and Clark, 1999). It is important to note that the repair of damaged dermis is dependent on the level of injury. During the healing process of the skin full-thickness wound, the formation of granulation tissue (combination of the macrophages, new capillary blood vessels, collagen, fibroblasts, and plasma cells) within the dermis at the injured site is a crucial phase (Singer and Clark, 1999).

1.5.1 Extracellular matrix

Collagen type III and I are the predominant components of the dermis, forming cross-linked fibrils of high tensile strength, while collagen types VI and VII are a small but functionally critical component (Keene et al., 1997, Keene and McDonald, 1993). In normal skin, collagen is a highly stable molecule with a reasonably low turnover; but after injury, dermal fibroblasts synthetize collagen via a complex intracellular and extracellular biosynthetic pathway. The elastic properties of the skin are conferred by elastin fibers, a combination of elastin and other complex glycoproteins. Elastin is also a very stable molecule in normal skin condition but, unlike collagen, it is not readily synthetized following injury (Broughton et al., 2006b).

1.5.2 Connective tissue cells

In the normal dermis fibroblasts are the principle cell type, and other dermal residing cells including mast cells and tissue macrophages are minor components (Slater and Goldsmith, 1993). As mentioned before skin is an immunologically active organ composed of B and T lymphocytes (Streilein, 1983). During the inflammatory process, including the wound healing, neutrophils and macrophages rapidly enter the connective tissue to participate in the wound healing response (Kanzler et al., 1986).

1.6 Hypodermis

Underneath the dermis is a thick layer of sub-cutaneous tissue; this is the connection point between the skeleton and skin (see Figure 1). This soft tissue is a type of connective tissue composed of fat. This layer provides a cushion and separates the dermis from underlying muscle and bone. Without the sub-cutaneous tissue skin would slide off the muscles (Slater and Goldsmith, 1993). Previous studies indicated that mesenchymal stem cells reside in the hypodermis and may contribute to dermal wound healing. Under normal conditions this type of skin cells do not readily undergo mitosis, and following injury the hypodermis is not regenerated but is replaced by scar tissue (De Ugarte et al., 2003).

1.7 Epidermal stem cells

Dermis is originated of embryonic mesoderm and is separated from the overlying ectodermal originated epidermis by basement membrane. During embryonic development under the stratification process, the outward movement of the proliferative basal cells towards the surface of the skin creates stratified squamous epithelium. The stratification process is repeated during the life of the organism and provides the protective and water barrier functions between the body and the environment (Kalinin et al., 2002). Such an accomplished continuous epidermis renewal process that directs the consequent production of the epidermal layer requires the involvement of the primary stem cells (SCs).

Previous studies have confirmed three locations of multipotent SCs within the adult epidermis: cells found in the bulge area of the hair follicle (Oshima et al., 2001,

Taylor et al., 2000), keratinocytes of interfollicular epidermis (IFE) and the sebaceous gland (Ghazizadeh and Taichman, 2001) (Figure 1.2.c). Reports have shown that the multipotent SCs within the bulge region of the hair follicle are the main source of epidermal SCs that replenish hair, IFE and sebaceous lineages (Cotsarelis et al., 1999, Fuchs and Raghavan, 2002, Watt, 2001).

Several lines of evidence indicate that there are sub-population cell types within the basal layer of IFE that are able to self-renew, undergo terminal differentiation and give rise to non-SCs known as transit amplifying cells (TA) (Figure 1.2). Limited division and proliferation capacity have been considered as indicators for TA cells (Barrandon and Green, 1987, Jones and Watt, 1993, Hunt et al., 2000, Tanaka, 2012).

Specification of epithelial stem cells in the skin during development enables them to form the three main epithelial lineages including epidermis, hair follicle and sebaceous gland. Each of these lineages has the ability to renew and maintain their own stem cell population. In response to injury due to the damage of stem cells at the injured site of the skin, resident stem cells from other sites within the epithelium have the capability of migration to the injured site and contribute to the repair of the epithelial tissue (Tanaka, 2012).

1.8 Wound healing

Wound healing is a complex process in which the injured tissue self-repairs. Different chronological phases of the healing process include: homeostasis, inflammation, proliferation and remodeling (Figure 1.3). The primary objective of the hemostasis phase of wound healing is to control bleeding. While the inflammatory phase is characterized by a host of cells that work together to mount an inflammatory response, synthesize granulation tissue, and restore the epithelial layer. The objective of the inflammatory phase of wound healing is to clean the wound of cellular debris. Accumulation of macrophages encourages fibroblast mitosis and migration into the wound. This phase is known as the proliferation phase of wound healing. At the final stage of wound healing collagen is remodeled and realigned under a process that is termed maturation and remodeling (Broughton et al., 2006b, Gantwerker and Hom, 2012).

The cellular events that take place during the wound healing are: infiltration of neutrophils and inflammatory components within the early days of healing (1-2 days);

migration of epithelial-mesenchymal cells to initiate re-epithelialization (within 1-2 or 3 days); migration of fibroblasts and myofibroblasts differentiation to the wound site to be involved in progressive alignment, collagen production and matrix contraction (between 4 and 15 days after wounding); increased angiogenesis (formation of new blood vessels) occurs within 4 to 5 days after wounding, as does the production of dermal matrix, which can last for almost 14 days. Two weeks after wounding the remodeling stage starts, which can last several weeks to months, depending on wound size (Figure 1.3).

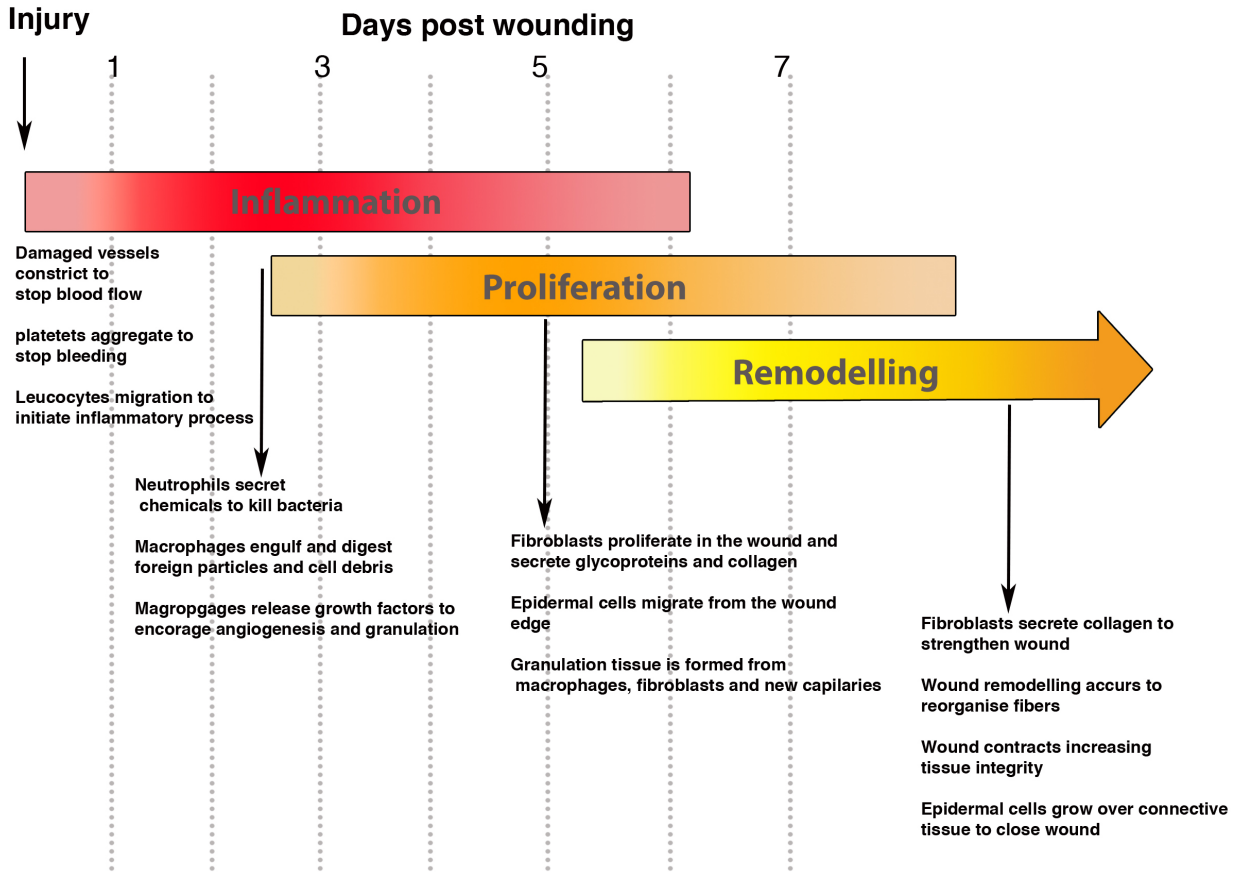


Figure 1.3. Wound healing phases.

Hemostasis immediate after wounding, inflammation 1-3 days post wounding, proliferation 3-5 days and remodeling from day 7 up to weeks post wounding (adapted from (Hunt et al., 2000, Tanaka, 2012).

1.8.1 Hemostasis

Hemostasis occurs right after the initial injury to approximately 3 hours post injury. At the time of injury epinephrine, a chemical that constricts peripheral blood vessels, is released in an attempt to minimize bleeding into the soft tissues. The key cell, which is responsible for this function, is the platelet, which causes the body to form a clot to prevent further bleeding. At this stage there is an increased aggregation of platelets to enable the wounded vessels to complete the clotting process. Platelets also release key cytokines such as platelet-derived growth factors (PDGF) that cause the accumulation of cells to participate in later phases of healing. Hence the objective of the hemostasis phase of wound healing is to control bleeding. (Certosimo et al., 1998, Gantwerker and Hom, 2012).

1.8.2 Inflammation

It is a well-known fact that inflammation is essential for the establishment of cutaneous and other tissues homeostasis, immediately following injury. The specific subsets of inflammatory cell lineages, and other inflammatory orchestrating networks involved with tissue repair including cytokines have been identified and examined in recent experimental research. Therefore, a more specified investigation into mechanisms controlling the inflammatory response during repair, and how inflammation controls the consequence of the healing process will help as an important milestone in the therapy of pathological tissue repair. Immediately after injury, the infiltrating leukocytes, principle cellular components of the inflammatory response, initiate the healing process. Infiltrating leukocytes are involved in stopping entering pathogens, tissue degradation and tissue formation. Previous studies have shown that reduction or induction of leukocytes infiltration into the damaged tissue may effect cell migration, proliferation, differentiation, and the quality of the healing response (Eming et al., 2007). It has been demonstrated in several experimental studies that inflammatory response during normal healing is characterized by changing patterns of various leukocyte subsets (Singer and Clark, 1999, Gantwerker and Hom, 2012); therefore the well-defined monitoring of these events is critical for optimal repair.

An extended inflammatory phase and any disruption in the decontamination process can lead to the continued elevation of pro-inflammatory and stretch the inflammatory

phase. Matrix metalloproteases (MMPs) accumulation is the result of elongated inflammatory phase that can degrade the ECM and delay wound healing (Edwards and Harding, 2004, Jones et al., 2004, Menke et al., 2007).

1.8.2.1 Polymorphonuclear leukocytes (neutrophils, PMN)

Immediately after injury, platelets and polymorphonuclear leukocytes (neutrophils, PMN) accumulated in the blood clot, release a wide range of chemoattractants for cells involved in the inflammatory phase (Szpaderska et al., 2003). Proinflammatory cytokines IL-1β, tumor necrosis factor-α (TNF-α), and IFN-γ at the wound site activate the endothelial cell wall of blood capillaries which leads to release of the bulk of neutrophils in the wound. A previous experimental research revealed that migration of PMNs to skin wounds induces a large transcriptional activation program, which may regulate cellular fate and function and promote wound healing (Theilgaard-Monch et al., 2004). The fact that neutrophils can be beneficial or detrimental to healing has been shown by depletion of neutrophils in wound healing assays since the 1970s. These studies have confirmed that although, dermal repair parameters were not affected by neutropenia, reepithelialization was significantly accelerated. Indirect regulation of the innate immune response during wound healing by neutrophils has been described in a recent *in vitro* study that demonstrated; neutrophils isolated from wound site can modulate the cytokines profile expression of macrophages (Daley et al., 2005). However, the fact remains unclear, whether the lack of PMNs has a direct beneficial effect on reepithelialization, or whether the relative increase in other subsets of inflammatory cells, such as the macrophages, might be responsible for accelerated epithelialization (Dovi et al., 2003).

1.8.2.2 Monocytes/Macrophages

Within 2 days after injury, the neutrophil infiltration at the wound site ends, and alongside resident macrophages, the major quantity of macrophages at the wound site is recruited from the blood. Platelets trapped in the fibrin clot, hyperproliferative keratinocytes at the wound edge, fibroblasts, and leukocytes, are introduced as the major source of growth factors, proinflammatory cytokines, and macrophage inflammatory protein 1-α(Mip-1α), that regulate the macrophage infiltration into the wound site (Werner and Grose, 2003). Activation and differentiation of monocytes

extravasate from the blood vessel; transform these cells into mature tissue macrophages that lead to significant changes in gene expression. Regulatory function of mediators present in the wound site, to control monocyte maturation procedure, has been confirmed previously (Gordon, 2003) (Figure 1.4).

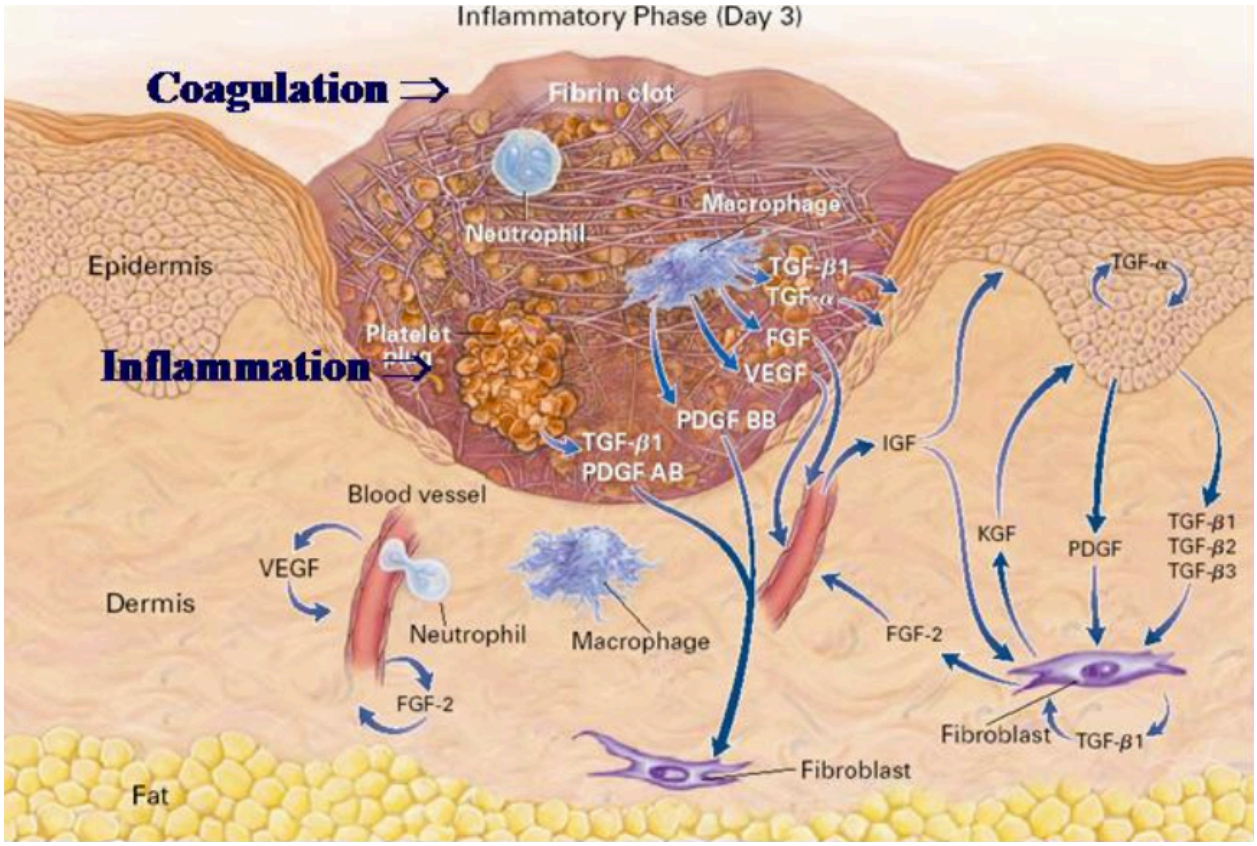


Figure1.4.Wound healing during Inflammation phase (3 days after injury).

Growth factors thought to be necessary for cell movement into the wound are shown. Adapted from (Singer 1999).

A fundamental role for macrophages in wound healing in guinea pigs was proven over 30 years ago, where depletion of macrophages showed a significant delay of healing (Leibovich and Ross, 1975). More recent studies not only have supported and extended these initial findings but also suggested more integral roles of macrophages in a successful outcome of the healing response. Immunological functions of

macrophages, as antigen-presenting cells and phagocytes, beside other functions including, synthesis of numerous potent growth factors, such as TGF-β, TGF-α, basic fibroblast growth factor, platelet-derived growth factor, and vascular endothelial growth factor, which promote cell proliferation and the synthesis of extracellular matrix molecules by resident skin cells during wound healing, has pronounced macrophages as strategic elements of skin wound healing (Figure 1.5) (DiPietro and Polverini, 1993).

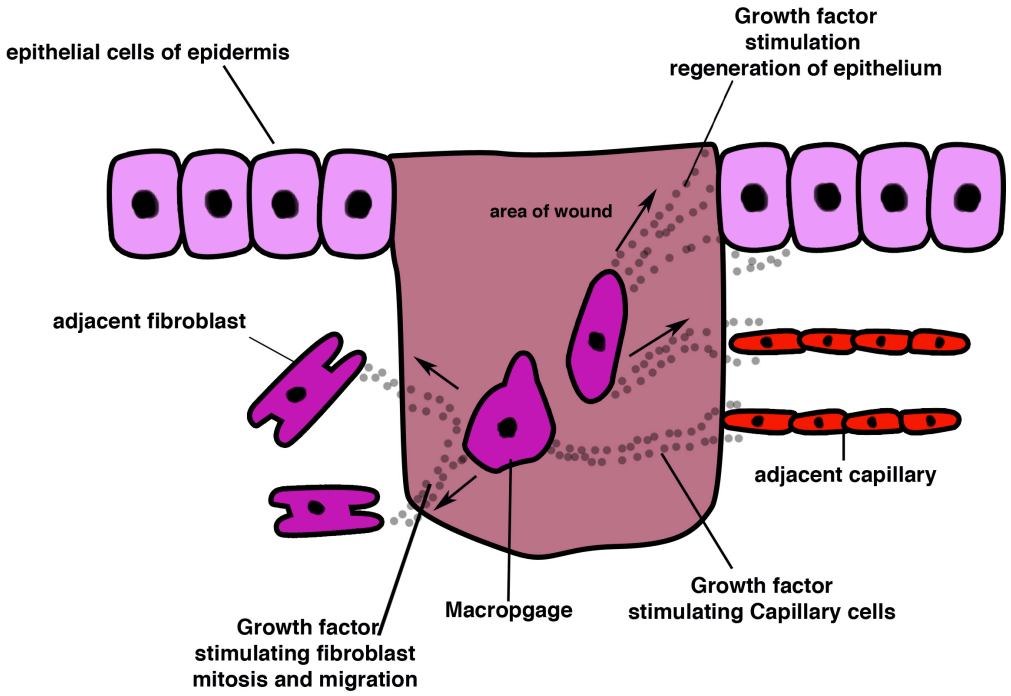


Figure 1.5.The production of bioactive mediators by macrophages.

Macrophages synthesize and release a vast array of regulatory molecules relevant to all the major tissues types involved in wound healing (adapted from DiPietro and Polverini, 1993).

1.8.2.3 T-cells

Cells of the adaptive immune response, in particular T cells, play a crucial role in wound healing (Fishel et al., 1987). However, a more definitive understanding of T-cell function during wound healing requires further investigation; a role for T-cells in tissue remodeling has been supported by previous studies. Secretion of cytokines by T-cells may also suggest the association of T-cells and remodeling (Azouz et al., 2004). Macrophages appear to be a major source for these cytokines. Using IFN-γ-deficient mice, in a wound healing assay demonstrated, significant reduction in the number of neutrophils, macrophages and T-cells at the wound site. These findings proved that there was a role for T-cells in tissue remodeling and, suggested a delayed wound closure and PMNs reduction occurred, as a consequence of the lack of the endothelial activation.

A previously discovered unique population of T-cells, termed γδ T cells, are also reported to be effective in epidermal repair in skin (Jameson et al., 2002). It has been confirmed that γδ T cells play a critical role in keratinocyte proliferation and differentiation during wound healing as they have been identified as a source of key growth factors such as fibroblast growthfactor-7, -10 (Jameson et al., 2002). It has also been shown that this population of T-cells are involved in macrophage infiltration into the wound site (Jameson et al., 2005). Secreting different cytokines profile from T-cell subsets including, Th1 and Th2, may propose different regulatory functions for these T-cells (Azouz et al., 2004). Releasing of IFN-γ, TNF-α and IL-2 are most likely to be regulated by Th1, whereas, Th2 release IL-4, 5and 10. The demonstrated novel functions of T-cells during the healing process may deliver a new perspective on T-cell regulation in wound healing.

1.8.3 Proliferation

One of the first cells that are attracted into the wound, as a result of the coordinating cytokine growth factor releasing activity of the macrophages, are fibroblasts. The cytokines released from macrophages stimulate the fibroblast, which then encourages the fibroblast mitosis and migration into the wound. The proliferation phase involves the migration of fibroblasts and beneficial cells and also the destruction of dead materials, the bacteria and cell debris at the wound site (Hunt et al., 1999).

The fibroblasts in the wound produce collagen that causes a significant tensile strength of the wound. As well as collagen, the fibroblast also produces a glycoprotein called Extra Cellular Matrix (ECM) that fills up the gaps between the cells and the collagen strands (Figure 1.6). Therefore, the activation of fibroblast causes the wound to be filled with matrix and also collagen strands have held wound edges together. Fibroblasts are identified as a key factor for secretion of growth factors that attract epithelial cells to the wound site (Table 1.1).

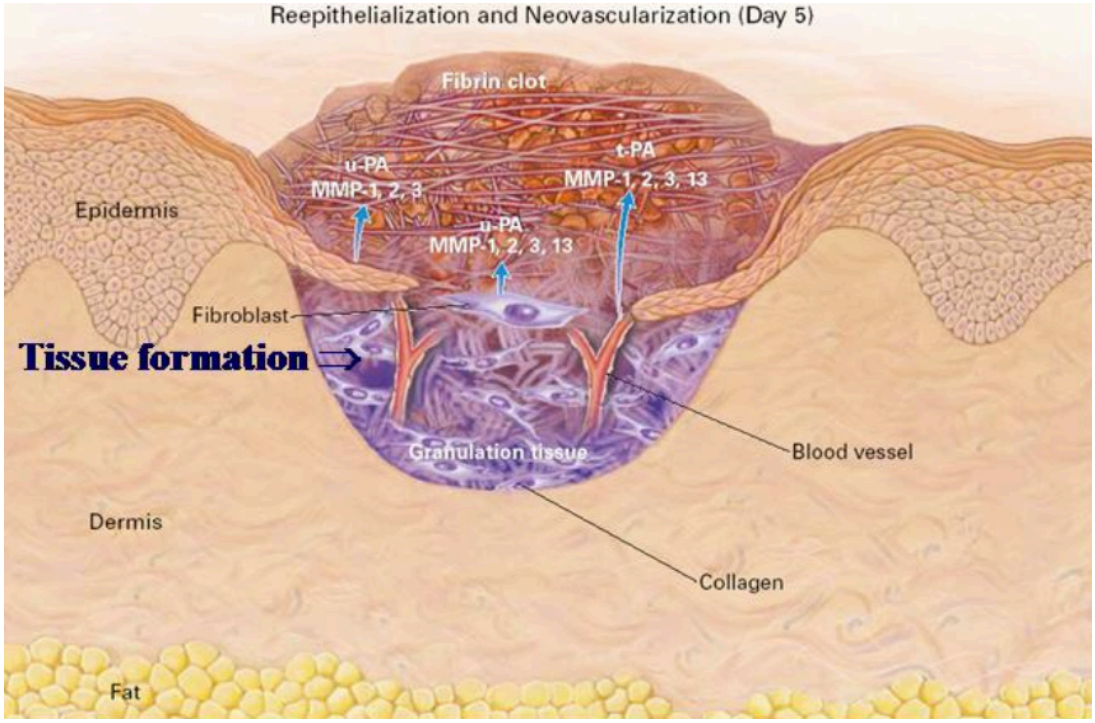


Figure 1.6. Wound healing during Proliferation phase (5 days after injury).

Blood vessels are seen sprouting into the fibrin clot as epidermal cells resurface the wound. Proteinases thought to be necessary for cell movement are shown. MMP-1, 2,3 and 13: matrix metalloproteinases 1,2,3 and 13(collagenase 1, gelatinase A, stromelysin 1, and collagenase 3, respectively). Adapted from Singer, 1999.

1.8.3.1 Angiogenesis and granulation

As previously mentioned, monocyte-derived macrophages are located around the wounded tissue and also into the wound site. Growth factor produced by the macrophages, affects the adjacent capillaries and causes the growth of capillaries into the wound. The growth of new blood vessels into the wound that is fundamental to

supply the wound with oxygen and nutrients is known as angioneogenesis. Regeneration of the epithelium is also stimulated by macrophage-derived growth factor. Two days after the injury, the bed of the wound will be infiltrated by helper T lymphocytes known as T-Cells. Secretion of cytokines from helper T cells causes the accumulation of more T-cells, which results in the increase of macrophages, and helps the angiogenesis process.

The combination of the macrophages, the new capillary blood vessels, the collagen the ECM, the fibroblast and the plasma cell is called the granulation tissue. Granulation tissue is the essential intermediate tissue, produced by the body to facilitate wound healing (Hunt et al., 2000, Quinn and Wells, 1998).

Growth factor	**Abbreviation**	**Main origins**	**Effects**
Epidermal Growth Factor	EGF	Activated macrophages Salivary glands Keratinocytes	Keratinocyte and fibroblast mitogen Keratinocyte migration Granulation tissue formation
Transforming growth factor-α	TGF- α	Activated macrophages T-lymphocytes Keratinocytes	Hepatocyte and epithelial cell proliferation Expression of antimicrobial peptides Expression of chemotactic cytokines
Transforming growth factor-β	TGF-β	Platelets T-lymphocytes Macrophages Endothelial cells Keratinocytes	Granulocyte, macrophage, lymphocyte, fibroblast and smooth muscle cell chemotaxis

		Smooth muscle cells Fibroblasts	Angiogenesis Fibroplasia Matrix metalloproteinase production inhibition Keratinocyte proliferation
Keratinocyte growth factor	KGF	Keratinocytes	Keratinocyte migration, proliferation and differentiation

Table1. 1Growth factors involve in wound healing (Hunt et al., 2000, Quinn and Wells, 1998).

Wounds can heal in one of two ways; they can heal by regeneration or fibrosis. In a good wound healing environment there is more regeneration because there is also more mitosis and migration of the original cells which are going to replace the damaged cells. In the case of fibrosis the accumulation of more collagen based fibers tissue results in scar tissue at the wound site. Regeneration and fibrosis in bigger wounds are combined and that is one of the reasons that larger wounds result in scar formation. Due to the regeneration as time goes on the wound gets smaller. The epithelial cells from the surface of the skin need to migrate in a moist environment underneath the blood clot and the epithelial cells migrate over the surface of the healthy living granulation tissue and re-epithelialiase the wound (Hunt et al., 1999).

1.8.4 Remodeling

The final stage of healing is called remodeling or the maturation phase. Over time the scar gets faint or smaller. Collagen fibers get organized and re-formed and that increases the tensile strength of the wound. Reduction in vascularity, re-ordering of the collagen, differentiation of the fibroblast in the wound from the being individual fibroblasts into being strips of fibroblasts (myofibroblasts) are all indicators for this phase of healing process (Gomez et al., 1991). Myofibroblasts are attached to the

edges of the wound and contract and reduce the size of the wound, which can end up with a shortened scar. At the end of the wound healing phase, collagen is remodeled and realigned under a process which is characterized as maturation and remodeling (Midwood et al., 2004). The myofibroblast is a cell that generates *in vivo* essential forces for wound contraction. Structural and features, such as being contractile and synthetic, have made myofibroblasts suitable to promote granulation tissue contraction and exerting fractional forces on surrounding connective tissue during wound healing. During wound contraction myofibroblasts establish a grip on the edges of the wound and under a contraction mechanism promote the closure of the wound (Midwood et al., 2004).

1.9 Skin Wound healing

An injured skin with the broken protective barrier undergoes the normal physiologic process of wound healing which initiates immediately after receiving an injury. The response of cells from different layers of the skin to injury is related to their capacity of basic cellular processes including, cell movement, cell replication and restoration of specialized function. At the injured site of the skin the barrier is compromised, cells and ECM are damaged or lost, the blood supply is disrupted and there is at least a partial loss of function. The coordinated sequence of events in response to skin injury involving **migration** (cell movement), **mitosis** (cell proliferation) and **maturation** (restoration of specialized function) is summarized in the flow diagram in Figure 1.7.

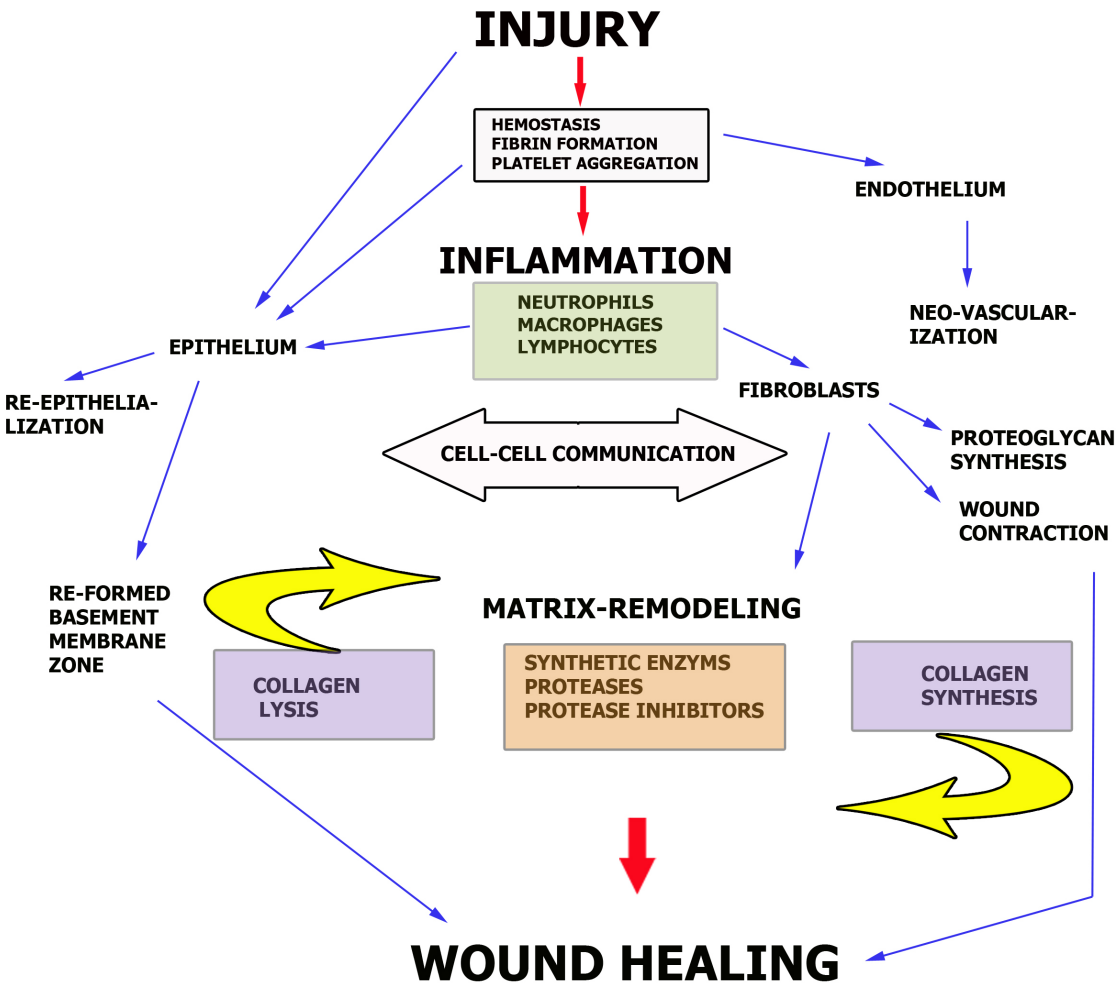


Figure 1.7. The orchestrated sequence of events in response to skin injury.

(Adapted from Singer, 1999)

1.9.1 Epidermal wound healing

The high regenerative capacity of basal keratinocytes is achieved by a regulated sequence of migration, mitosis and maturation of these cells (Figure 1.8).

Cytokines, growth factors, and other wound signals acting on basal keratinocytes at the site of injury are known as essential key factors in epidermal wound healing .To initiate the healing and achieve the normal epidermal structure and barrier function of the epidermis, epidermal cells in close proximity to the wound margins undergo structural and functional changes to facilitate motility while retaining cell-cell association to move as an epithelial sheet (Clark, 1993). During the healing process, proliferation of epidermal keratinocytes, increase the cell mass and replace tissue volume at the injured site; and following the epithelialization complete resurfacing of the wound will be achieved. Finally, the terminal differentiation of the cells in the re-epithelialized wound reestablishes the normal epidermal structure and barrier function. All these coordinated degradation and regeneration processes during the healing cascade require a delicate balance of biochemical signaling, which is mediated by local release of growth factors and cytokines (Hebda et al., 1990).

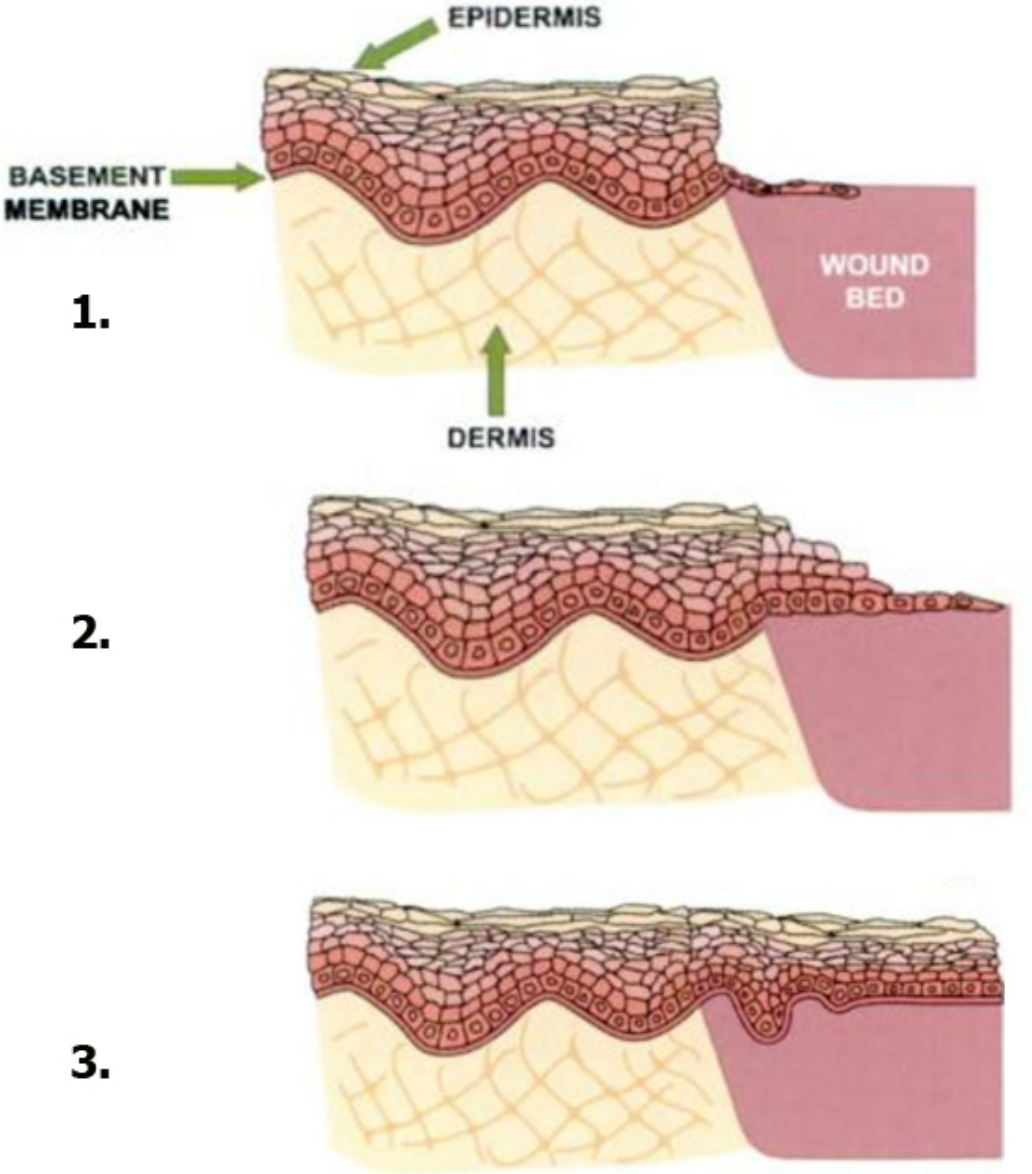


Figure1.8.Epidermal wound healing.

Injury to the epidermal layer induces epidermal keratinocytes to undergo a process of migration (1), mitosis (2) and maturation (3) to reconstitute the epidermis and restore barrier function. At the wound edges, these keratinocytes form the socalled hyperproliferative epithelium, which strongly proliferates and migrates to replenish the wounded area with new tissue. Cells from the hyperproliferative epithelium overtime displace the fibrin clot. Adapted from (Singer, 1999).

1.9.2 Dermal wound healing

Dermal injury occurs when the injury crosses the BM, and causes damage to blood vessels is the dermis. Preventing the blood flow by released platelet and formation of a fibrin clot is described as platelet-induced hemostasis; the fibrin clot is the first provisional matrix for wound healing that forms at the site of the injury. Additional agents released from platelets and mast cells produce inflammation and edema at the injured site (Clark, 1993, Singer and Clark, 1999). Chemokines attract neutrophils to the site during the early inflammatory phase of healing; neutrophils phagocytose bacteria. Circulating monocytes are also attracted to the site by chemokines. Monocytes rapidly activated to developed macrophages and phagocytose` any remaining contamination, as well as tissue debris. Macrophages are involved in releasing growth factors that serves as important signals for and mediators of wound healing (Leibovich and Ross, 1975, Martin and Leibovich, 2005). Inflammatory response induces the formation of granulation tissue; this loose connective tissue is the second provisional matrix forms in wound healing and is named for its granular appearance. Granulation tissue forms the scab (the dried, compacted fibrin clot) that is the third final matrix of dermal repair and maintains the collagen deposition (Singer and Clark, 1999).

1.9.3 Connective tissue repair

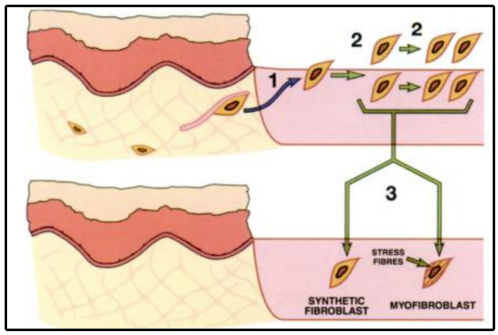
Establishment of granulation tissue in a loose connective tissue provides a nutrient-rich environment for fibroblast proliferation and collagen synthesis. Replacement of granulation tissue with a collagenous matrix forms the mature connective tissue of the healed wound. Although, the newly formed matrix is functionally sufficient, the original structural organization and function are not perfectly restored.

Following dermal injury, key factors for collagen remodeling including the collagen production by fibroblasts and matrix metalloproteinases (MMPs) activity up-regulate; however the maximal of tensile strength achieved after healing is lower than in

uninjured skin (Lorenz et al., 1993). In the case of dermal injury, normally resting dermal fibroblasts become activated and chemotactic signals direct the migration of this cell population into the wound. At the wound bed, following proliferation some of the fibroblasts recommit to the collagen synthesis and other ECM molecules, although other fibroblasts morphologically change to become myofibroblasts and contribute to contraction and remodeling of the healing wound as it matures (Figure1. 9(A) (Darby et al., 1990, Lorenz et al., 1993).

The skin injury disturbs the blood vessels within the dermis. As the viability and metabolism of all tissues, including skin, depend on sufficient blood supply, any disruption to this part of the skin must be restored as part of the healing process. Endothelial cells migrate through weak spots in the BM in response to chemotactic agents, at the vessel margins. Endothelial cells proliferate in response to released growth factors in the wound bed (Figure 1.9 (B). Finally, the remodeling of blood vessels takes place in coordination with the decreasing demands of the wound as it matures (Clark, 1993, Hunt et al., 2000).

A.



1. Fibroblasts into the wound
2. Synthesizing collagens and other ECM molecules
3. Fibroblasts become two distinct types

B.

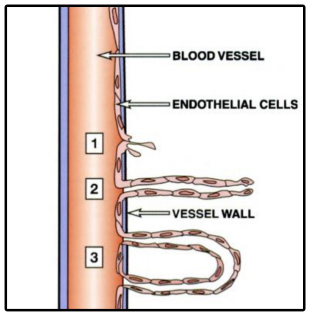


Figure1.9. Dermal wound healing.

A) Injury to dermis induces an inflammatory response that triggers fibroblasts in the adjacent tissue to undergo a process of migration, mitosis and maturation to repair the dermal connective tissue and restore structural integrity. B. Wound angiogenesis. Injury of the connective tissue includes damage of the vascular supply. The resulting inflammatory response induces endothelial cells in vessels in the wound margins to undergo a process of migration (1), mitosis (2), and maturation to bring vessels and a blood supply into the wound bed (Darby, Skalli, & Gabbiani, 1990; Hunt, Hopf, & Hussain, 2000).

1.10 Factors that effect wound healing

The immediate goal in repair is to achieve tissue integrity and homeostasis (Singer and Clark, 1999). Three phases overlap in time during the healing process, involving inflammation, tissue formation, and tissue remodeling, to achieve a perfect repair. infiltration of leukocytes into the wound site at the initial stages of healing, epithelialization and newly formed granulation tissue and conclusive deposition of structural extracellular matrix molecules are indispensable for initiating repair and progression into the healing state. Direct cellular interaction, as well as the indirect crosstalk between different cell populations, by soluble mediators, acts as essential cellular responses to injury. During skin wound healing, an organized communication between the epidermal and dermal compartment is critical. Numerous factors have been recognized that are involved in a complex communication between epidermal and dermal cells to facilitate wound repair. The balance between stimulating and inhibitory mediators during different stages of repair is necessary in achieving tissue homeostasis following injury (Werner and Grose, 2003).

Impaired healing of wounds can be induced by numerous factors both local and systemic. Local factors include the presence of foreign particles or microorganisms, ischemia, pressure and infection whereas systemic factors comprise age, vascular insufficiencies, immune suppressive medication and underlying conditions such as diabetes mellitus(Hess and Kirsner, 2003, Nzietchueng et al., 2002). Identification of a delayed wound healing and managing wounds of all types, may be achievable with an understanding of the various essential factors such as anatomy and physiology of the skin, different stages of the wound repair progression and the type of wound.

1.10.1 Enhanced inflammatory responses

Although pro-inflammatory responses are essential for wound healing, they become detrimental in wounds where inflammation persists. In the cases where bacteria cannot be eliminated, leucocytes in the wound continue to produce pro-inflammatory mediators. Consequently the influx of new leucocytes, such as neutrophils, monocytes and macrophages increases (Wetzler et al., 2000). This leads to excessive pro-

inflammatory responses in these wounds, which attract even more cells that also produce pro-inflammatory cytokines(Hirano et al., 2007). Phagocytes are activated to release proteolytic enzymes and also to produce large amounts of reactive oxygen species (ROS)(Gauss et al., 2007) as a consequence of pro-inflammatory cytokines and/or bacterial products present in the wound. In agreement with this, chronic wounds are associated with elevated expression of pro-inflammatory cytokines, such as TNF-α, compared to normal healing wounds(Goren et al., 2003); the levels of these cytokines decrease when the wound begins to heal.

1.10.2 Altered growth factor production and/or signaling

Chronic wounds may differ in the levels of growth factors and/or in the cellular responses to these factors from normal healing wounds. It has been reported that growth factors such as PDGF, TGF-β, FGF and VEGF, which are involved in the recruitment and stimulation of cells that are responsible for repair, are decreased in chronic wounds(Pierce et al., 1995). However, other reports mention no local growth factor deficiency in chronic wounds, whereas increased levels of PDGF and VEGF have been found as well (Trengove et al., 2000). Of note, increased levels of VEGF in chronic wounds were accompanied by increased levels of the VEGF inhibitor and/or its degradation(Trengove et al., 2000). These contrasting results for the levels of growth factors may be caused by differences in wound pathology and cell types or localisation of the cells within the wound and should be further investigated. Furthermore, the mechanisms underlying the imbalances in growth factors and their inhibitors in chronic wounds remain to be elucidated. Excess levels of proteinases may cause growth factor degradation/inactivation. Furthermore, growth factors may bind to protein macromolecules and become ‘trapped’ so that they are unable to bind and activate cells (Lauer et al., 2000). Another possible explanation is that an impaired ECM composition diminishes the actions of growth factors *via* a decrease in integrin binding. Finally, intracellular signal transduction may be dysfunctional in the cells(Chen et al., 1997).

1.10.2.1 Wound healing and Type β-Transforming Growth Factor (TGF-β)

Type β-Transforming Growth Factor 1 (TGF-β1) is a low-molecular-weight protein with a broad variety of biological effects. TGF-β1 is a dimer of two identical subunits and binds to a plasma-membrane receptor and has been detected from a wide variety of normal adult tissue (Roberts et al., 1983). The hypothesis that TGF-β1 might play a role in wound repair was surprising, especially when studies suggested the presence of TGF-β1 activity in serum derived from platelets (Childs et al., 1982). It is also important to note that lymphocytes with established functions in inflammation and wound repair are a source of TGF-β1. It has been confirmed that transcription of the TGF-β gene and secretion of the growth factor can be induced by lymphocyte activation (Kimble and Simpson, 1997).

1.10.2.2 Wound healing and tumor necrosis factor-alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is a cytokine produced by macrophages, CD4 positive lymphocytes and many other cell types involved in inflammation. Documented results indicate the involvement of TNF-α in increasing the expression level of inflammation genes, cytokine, Toll-like receptor and also nuclear factor-kB pathways. Previous data suggest that this growth factor may be involved in enhancement of apoptosis and reduction of proliferation of fibroblast. Also an in vitro investigation showed that TNF-α induced gene sets regulate a number of pathways that could influence inflammation and apoptosis (Siqueira et al., 2010).

1.11 Further effective local factors during wound healing

1.11.1 Enhanced proteinase activity

Increased levels of pro-inflammatory cytokines enhance the synthesis and/or release of several matrix metalloproteinases and serine proteases (Agren and Werthen, 2007). In agreement with this, increased proteolysis has been observed in chronic wounds. Elevated levels of MMP-1, MMP-2, MMP-8 and MMP-9 have been reported for delayed healing wounds(Lobmann et al., 2002), as compared to normal healing wounds. Altered distribution of proteinase-producing cells in specific wound areas has been observed. Other proteinases, such as elastase, have been reported to be

elevated as well in chronic wounds due to the large numbers of activated neutrophils. Interestingly, one study showed that elastase degrades MMPs *in vivo* and the authors suggested that elastase is the main cause of ECM destruction(Grinnell and Zhu, 1996).

1.11.2 Pressure and Tension

Previous studies have shown that by 5 to 7 days post wounding, the tensile strength of wounds closed under tension in rats is significantly higher than the tensile strength of wounds closed without tension(Wang and Zhao, 2006). Moreover, the beneficial effect of platelet derived growth factors (PDGF) in accelerating healing in wounds closed under tension has been demonstrated (Tzikas et al., 1996). Additionally a strong relationship between the rate of contraction and the maximum thickness of the granulation tissue was demonstrated. In this study fibroblasts, blood vessels and connective tissue fibers, were introduced as effectively a contractile engine. An in *vitro* quantification of granulation tissue samples confirmed that total cellularity paralleled the tension developed. Pervious research suggested the reduction in cellularity preceded by approximately 4 days post wounding, the decrease in the tension developed that led to an increase in the collagenous scaffolding. Also it has been indicated that the portion of fibroblast with the myofibroblasts characteristics primarily increases fast, and then remains constant throughout the contraction phase and decreases as contraction terminates (Snowden and Cliff, 1985).

1.12 Introduction to Notch signaling

One crucial factor for the development of multicellular three-dimensional structure is the ability to form complex biological patterns from a single cell. Pattern establishment is the consequence of coordinated gene action that targets the developmental fate of individual cells. Essential, cell-autonomous factors as well as non-autonomous, guide and orchestrate the cell proliferation, migration, growth, differentiation, and death, through developmental path to bring together cellular groups in a defined manner. The mechanism of contribution of the essential

developmental factors in specification of cell fates describes the main issue of developmental biology. Notch signaling is an evolutionary conserved mechanism that is used by metazoans to control cell fates through local cell interactions (Artavanis-Tsakonas et al., 1999).

1.12.1 Notch history

In 1914, John Dexter identified and discovered Notch in the fruit fly (*Drosophila Melanogaster*) for the first time. The name 'Notch' is derived from the characteristic notched wing found in flies with haploinsufficency of the Notch gene (Okuyama et al., 2008). Further research in the 1970s demonstrated that the homozygous mutations result in lethal phenotypes in fruit flies, due to neurogenic aberrations, where cells destined to become epidermis switch fate and give rise to neural tissue (Wright, 1970).The involvement of Notch in several other developmental processes in Drosophila, such as bristle formation (Heitzler and Simpson, 1991)maintenance of muscle founder cells (Bate et al., 1993), midgut progenitor cells and cell fate regulation decisions in stem cell progeny (Heitzler and Simpson, 1991) have also been shown. The functional roles of Notch in cell specification has been credited by a study of the nematode *Caenorhabditis elegans* (*C.elegans*); where two Notch homologues, LIN-12 and GLP-1, were reported in *C.elegans* (Kimble and Simpson, 1997). Further studies that applied to model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have revealed a steady path to gene cloning, functional and genetic characterization of Notch signaling pathway in the 1980s (Kimble and Simpson, 1997). The Notch gene encodes a single Notch receptor and two ligands, which are present on adjacent cells in Drosophila (Fehon et al., 1990). Notch receptors have been identified in all vertebrate species. In mice and humans, four Notch proteins (Notch1-4) and five Notch ligands (Delta-like 1,3 and 4; Jagged 1 and 2 have been identified (Okuyama et al., 2008, Wang, 2011) (Figure 10). The function of the Notch receptors and ligands are briefly discussed below.

1.13 The Notch-pathway participants, structure and function

1.13.1 Notch receptors

The intermediary function of Notch in cell fate decisions has been well known and documented. Due to the conservation of Notch molecular structure the general function of Notch has been highly conserved during evolution (Artavanis-Tsakonas et al., 1995). During biosynthesis a furin cleavage produces the mature Notch receptor. Figure 10 shows the molecular structure of Notch signaling participants. Notch is a transmembrane protein; the extracellular domain of Notch that functions as a receptor in cell-cell interactions, has 29 to 36 tandem epidermal growth factor (EGF) repeats and three cysteine rich lin-12/Notch repeats (LNR) and a region that links to the transmembrane and intercellular fragment. This linker is critical in preventing premature activation of Notch receptors. Six cdc10/SWI6/ankyrin repeats, putative nuclear localization signals (NLS), and a C-terminal OPA/PEST region, make the Notch intracellular domain (NICD). Cdc10 region of the molecule is very critical for intercellular signal transduction and also is the extremely conserved portion of Notch molecule (Bigas et al., 1998, Coffman et al., 1993, Fortini et al., 1993) (Figure1.10).

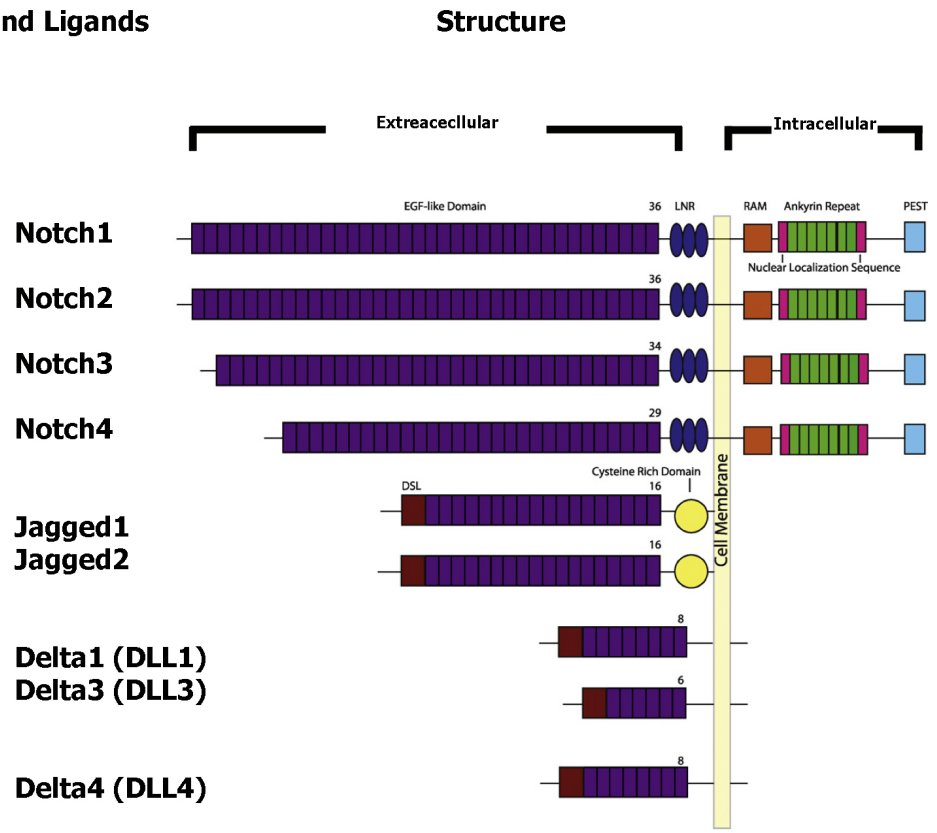


Figure1.10.Canonical regulators of Notch signaling.

Protein structures of canonical Notch pathway receptors and ligands are shown. All proteins contain 6–36 EGF-like domains and are type I transmembrane proteins.
The total number of EGF-like domains in each protein is shown above the last EGF-like domain. The four Notch receptors include LIN-12-Notch repeats (LNR) adjacent to the plasma membrane and also contain large modular intracellular domains (NICDs) that act in the nucleus. The ligands all contain DSL domains at the N-terminus. Their intracellular domains are very short. Dll should not be confused with Dlk, a pair of non-canonical ligands (Wang, 2011).

1.13.2 Notch Ligands

Notch ligands are transmembrane proteins that are illustrated by an N-terminal DSL (DSL proteins, such as Delta, Serrate, and Lag-2) an extracellular domain that is crucial for interaction with the Notch receptor (Figure1.10). Ligand binding of Notch molecule results in activation of this receptor. It has been reported that among a group of cells having comparable cell fate potentials, expressing higher levels of Notch in some cells inhibits differentiation and directs cells to remain multipotent. Functions of different parts of Notch molecules in several systems have been elucidated (Lieber et al., 1993).

1.13.3 Nuclear effectors

CSL (CBF1, Su (H), Lag-1) that is a DNA-binding protein acts as a key transducer of Notch signaling pathway. CSL is characterized by a β-trefoil domain (BTD) between the two Rel-homolohy regions (RHR-N, RHR-C) (Kovall and Hendrickson, 2004). Most of the DNA contacts are mainly made through the RHR-N and BTD domain. It has been reported that a hydrophobic pocket within the BTD domain facilitates the interaction with the NICD. Mastermind (Mam) is a co-activator that is essential to activate transcription .

1.14 Notch signaling cascade

The cell-to-cell contact that leads to interaction between Notch receptors and their ligands (Delta or Jagged) promotes two proteolytic cleavage events in Notch receptor (Figure1.11). The ADAM-family of metalloproteases mediates the first cleavage and an enzyme complex that contains presenilin, nicastrin, PEN2 and APH1 that is known as γ- secretase controls the second proteolytic cleavage (Bray, 2006) . The second cleavage releases the Notch intracellular domain (NICD), which then translocate to the nucleus and cooperates with the CSL (DNA-binding protein RBP-J) and its co-activator Mastermind (Mam), and acts as a transcriptional co-activator. This activation leads to the conversion of CSL (CBF1, Su (H), Lag-1) proteins from repressors to transcriptional activators and triggers the up-regulation of target genes

such as HES (Hairy/Enhancer of Split genes). The Notch signaling pathway is illustrated in Figure 1.11.

Binding NICD to RBP-J in the nucleus removes corepressors from RBP-J, and leads to the derepression of promoters containing RBP-J binding sites and consequently recruits a coactivator complex by which the transcription of Notch target genes gets activated (De Strooper et al., 1999).

The mechanism that regulates Notch activation as a result of an interaction between a ligand and the Notch extracellular domain (ECD) is still unclear (Bray, 2006). The factor that determines in which cells the ligand and the receptor are active has been the ambition of numerous different studies as an interesting subject to be well defined. It has been described that dramatic differences presented in signaling and signal reception between cells are not linked with recognizable alterations in the expression levels of ligands or the receptors. However the full complexities of Notch regulation are still to be clarified, the post-translational modifications and trafficking of the Notch ligands and receptor are the main factors by which the pathway activation can be affected (Bray, 2006).

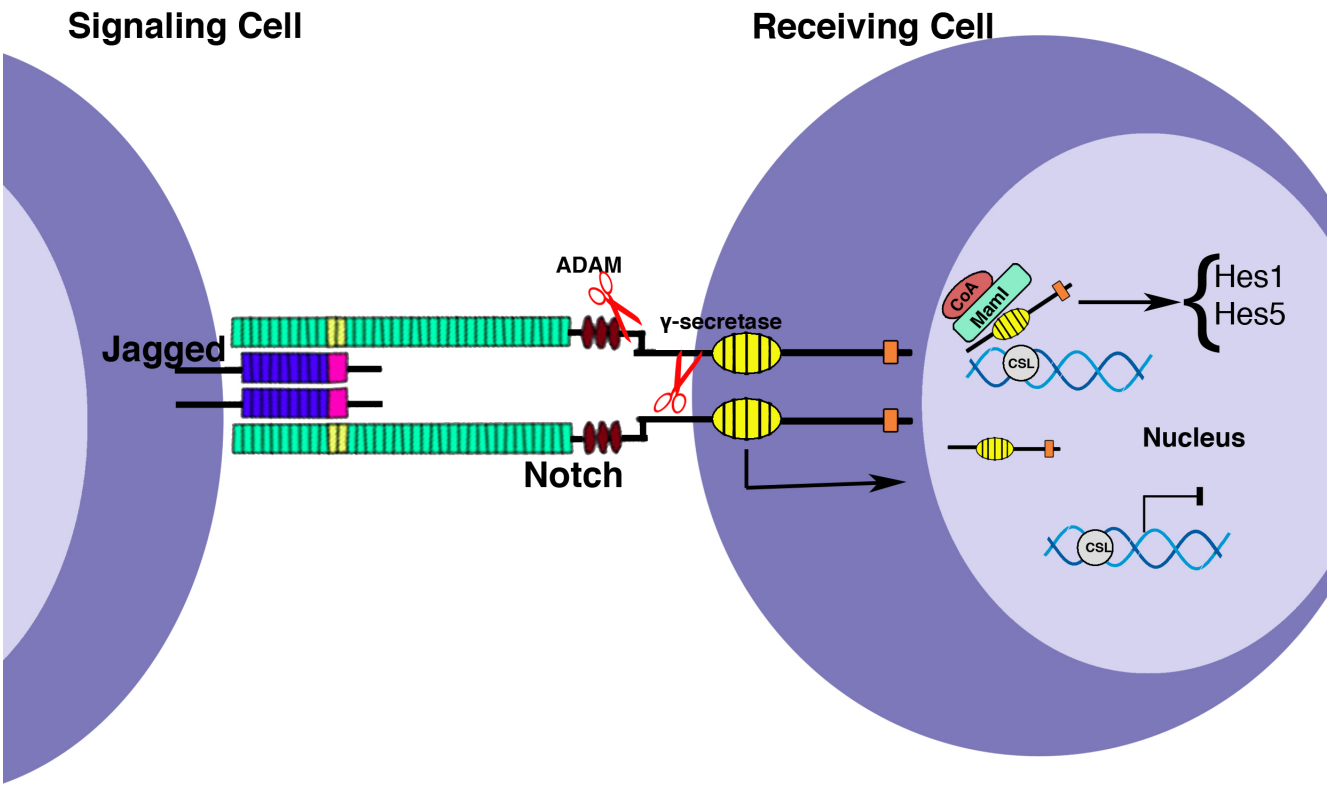


Figure 1. 11.Core Notch signaling pathway.

The core elements of Notch signaling pathway are shown. Notch and most of its ligands are transmembrane proteins, so the cells expressing the ligands typically must be adjacent to the notch-expressing cell for signaling to occur. These include the Notch receptor, DSL ligands, CSL transcriptional factors and target genes such as HES family of basic helix-loop-helix transcriptional regulators. Upon binding ligand the Notch signaling converts CSL from a transcriptional repressor to a transcriptional activator. This conversion is via direct protein-protein interactions between the Notch intracellular domain and the CSL. A metalloprotease (ADAM) controls the first cleavage of Notch after ligand binding and the second cleavage controls by γ-secretase complex. The release of intracellular domain of Notch (NICD) is the result

of these cleavages that can be transported to the nucleus, where it cooperates with transcription factors to regulate gene activity (Adapted from Tsakonas, 1995).

1.14.1 Proteolytic cleavages of Notch

Three different cleavage sites are reported during Notch receptor activation known as S1, S2 and S3 (Figure1.12). First cleavage (S1) is a non-activating cleavage that occurs during Notch receptor maturation. Furin-like convertase mediates this cleavage that is required for cell surface expression of heterodimeric full-length Notch receptor-Ca^{++} coordinated bond between Transmembrane and Intercellular domain (TMIC) and Extracellular Notch (ECN). The second cleavage is an activating cleavage that is mediated by two metalloproteases known as ADAM10 and tumour-necrosis factor-α (TNF-α)- converting enzyme (TACE; also known as ADAM17). This cleavage occurs at an exracellular site (S2) between Ala ^{1710} and Val ^{1711} residues, almost 12 amino acids outside the transmembrane domain (Figure1.12). S2 is a ligand-induced proteolytic cleavage that ends to a carboxy product called NEXT (for Notch EXternacellular Truncation) that is an intermediate precursor to S3 cleavage (Mumm et al., 2000). The third cleavage site termed site 3 (S3) is an activating cleavage and occurs within the transmembrane domain following S2 cleavage. S3 results in the release of NICD and activation of CSL-associated downstream targets. This cleavage is dependent upon the Presenilins that is a γ-secretase-like protease. The proteolytic fragment found to be the result of proteolysis between glycin 1743 and valine 1744 of mouse Notch1(Schroeter et al., 1998)(Figure1.12).

1.14.2 Notch-ligand activity

Contribution of Notch ligands to differential activity of the pathway, expression of Notch ligands by a distinct population of cells, and the involvement of Notch ligands during development are well established.

Identification of an adaptor protein known as E3ubiquitin ligases that is essential for ligand activation and interacts directly with Notch ligands describes the regulation of pathway and the link between ligand activity, ubiquitylation and endocytosis (Chitnis, 2006).

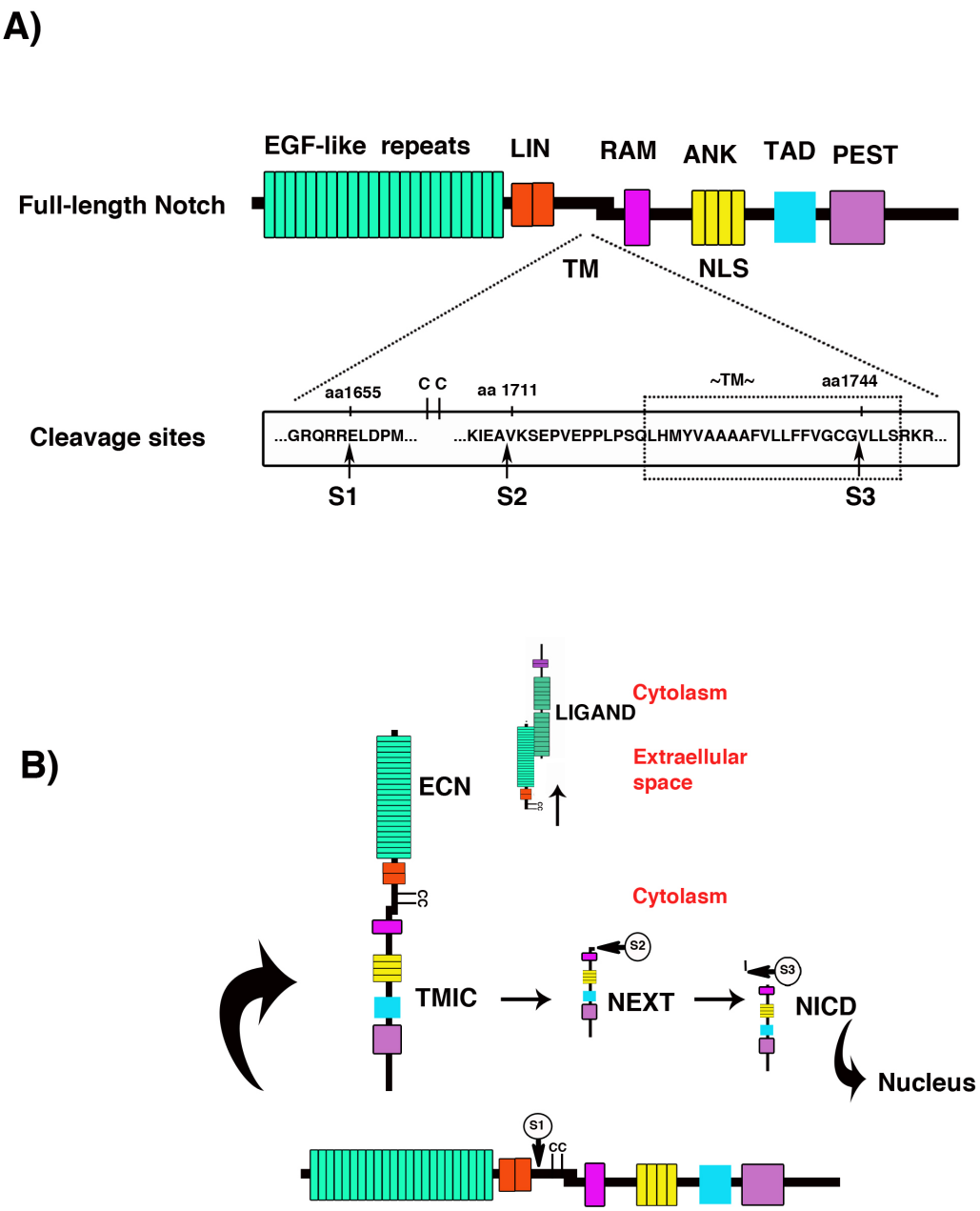


Figure.1.12. Diagram of Notch protein and the proteolytic cascade of Notch activation.

A) Schematic of Full-length Notch protein, and amino acid sequence of mouse Notch1 encompassing each known cleavage site is shown within the open rectangle. Precise sites of S1,S2 and S3 cleavage are shown by arrows and the amino acid number of the respective C-terminal ends. The shaded rectangle shows an approximated transmembrane domain with a 24 amino acids. B) A diagram of the

proteolysis-mediated model of ligand-induced Notch activation. Abbreviations: ECN, Extracellular Notch; TMIC, Transmembrane and Intracellular domain. NEXT, Notch Extracellular Truncation; NICD, Notch Intercellular Domain. Figure is adapted from (Mumm, 2000).

1.14.3 Notch target genes

In various cellular and developmental contexts a limited set of Notch target genes have been identified. The hairy/enhancer of split (Hes) family genes that are described as neurogenic genes in Drosophila are some of the most critical highly conserved proteins that are regulated by Notch in multiple cell types. The transcription factors Hes1, Hes5 and Hey1 (subfamily of Hes) that function as transcriptional repressors are described as the main Notch target genes in mammals (Fischer and Gessler, 2007, Kageyama and Ohtsuka, 1999). CD25 and GATA3 were also identified to be Notch target genes in T-cell and T-cell later developmental stage transcription factors respectively (Amsen et al., 2007, Fang et al., 2007, Reizis and Leder, 2002). Notch target genes, which are, involved in cancer, are described as c-Myc (Palomero et al., 2006, Satoh et al., 2004), cyclinD1 (Ronchini and Capobianco, 2001) and p21/Waf1 (Rangarajan et al., 2001). Furthermore a number of other Notch target genes have been reported including NFkB2 (Oswald et al., 1998), Ifi-202,Ifi204,Ifi-D3 and ADAM19 (Deftos et al., 2000).

1.14.4 Termination of Notch signaling

Notch activation releases the most important part of the signal in the form NICD from the transmembrane. It has been reported that the optimization of signal strength is under the control of different mechanisms involved in NICD production. It has been also mentioned that NICD acts in the nucleus and must be removed from the mitotic cells(Kopan, 1999). The significance of the proteasome in Notch signaling has been demonstrated (Schweisguth, 1999). Previous studies reported post-transcriptional modification of NICD on its PEST domain by the CDK8 kinase, and proteasomal degradation by the E3 ubiquitin ligase Sel10/Fw7. This modification removes NICD from the DNA-binding complex and releases the cell ready for the next round of signaling. Additional phosphorylation sites that regulate Notch signaling still remain to be identified (Hubbard et al., 1997).

1.15Notch1 and Notch2 association

Both intercellular and extracellular domains of Notch act equivalently in Notch activation mechanism. It has been demonstrated that a segment of Notch enters and functions in the nucleus after cleavage of the transmembrane protein. It has been established that the Notch intercellular domain (NICD) functions directly within the nucleus as a transcriptional adapter protein and the nuclear localization signal (NLS) is the NICD site of action, which is functional in the nucleus (Lieber et al., 1993).

Observation of the function of the Notch molecules lacking the extracellular domain, demonstrated a behavior of these molecules as constitutively as activated forms of Notch. Therefore these studies reported that ligand binding Notch activation results can be comparable to the form of Notch activity achieved by the expression of only the intracellular domain (Fortini et al., 1993). These studies endorse the significance and critical function of Notch intracellular domain. The essential roles of Notch1 and Notch2 structurally conserved genes for the development of various organ systems in mammals are well established. A study by Kraman and his group hypothesized that the differences between the amino acid sequence within the intracellular domains (ICDs) of Notch1 and Notch2 could be responsible for exerting the functional differences observed between these two genes *in vitro* (Kraman and McCright, 2005). To investigate the Notch ICD functions *in vivo*, transgenic mice were created that contained a replaced non-conserved region of the Notch2 ICD comprises 426 amino acid with the homologous region of Notch1. The main aim of this study was based on identification differences in the signaling and functional capabilities of the Notch1 and Notch2 ICD. Unexpected results from this experiment demonstrated no native Notch2 message, however the organ (the kidney glomeruli and intrahepatic bile ducts) structural development, which required Notch2, was perfectly formed. This report has shown that the activation domain on Notch receptor (ICD) between Notch1 and Notch 2 in mice is functionally conserved. As the amount of conservation in the ankyrin repeat region of NICD is high, hence it has been hypothesized that the replacement of the entire Notch2 ICD would have presented the same results (Kraman and McCright, 2005). Nevertheless, another *in vitro* investigation has shown that the C-terminus of both Notch1 and Notch2 could activate transcription; another study has reported potential distinct functions of mice Notch1 and Notch2 in hematopoietic differentiation due to transcriptional activating differences between the different

Notch ICDs (Bigas et al., 1998). This study spotted a region C-terminal of the ankyrin repeats that presented differential cytokine response to the Notch1 and Notch2 ICDs in myeloid progenitor cells undergoing differentiation. Association of the structural differences between Notch1 and Notch2, within Notch cytokine response (NCR) regions, contribute to functional specificity in this system (Bigas et al., 1998). It can be concluded from previous studies that Notch1 and Notch2 ICDs are functionally redundant, however the differential ligand interaction or extracellular domain modifications could be termed as the main factors that can effect Nocth1 and Nocth2 activation. As Notch1 and Notch2 ICDs apply the same downstream effects, to active the signaling pathway to control the cell fate decision and developmental functions, Notch1 and Notch2 ICDs can be used.

1.16 Notch signaling function

The Notch signaling pathway is significantly involved in cell-cell communication, cell fate decisions and regulation of the embryonic developmental cascade (Austin and Kimble, 1987). The Notch signaling pathway is a highly conserved network that plays a key role in the proliferation and differentiation of many tissues. Notch signaling pathway orchestrates cell-fate decision in the different systems of many organisms ranging from insects to human (Bray, 2006).

1.16.1 Notch Signaling and embryogenesis

Involvement of Notch signaling in embryonic polarity has been confirmed by demonstrating the abnormality in anterior-posterior polarity in somites in the absence of Notch signaling (Feller et al., 2008). Furthermore, the functional role of Notch signaling during left-right asymmetry determination in vertebrates has also been reported(Levin, 2005). It has been demonstrated that Notch signaling is required in segmentation of somities as abnormal somitogenesis and loss of anterior-posterior polarity were reported in Delta mutant mice(Conlon et al., 1995).

1.16.2 Notch signaling and systematic development

Contribution of Notch signaling pathway has not only been reported in the development of the Central Nervous System (CNS) in *Drosophila*, but it has also been found that Notch pathway is critical for maintaining neural progenitor cells (NPC) in developing brain, glial cell specification (Furukawa et al., 2000, Scheer et al., 2001), regulation of dendritic development (Redmond et al., 2000) as well as adult brain function such as learning and memory (Costa et al., 2003).

It has been presented that Notch signaling performs key functions in mouse cardiovascular formation and morphogenesis not only during the developmental process but also in disease. It has been reported that three main cardiac developmental cascades known as: Atrioventricular canal development, myocardial development and cardiac out flow tract (OFT) in mice are all regulated by the Notch signaling pathway (Niessen and Karsan, 2008). It has been stated that differentiation of mouse vascular smooth muscle cells during embryonic and adult arteriogenesis requires Notch signaling (Chang et al., 2012). In addition endothelial cellular behavior during blood vessel development that occurs throughout angiogenesis, likewise is coordinated by Notch signaling (Leslie et al., 2007).

1.16.3 Notch and Regulation of tissue homeostasis in adults

There is much evidence to suggest that Notch signaling has an effect on the regulation of tissue homeostasis in adults in mice. Involvement of Notch signaling in angiogenesis has been confirmed by the mutation of Notch receptors and ligands in mice, by which the abnormality in the vascular system and many other tissues was detected (Krebs et al., 2000).

It has been confirmed that the effects of Notch signaling regulate a wide range of cellular process including the maintenance of stem cells, cell fate specification, differentiation and proliferation in many different organs in various mammalian stem and early progenitor cells (Chiba, 2006). Involvement of Notch signaling in the vertebrate hematopietic system has been shown by the effect of Notch on balancing the differentiating stem cells population by inducing these cells to retain the undifferentiated state (Jones et al., 1998).

The participation of Notch signaling in specification of cell fate to preserve undifferentiated states has been reported during the development of neuronal

precursor cells in *Drosophila.* A group of original equipotent cells that have the potential to be either epithelial cells or neuronal cells were monitored. The epithelial cell fate was reported in cells that received Notch signals whereas neuronal cell fate was observed in cells that express ligands (Kimble and Simpson, 1997). Furthermore Notch signaling can also influence cell fate choice during mice lymphocyte development (Radtke et al., 2004, Wilson et al., 2001) and induce terminal differentiation during epidermis formation (Radtke and Raj, 2003). There is also substantial evidence of involvement of Notch1 activation in leukemia and a variety of cancer cells in human (Purow et al., 2005). Although previous studies demonstrate that the involvement of Notch activation during cancer cell growth depends on cell types, the role of Notch signaling in tumorigenesis and cancer are still far from being elucidated (Sriuranpong et al., 2001, Talora et al., 2002).

1.17Notch and skin

Mammalian skin undergoes constant regeneration throughout homeostasis, and, in addition, must be primed to undergo wound repair in response to injury. Skin capability for maintaining tissue homeostasis, regenerating hair, and repairing the epidermis after injury is controlled by its stem cells, which reside in the adult hair follicle, sebaceous gland, and epidermis. The generation of the interfollicular epidermis (IFE), hair follicles (HF) and sebaceous glands (SG), is the consequence of the well-regulated terminal differentiation of epidermal stem cells. The differentiation pathway selected by these stem cell populations is principally determined by local micro-environmental signals (Jones et al., 2007, Watt et al., 2006). Understanding the mechanism by which these stem cell populations respond to activate migration, signaling networks, proliferation, differentiation and maintaining the normal homeostasis and wound repair in the skin along a specific lineage is particularly critical. Nevertheless the principles of these mechanisms are not clearly described yet; there is much evidence that support the involvement of signaling pathways in the regulation of skin SCs. It has been documented that a set of molecular archetypes including the Wingless (Wg/Wnt), Hedgehog (Hh), Transforming Growth Factor-β(TGFβ), Receptor Tyrosin Kinase/Phosphotase and Notch pathway perform to interpret and transmit signals as distinct cellular transcriptional responses (Gerhart,

1999). These signaling pathways appear to be also critical in the regulation of the skin SCs behavior (Fuchs and Horsley, 2008).

It has been shown that Wnt signaling regulates SCs in the HF during differentiation, although limits the SG maintenance and differentiation. BMP signaling has a positive function in HF SCs quiescence while it has a negative effect on these SCs activation. There is reported effect of BMP signaling on the epidermis or SG linages. TGFβ signaling pathway performs a critical function in controlling epidermal homeostasis whereas Notch signaling has been introduced as an universal regulator of all SC linages in the skin epithelium (Fuchs and Horsley, 2008).

Although the identification of these fundamental signaling pathways that regulate the differentiation program of the stem cell compartment of many tissues have been considerably progressed in recent years (Blanpain et al., 2007, Watt et al., 2006), the mechanism that controls the epidermal highly organized differentiation program has not been established in detail. The basal layer localizes to the basement membrane and consists mostly of transient amplifying cells with a few SC interspersed. The basal cell layer gives rise first to the spinous layer followed by the granular layer and then the cornified layer. Notch1 signaling induces the expression of early differentiation markers such as Keratin1 and Involucrin, and partially represses the expression of Loricrin and Filaggrin, two late differentiation markers (Watt et al., 2008, Koch and Radtke, 2010) (Figure 1.13).

The Notch pathway plays an important role in the regulation epidermal differentiation. The expression of Notch ligands, receptors and effectors in adult and embryonic skin in mice has been previously reported (Okuyama et al., 2008, Watt et al., 2008). The effects of cell-autonomous and non-autonomous of Notch on epidermal differentiation have also been the focus in recent studies. Here the diverse ways in which Notch signaling regulates epidermal differentiation are described.

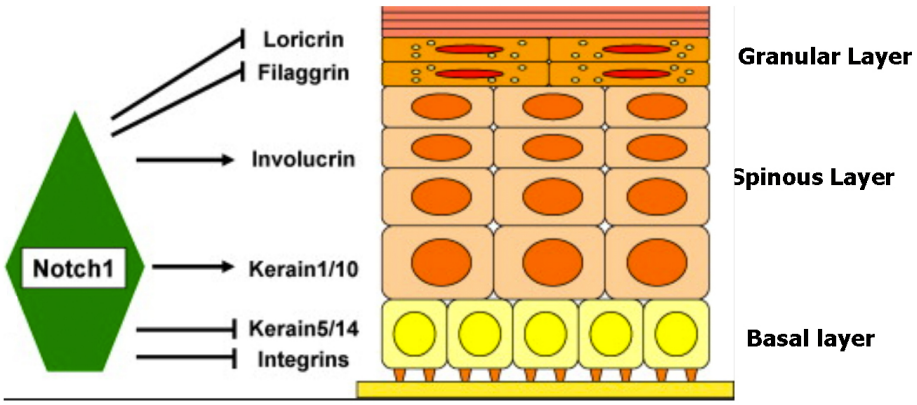


Figure1.13. Notch1 function in epidermis.

The epidermis is composed of several layers, each in different stages of differentiation. Specific proteins are expressed in each cell layer. Notch1 enhances the expression of keratin1 and involucrin, and prevents the induction of filaggrin and loricrin, which are induced in much later stages (Koch, 2010).

1.17.1 Sites of activated Notch pathway in developing and adult skin

Several Notch receptors and ligands are expressed in both developing and adult skin (Kopan and Weintraub, 1993). In epidermal development Notch receptors 1-3 and their ligand, Jagged1, expression is detected suprabasaly, whereas Jagged2 expression has been reported in basal cells during skin development in mice (Powell et al., 1998)..

The up-regulated Notch1, Notch2, Notch3 and Notch4 expression in both suprabasal cells of IFE (Blanpain et al., 2006, Nickoloff et al., 2002) and hair follicles (Kopan and Weintraub, 1993) have been detected in cells that are initiating or are undergoing terminal differentiation in adult skin in mice. Notch ligands (Delta1, Jagged1 and Jagged2) have also been detected in the adult epidermis. Previous studies reported the expression of Jagged1 primarily in the suprabasal layer of the upper outer root sheath and bulb pre-cortex and in some basal cells; Jagged2 expression was detected in the hair follicle bulb cells next to the dermal papilla and in the basal layer of the outer root sheath (Favier et al., 2000, Powell et al., 1998) (Figure 1.14). Recent studies have provided evidence that in the mouse skin, Delta1 is expressed in the dermal papilla and in the epithelial cells at the base of the hair follicle, and in clusters of cells in the basal layer of the IFE in embryonic but not adult epidermis (Estrach et al., 2008). Previous investigations suggest that the expression of Notch pathway elements is noticeable in both embryonic and adult epidermis and this pathway is primarily active in cells of the IFE or HF that are committed to, or undergoing, terminal differentiation(Watt et al., 2008).

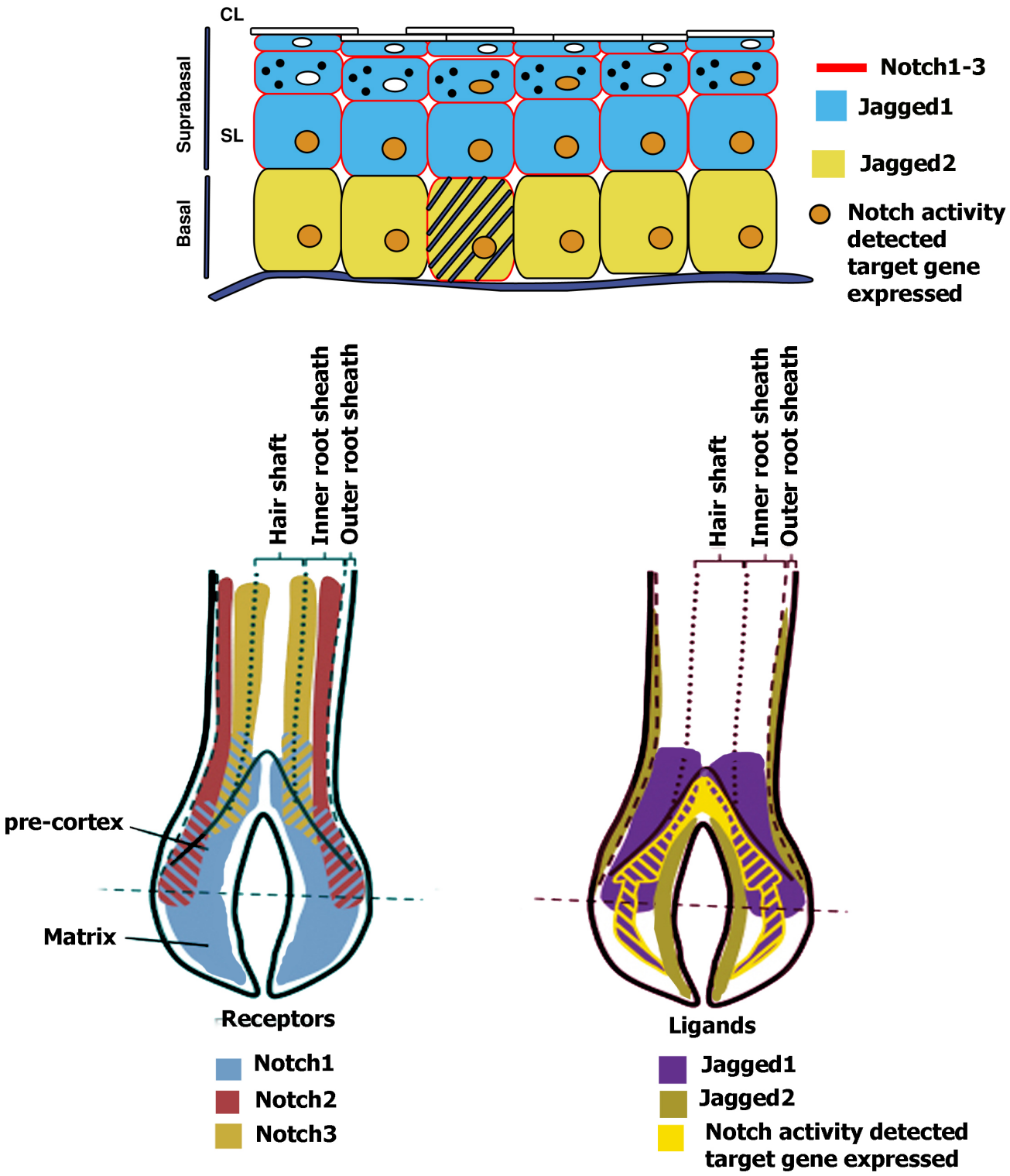


Figure 1.14.Expression of Notch ligands and receptors.

Neonatal interfollicular epidermis (top panel) and hair follicles (bottom panel) in mouse are shown. Hatched shading indicates co-expression. CL: cornified layer; GL: granular layer; SL: spinous layer (Watt, 2008).

1.17.2 Postnatal maintenance of the epidermis and Notch receptors

A previous study by applying the postnatal ablation of Notch1 has confirmed a hyperproliferation of epidermis and suggested the tumor suppressor function for Notch1 in epidermis in mouse (Rangarajan et al., 2001, Okuyama et al., 2008). Deletion of Notch2, Notch3 or Notch 4 alone, did not suggest any effect on the mice embryonic or adult epidermis (Krebs et al., 2000, Krebs et al., 2003, Pan et al., 2004). Deletion of Notch1 or Jagged1, only, has suggested similar effects including the normal HF formation in the embryo, whereas in adult, disturbed HF and immature sebocytes were observed (Pan et al., 2004). Deletion of Delta1 results in a delay of hair growth in postnatal, but no further effects on the HF (Estrach et al., 2008). These studies have suggested that Jagged1 is contributing in regulation of hair follicle differentiation, and Delta1 controls the IFE proliferation and differentiation.

The effects of activation the Notch pathway by overexpressing NICD in different epidermal layers has been determined in recent studies. Expansion of the differentiated cell compartment of the IFE, disturbed differentiation of HF lineages and hair loss are the reported consequences of the overexpressing of NICD in suprabasal layers of the IFE and the HF inner root (Uyttendaele et al., 2004). Increasing the epidermal thickness due to an expansion of the IFE spinous layers and reduced granular cell differentiation are described as the results of the overexpression of NICD in the basal layer of the epidermis, SG and HF(Blanpain et al., 2006). These studies have delivered evidence of an essential postnatal role of Notch signaling for HF maintenance and the regulation of SG terminal differentiation within the IFE, while suggesting that Notch is not required for embryonic development of the epidermis (Watt et al., 2008).

1.18 Non-cell autonomous effects of Notch in skin

Numerous previous studies have acknowledged the cell-autonomous effects of Notch signaling in epidermal development and epidermal stem cells differentiation (Ambler and Watt, 2010, Blanpain and Fuchs, 2006, Estrach et al., 2006, Estrach et al., 2008, Moriyama et al., 2008).

There is clear evidence that Notch has non-autonomous effects on epidermal differentiation. Contribution of Notch pathway in Drosophila development has been explained as one of the most important non-cell autonomous effects of Notch (Bray, 2006). Another study confirmed that, epidermal deletion of Notch leads to increased epidermal production of thymic stromal lymphopoietin (TSLP), which triggers a B lymphoproliferative disorder with massive dermal accumulation of B cells(Demehri et al., 2008). Introducing Notch as an epidermal tumour suppressor, has also confirmed the effects of non-cell autonomous effects of Notch in epidermis (Demehri et al., 2009b, Estrach et al., 2008). Involvement of Notch signaling in wound healing and tissue repair, suggests more evidence of non-cell autonomous effects of Notch (Chigurupati et al., 2007, Nickoloff et al., 2002).

Previous research exhibited that, in adult skin, Notch signalling via Jagged 1 in the epidermis endorses downstream global changes to the dermal stroma (Ambler and Watt, 2010). In this study Jagged 1 has been described as a key component of cell autonomous and non-cell autonomous Notch signaling in the skin. Examples of non-cell autonomous effects of Notch in this study, extended to the up-regulation of TNFα following Notch activation by which the contribution of Notch to the inflammation and barrier defects might be explained (Ambler and Watt, 2010).

1.19 Notch Non-autonomous effects and inflammatory responses

Innate and adaptive arms of the vertebrate immune system need to be functionally organized to support immunity and to prevent immunopathology. Although, the Notch pathway was initially described as a pleiotropic mediator for cell fate in invertebrates, the regulation of immune cell development and function, by the Notch

pathway has recently arisen by different researches (Mochizuki et al., 2011, Radtke et al., 2013, Ambler and Watt, 2010).

The roles of Notch in developing lymphocytes were initially reported, although recently Notch has been demonstrated to be key in controlling the homeostasis of several innate cell populations (Mochizuki et al., 2011, Radtke et al., 2013). A non-cell autonomous effect of Notch in adaptive immunity has been expanded, by involving in regulation of T cell differentiation and function. The inflammatory T-cell response is important for protecting the organisms against infections throughout wound healing. Thus, the regulation of effector T cells is essential during the inflammatory phase of repair. Accumulating evidence indicates that Notch plays critical roles in regulating the differentiation of antigen activated T cells into distinct lineages of effector T cells. Furthermore, significant progress has been made in the development of new methods in order to modulate Notch for inflammatory responses. Previous research showed that Notch activity, via Jagged1, stimulates epidermal production of proinflammatory TNFα and 14-21 days of persistent Notch activity leads to $CD3^{+}CD4^{+}$ T cell recruitment in mice (Ambler and Watt, 2010).

Innate Lymphoid Cells (ILCs) is an essential population of lymphocytes, for secondary lymphoid tissue formation, hemostasis and cytokines production in response to infection. ILC22s as a subset of ILCs is explained as key factors in production of IL22 induced by IL23 from dendritic cells, in adult thymus regeneration. Recent wound healing research (Ambler and et al., unpublished data) demonstrated that, epidermal Notch1 activation results in the up-regulation of TNFα, which directly induces CCL20 expression recruiting ILC22 into the dermis. These data provide evidence that epidermal proliferation and macrophage entry into the dermis and production of essential wound healing cytokines is depending at the accumulation of ILC22, as a non-cell autonomous effect of Notch activation. A previous study (Ambler and et al., unpublished data) showed that forced, ectopic epidermal Notch1 activity resulted in extensive, epidermal proliferation and severe inflammation, two phenotypic hallmarks of skin wound healing (Ambler and Watt, 2010).

In this project, we will discuss recent findings that help to gain insight into a better understanding of the mechanisms by which epidermal Notch activity may benefit

wound healing, via infiltration of immune regulatory factors to the injured mouse skin.

1.20 Notch Signaling and wound healing

The functional roles of the Notch receptor family in cell fate decision in many developmental systems are documented (Maillard et al., 2005). Results from a previous study indicate that Notch family members induce T lymphopoiesis in a lineage-specific manner(Hozumi et al., 2003). Involvement of Notch signaling has been also reported in the regulation of lymphocyte expansion and immune function (Kared et al., 2006). The outcomes of a research that focused on involvement of Notch in regulation of the neural cell responses to injury suggest that Notch signaling may be a target for regeneration of damaged neural cells (Arumugam et al., 2006). Participation of Notch signaling in angiogenesis, formation of new blood vessels, (Xue et al., 1999, Krebs et al., 2000) and also in regulation of epithelial cell proliferation and migration during angiogenesis in normal tissue and tumors has previously been confirmed (Nakajima et al., 2003, Sainson et al., 2005). Notch has also been shown to influence the comportment of different cell types such as keratinocytes, fibroblasts and platelets(Rangarajan et al., 2001). Although there is not much evidence indicating a direct role of Notch in wound healing, the Influence of Notch signaling in the regulation of tissue homeostasis, angiogenesis, regulation of inflammatory functions, re-epithelialization and regulatory functions on keratinocytes, fibroblasts in epidermis, all provide a proposal of hypothetical contribution of Notch signaling during skin wound healing. Skin wound healing involves an initial inflammatory response that requires the release of chemokines and growth factor from stromal and epidermal compartments of skin. As mentioned before, during cell fate decision and pleiotropic functions in skin, Notch plays a key role. Previous studies that have confirmed an extensive epidermal proliferation, and also inflammation, after forced ectopic Notch activity (Ambler and Watt, 2010). Elucidated models that have inhibited or activated Notch during the healing process, all suggest that Notch might be involved in wound healing. Hence in this project we used the K14NICDER transgene contains a truncated, Notch1 intercellular domain (NICD) that can be temporally and spatially activated by 4-hydroxy-tamoxifen (4OHT) in the basal, keratin 14-expressing epidermis.

A jagged1-dependent mechanism of Notch1 activity was previously reported in 4OHT-treated NICDER transgenic skin (Ambler and Watt, 2010), in this project we investigate if the Notch2 activation is also jagged1-related. The relative regulatory of inflammation response in wound healing by Notch2 activity and the relation of this activity and jagged1 function will be also determined by applying triple transgene K14NICDER/K14CreER/*jag1*$^{flox/flox}$ mice lacking jagged1.

An extensive epidermal proliferation and inflammation after forced ectopic Notch activity was reported in 4OHT-treated NICDER transgenic skin (Ambler and Watt, 2010). Notch signaling activation in the absence of a wound in the transgene model K14NICDER after one week of applying 4OHTon mouse back skin, confirmed the infiltration of CD4 and CD3 positive T-cells to the epidermis and dermis (Ambler and Watt, 2010). A natural wound healing response in the early inflammatory phase is the accumulation of $CD4^{+,}$ CD^{3+} and $CD8^{+}$; to examine whether inducing Notch activity before wounding will enhance the wound healing rate and condition, the accumulation of early immune response cells before wounding in the back skin was hypothesized. To investigate the relative skin inflammatory response and wound healing may suggest the fundamental role of Notch signaling during skin wound healing. Before wounding a week 4-OHT treatment of back skin of K14NICDER mice will cause the Notch activity in epidermis and also will provide us with the prospect of Notch activation and skin inflammatory relation.

Therefore in this study we would like to demonstrate whether induced Notch activity effects the TNF-α expression in prior to wound 4OHT-treated K14NICDER, which the inflammatory responses are enhanced. In this study the continuing of 4OHT treatment after wounding is hypothesized to encourage the filtration of more immunocytes and other induced Notch activity consequences to the wounded skin, and monitoring the healing process.

1.21 Hypotheses

I hypothesis that epidermal Notch signalling may be active in the skin as a response to wound healing. In addition, I hypothesis that forced epidermal Notch1 activity results in extensive, epidermal proliferation and severe inflammation; persistent Notch activity may lead to chronic inflammation and poor wound healing. I also hypothesise if the anatomical location of the wound affects the rate of wound closure on mouse back skin due to possible site dependent proliferation and differentiation rate in mouse back skin.

2 Materials & Methods

All experimental procedures were conducted in compliance with Durham University and the School of Biological and Biomedical Sciences safety policy according to the control of substances Hazardous to Health (COSHH) regulations. All reagents were sourced directly through the relevant suppliers and COSHH regulations were followed throughout their usage.

2.1 Mice

Experimental procedures were performed under a UK government Home Office licence (Licence number: 60/3941). The generation and characterisation of transgenic mice (K14NICDER, K14CreERJag1 $^{flox/flox}$) has been previously described (Brooker et al., 2006, Jensen et al., 2009, Kawamoto et al., 2000, Lo Celso et al., 2004, Estrach et al., 2006).

The NICDER transgene model used in this project consists of amino acids 1751-2290 of the mouse Notch1 intercellular domain (NICD). There is a FLAG sequence at the N-terminal of this gene and a modified oestrogen receptor (ER) fused in frame at the C terminus(Schroeder and Just, 2000). The construct that was made of NICD was in to the Keratin14 (K14) promoter cassette (Vasioukhin et al., 1999) and injected into the pronucleus of (CBA×C57bl) F1 embryos. Potential founder lines were screened by PCR (forward: rabbitβ -globin intron 5′-TACTCTGAGTCCAAACCGGGC-3′ and reverse: mouse Notch1 5′-CACTCGTTCTGATTGTCGTC-3′) (Figure 2.1).

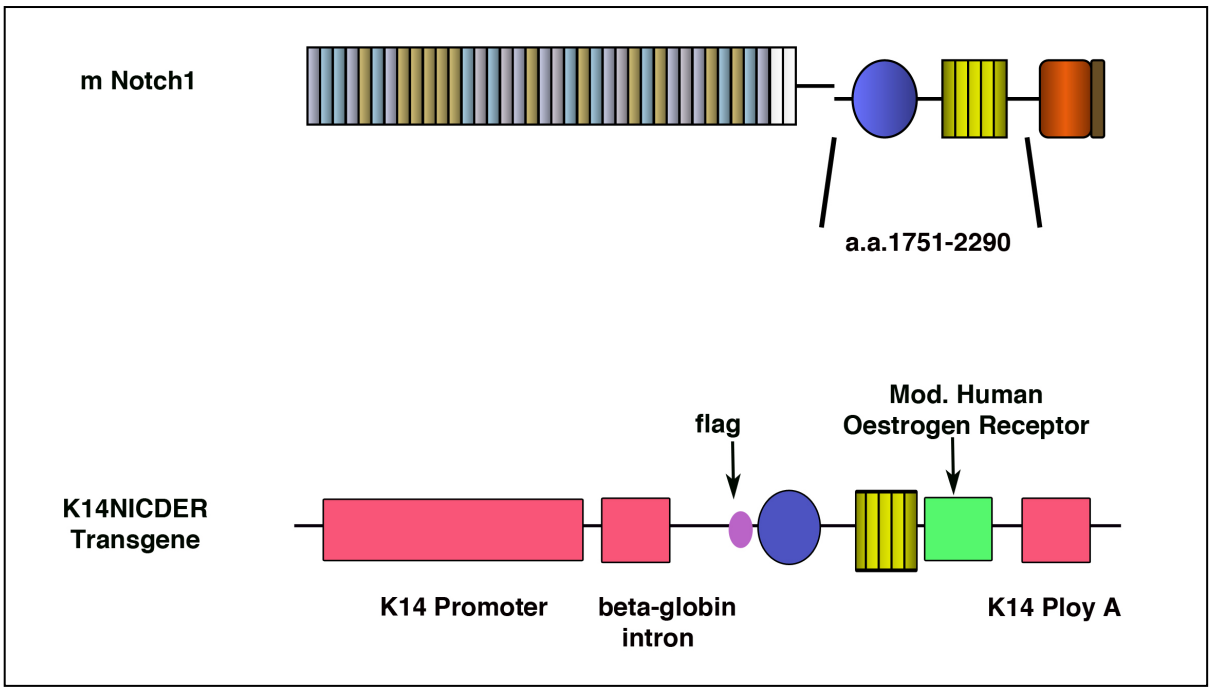


Figure 2.1 The construct that was made of NICD was in to the Keratin14 (K14) promoter cassette.

A NICD region is shown as part of the full-length (top). At the bottom, the FLAG sequence at the N-terminal of Notch1 and a modified oestrogen receptor (ER) fused in frame at the C terminus.

2.2 Dermal wounds and quantification of healing

Mice were shaved at the back skin and anesthetized using 2 to 2.5% vaporized inhaled isoflurane. Dorsal skin was cleaned with iodine povidone solution (10%), and wiped with distilled water. Full-thickness dermal wounds were created in the skin of the back of mice (Figure 2.2) using a 4-mm biopsy punch (Stiefel, manufactured, USA) pressed into skin with twisting motion until punched through both layers of skin.

In a normal wound-healing assay it is quite important to have the wounds always at equal distance from the midline, To determine the rate of healing at different locations, wounds were created at three different parts of the back skin (Figure 2.2).

To active NICDER transgenes, 7-week-old mice were treated topically with 2mg 4OHT(Sigma) dissolved in acetone (Estrach et al., 2006) or treated with vehicle (200µl of acetone) applied directly to the wound site three times a week.

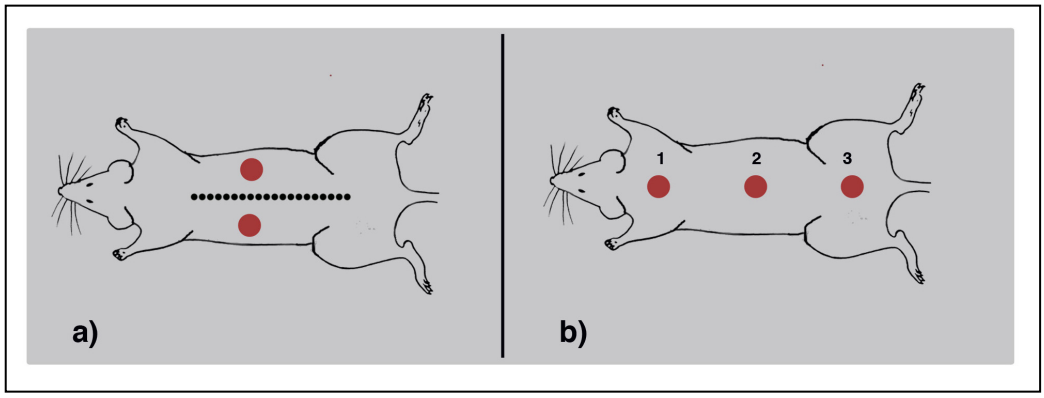


Figure 2.2.Schematic diagram of the punch wounds model.

A) Punch wounds, 2 punch wounds on each side, in the center of the mouse back skin. This method was used for normal wound healing and K14NICDER wounding experiments. **B)** Punch wounds, 3 punch wounds at different locations of mouse back skin (Upper (1), middle (2) and lower (3)). All wounded samples were harvested at different assayed time points after wounding and processed for histological analysis by cutting each sample.

At the desired time point (1-day, 4 and 7days old wounds for a normal wound healing experiment and 2,5 and 8 days for the wound location experiment) required numbers of the mice in each group were sacrificed. Skin tissue samples were cut out at the wounds including a few millimeters of the surrounding skin from all of the mice for histological and biochemical analyses. For RNA extraction and protein lysates the same amount of intact tissue was collected from each mouse. Digital photographs of the injury site were taken; wound size was measured and quantified.

2.3 Histology

To assess cellular infiltration into the wounded site of the skin, samples from three mice per group were collected at the desired time point. To obtain skin sample from the biopsied area mice were scarified and tissue removed by dissection. Wounded area of skin tissue were subsequently placed on a millipore filter for stabilization (any membrane that is resistant to organic solvents such as nitrocellulose) and cut exactly in two. Half the wound tissue were embedded either directly in Optimal Cutting Temperature (OCT) tissue freezing medium (for cryo-sections) or fixed overnight with 4% PFA and embed in paraffin, so that the sectioning can start in the middle of the wound. Formalin-fixed samples were sectioned at 8μm and stained with hemotoxylin and eosin.

2.3.1 Hemotoxylin and Eosin (H&E) staining

This procedure was performed with the slides in glass staining racks and the solutions in square glass staining jars.

Formalin fixed tissue was embedded in paraffin blocks before being sectioned and placed on a glass slide. 8 μm paraffin section of skin tissue, processed, sectioned and de-waxed and rehydrated by serial incubation in Xylene (2x3min), 50:50Xylene/100%ethanol (1x3min), 100% ethanol (2x3min), 95% ethanol (1x3min), 70% ethanol (1x3min), 50% ethanol (1x3min) and finally in H2O (1x5min). Slides were stained with Hematoxylin (Harris Hematoxylin Solution Sigma, HHS32) for 5 minutes at room temperature and subsequently were rinsed under running tap water in the staining jar until the water was no longer colored (~5 min.). Slides were immersed into acid alcohol (1%HCl in 70%ETOH) 2-3 times until the sections turn pink. Slides were rinsed with tap water for 3-5 minutes, and then were soaked in Ammonia Water (1mL NH_4OH in 1L H_2O) for 5-6 immersing until sections were darkened noticeably. Slides were rinsed with tap water for 3-5 minutes, followed be adding Eosin (Eosin Y Aqueous Solution Sigma, HT110232) to slides for 1minute. Slides were again rinsed under running tap water for 3-5 minutes. Slides then were dehydrated by serial incubation in 95% ethanol (2x3min), 100% ethanol (2x3min), 50:50 Xylene/100%ethanol (1x3min) and Xylene (2x3min). Slides were kept in Xylene (no longer than one hour) until they were cover slipped using Permount or a Xylene-based mounting medium.

2.3.2 Elastic Tissue Fibers -Verhoeff's Van Gieson (EVG) staining

To demonstrate atrophy of elastic tissue in wounded skin EVG staining was done. This procedure was also performed with the slides in glass staining racks and the solutions in square glass staining jars. These reagents were prepared before starting the staining procedure.

Alcoholic Hematoxylin: Hematoxylin 5.0 gm, Absolute alcohol 100.0 ml. Dissolved hematoxylin into alcohol with the aide of gentle heat, not boiled.

10% Ferric Chloride: Ferric chloride 10.0 gm, Distilled water 100.0 ml, mixed well.

Van Gieson's Solution: 1ml1% Acid fuchsin, 45ml Picric acid, saturated.

Formalin fixed tissue was embedded in paraffin blocks before being sectioned and placed on a glass slide. 8-µm paraffin section of skin tissue, processed, sectioned and de-waxed and rehydrated by serial incubation as described in the section 2.3.1 (H&E) staining. Sections were incubated in Verhoeff's hematoxylin(20ml Alcoholic hematoxylin,8ml10% ferric chloride,8ml Lugol's iodine).slides were rinsed with tap water for 5 minutes and then slides were differentiated in 2% ferric chloride solution and sections were checked microscopically for black fibers on a gray background. Slides were rinsed with tap water and were kept in 5% Hypo solution for 1 minute. Slides were washed under running tap water for 5 minutes and then counterstained in Van Gieson's for 5 minutes. Slides were dehydrated by serial incubation in 95% ethanol (2x3min), 100% ethanol (2x3min), 50:50 Xylene/100%ethanol (1x3min) and Xylene (2x3min). Slides were kept in Xylene (no longer than one hour) until they were cover slipped using Permount or a Xylene-based mounting medium.

2.4 Immunohistochemistry

2.4.1 Antigen retrieval

Formalin fixed tissue was embedded in paraffin blocks before being sectioned and placed on a glass slide. 8 µm paraffin section of skin tissue, processed, sectioned and de-waxed and rehydrated by serial incubation in Xylene (2x3min), Xylene: ethanol (1x3min), 100% ethanol (2x3min), 95% ethanol (1x3min), 70% ethanol (1x3min), 50% ethanol (1x3min) and finally in H_2O (1x5min). Thereafter, antigen retrieval was performed by immersing the tissue sections in Tris-EDTA buffer (10 mM Tris, 1 mM

EDTA pH 8) or in 0.01 M citrate buffer (pH 6) that had been pre-heated for 3 minutes in a microwave (850W). The slides were heated for 10 minutes at 360W. Following this the slides were allowed to cool to room temperature (RT) for 30 minutes before washing in 1 x PBS. Sections were incubated with 0.1% Triton in PBS for 5 min and then washed in PBS in 3x5 min washes. Sections were taken through to the blocking step immediately. Non-specific binding of the primary antibody was blocked by incubating sections with 10% Goat serum, 1%BSA, 0.01%triton, diluted in PBS for 1 hour at room temperature prior to antibody incubation. After 1 hour of blocking, slides were gently tapped onto blotting paper to remove the blocking solution.

2.4.2 Antibody incubation and antigen visualisation

Primary antibodies were diluted in 0.5% BSA in PBS and each section was incubated with 120 µl primary antibody at 4°C overnight in humidity chamber. Negative controls were incubated in 0.5% BSA/PBS, omitting the primary antibody. Post incubation, unbound primary antibody was removed by washing 3 x 3 minutes in PBS. Thereafter, each slide was incubated with 120 µl poly-HRP-anti-mouse/rabbit/rat IgG for 1 hour at RT. After washing in PBS (3x5min), coverslips were mounted using Mowie Oil and left to harden at room temperature. Results were visualized and photographed on a Confocal Leica microscope.

2.4.3 Confocal Microscopy

All images were acquired on a Leica TCS-SP5 confocal microscope at X40 or X20 magnification. Excitation lasers for fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) dyes were standardized for each experiment, with DAPI dye set by eye for each image as required. Image processing was carried out using Adobe® Photoshop® 7.0 imaging software (Adobe Systems Inc, San Jose, CA, US).

Antibody	Immunoglobulin Subtype	Supplier and Code	Specificity of 1° Antibody	Source of 1° antibody	Working conc. of 1° Antibody	2° Antibody/ Supplier/ Code/ Working conc.
Anti-Rabbit Notch1	IgG	Abcam (Ab8925)	Reacts with mouse Notch1	Rabbit	1:200	Appropriate fluorescent Invitrogen/1:1000 Counterstained with DAPI
Anti-Rabbit Notch2	IgG	Abcam (Ab8926)	Reacts with mouse Notch2	Rabbit	1:200	Appropriate fluorescent Invitrogen/1:1000 Counterstained with DAPI
Goat anti Jagged 1	IgG	Abcam (Ab7771)	Reacts Mouse Jagged1	Rabbit	1:100	Appropriate fluorescent Invitrogen/1:1000 Counterstained with DAPI
Anti-Ki67	IgG	Abcam (Ab16667)	Reacts with Mouse, Rat, Human	Rabbit	1:400	Appropriate fluorescent Invitrogen/1:1000 Counterstained with DAPI
Anti F4/80		eBiosciences Clone BM8	Reacts with mouse F4/80 antigen	Mouse	1:100	Appropriate fluorescent Invitrogen/1:1000 Counterstained with DAPI
Anti CD3		BDBiosciences Clone 17A2	Reacts with mouse	Mouse	1:100	Appropriate fluorescent Invitrogen/1:1000 Counterstained

						with DAPI
Anti SM22a	IgG	Abcam (ab14106)	Reacts with Mouse, Rat, Human	Rabbit	1:100	Appropriate fluorescent Invitrogen/1:1000 Counterstained with DAPI
Anti MMP1	IgG	Millipore Clone 4E7.2 (Mab13439)	Reacts with Human and non-human MMP1	Human	1:200	Appropriate fluorescent Invitrogen/1:1000 Counterstained with DAPI

Table 2.1. List of primary antibodies used in Immunohistochemistry

2.5 Protein Analysis SDS-PAGE and Western Blotting

2.5.1 Tissue preparation

0.5-1 cm2 sections of back skin were collected and immediately snap-frozen and stored in liquid nitrogen. To denature the protein samples a combination of physical and biochemical techniques was applied. RIPA buffer was applied because the target proteins in this project were mostly located in the nuclei and cytoplasm. 600 µl of RIPA (150 mMNaCl, 50 mMTris-HCl, pH 7.5, 1% Nonidet P-40, 0.25% Na deoxycholate) and one protease inhibitor cocktail tablet (Roche Diagnostics, GmbH, Cat. no. 11 836 170 001, Mannheim, Germany was added to 10ml of RIPA buffer) was used for preparation of the lysate. After adding RIPA buffer to the frozen tissues, samples were homogenised using a Polytron tissue homogeniser for 2 minutes. These events are slowed down by keeping the samples on ice (4°C) throughout the lysis

procedure and appropriate inhibitors were added fresh to the lysis buffer. The homogenized tissue-lysis buffer mixture was incubated on ice for 30 minutes with gentle regular mixing with a pipette. The lysed sample was then centrifuged at 12000 rpm for 20 minutes at 4°C. The supernatant was then aspirated and transferred to a new tube and the pellet discarded. The samples were then stored on ice before proceeding to the next stage.

2.5.2 Bicinchoninic Acid Protein Assay

Protein concentration was determined by the Bicinchoninic Acid Protein Assay (BCA – Pierce: #23225). Prior to the assay lysates isolated as in **2.5.1** a standard curve derived from serially diluted 2mg/mL BSA was also prepared. Samples were loaded onto a 96 well plate as per manufacturer's instruction and incubated at 37°C to improve assay sensitivity (Olson and Markwell, 2007) The plate was read at 570nm.

2.5.3 Denaturation and reduction of protein

To enable antibodies to recognize and access the epitope fraction of the target protein, the 3D conformation of the protein needs to be unfolded. Consequently denaturation and reduction of protein samples was required prior to immunoblotting. This procedure was carried out by preparing samples in 3x loading buffer (30mM NaH2PO4, 30% (v/v) glycerol, 0.05% (w/v) Bromophenol blue, 7.5% (w/v) sodium dodecyl sulphate (SDS), made up in dH2O), DTT (23.5mM final concentration, from a 200mM stock solution made with dH2O) with 10-50µg of protein and made up to a final volume of 15µl of dH2O. Samples were mixed by vortexing (both before and after heating for good resolution), centrifuged at 13,000 rpm for one minute, incubated for five minutes at 95°C, centrifuged at 13,000 rpm for one minute and thoroughly vortex again. Samples were then stored at -80°C or applied to an SDS-PAGE gel for electrophoresis.

2.5.4 Gel electrophoresis

The proteins in the samples were separated using one dimensional polyacrylamide gel electrophoresis. SDS-PAGE (SDS Polyacrylamide Gel Electrophoresis) maintains

polypeptides in a denatured state as they run along the gel. In this project a 4% stacking gel was used, which allowed the proteins to form a nice tight band at the interface between the stacking gel and the 9% separating gel.

Reagents	**Stacking Gel (4%)**	**Reagents**	**Separating Gel (9%)**	**Supplier**
(Double distilled) Water	6.3ml	**(Double distilled) Water**	5.1ml	
0.5M Tris pH 6.8 (60.5g Trizma base in 1L dd water, pH to 6.8 with conc. HCL)	2.5ml	**1.5M Tris pH 8.8** 181.65g Trizma base in 1L dd water, pH to 8.8 with conc. HCL)	2.5ml	Trizma base Sigma-Aldrich T1503
10% SDS	100µl	**10% SDS**	100µl	Sigma-Aldrich L3771
Acrylamide/Bis-acrylamide 49:1 ratio	1ml	**Acrylamide/Bis-acrylamide 49:1 ratio**	2.25ml	Sigma-Aldrich AO924
Ammonium persulfate (APS)	100µl	**Ammonium persulfate (APS)**	50µl	Sigma-AldrichA3678
N, N, N', N'-Tetramethylethylenediamine (TEMED)	10µl	**N, N, N', N'-Tetramethylethylenediamine (TEMED)**	5µl	Sigma-Aldrich T9281

Table 2.2. Composition of Polyacrylamide gels

The wells of the gel were gently flushed with running buffer; Protein samples were loaded into the wells in the gel. 10-15µg of protein was loaded into each well. The gels were run at a voltage of 120 Volts for 20 minutes (until the protein ladder (Amersham ECL full-range rainbow marker) appeared to separate) and then at a constant voltage of 110 volts for 40-50 minutes (until the bromophenol blue had

migrated to the bottom of the gel ensuring that the proteins have separated into various bands in each well.

Reagents	X10 Running Buffer
(Double distilled) Water	1L
Trizma Base	30g
Glycine	144g
SDS	10g

Table 2. 3Running buffer

2.5.5 Coomassie blue staining

To visualise proteins post-SDS-PAGE, colloidal Coomassie staining was used. First, SDS-PAGE gels were fixed in 7% glacial acetic acid in 40% (v/v) methanol for 1 hour. The gels were transferred to colloidal Coomassie solution (Sigma product B2025) activated in 20% methanol and incubated with rocking for 4 hours or overnight. Gels were destained with 10% acetic acid in 25% (v/v) methanol for 60 seconds, rinsed twice with 25% methanol and destained for up to 24 hours. The destained gels were briefly rinsed with distilled water prior to drying down onto Whatman paper (GE Healthcare) with a gel drier for 40 minutes at 80°C.

2.5.6 Transfer of protein bands from gel onto PVDF membrane

To make the proteins accessible to antibody detection they are moved from within the gel onto a membrane made of polyvinylidenedifluoride (PVDF). Transfer was done in wet conditions during this project. PVDF membranes (Amersham) were pre-treated by soaking the membrane in methanol for 1-2 minutes. This was followed by a quick soak in water and then incubation in ice cold transfer buffer for 5 minutes. The wet

membrane was then placed on top of the gel, and a stack of transfer buffer soaked filter papers placed on top of that. This stack was sandwiched between two buffer soaked sponge pads and transferred to a tank filled with transfer buffer. The electro-transfer was performed in a cold room at a constant voltage of 30 volts over night.

Reagents	**(x1) Transfer Buffer**
(Double distilled) Water	2L
Trisma Base	6.06g
Glycine	28.8g
Methanol	400ml

Table 2.4. Transfer buffer

2.5.7 Visualization of all separated proteins after migration

Ponceau S dye (P7170-IL, Sigma-Aldrich) was used to visualize the uniformity and overall effectiveness of protein transfer from the gel to the membrane. The membranes were incubated with Ponceau S dye (1:10 dilution with water) for 3-5 minutes at room temperature on an agitator. The protein bands, if transferred effectively, appear as well-defined reddish-pink bands. The membranes were then washed extensively in (x1) Tris Buffered Saline-Tween (TBS-Tween) until the dye was washed off.

Reagents	**(x20) Tris Buffered Saline Solution (TBS)**

NaCL	234g
Trizma base	48.4g
(Double distilled) Water	1L

Table 2.5 (x20) TBS

Reagents	**(x1) Tris Buffered Saline Solution (TBS) 1Litre**	**Supplier**
(x20) TBS	50ml	
Tween-20	1ml	Sigma-Aldrich 27,434-8

Table 2.6. (x1) TBST

2.5.8 Blocking of the PVDF membranes

The membranes with the transferred protein bands were then blocked in blocking buffer for 1 hour at room temperature on an agitator. Blocking the membranes prevented non-specific binding of the primary and/or secondary antibodies to the membrane.

Reagents	**Blocking buffer**	**Supplier**
(x20) Tris buffered Saline (TBS)	2.5ml	
Tween-20	0.6ml	Sigma-Aldrich

		27,434-8
Low-fat milk (Marvel) or BSA	2.5g	Dried skimmed milk, R. No. 92962, Marvel, Dublin, Ireland. BSA (Fisher BPE9702-100)

Table 2.7. Blocking buffer

2.5.9 Antibody staining

The membranes were incubated with the primary antibody (diluted in incubation buffer) (table 8) overnight at 4°C on an agitator to enable adequate homogenous covering of the membrane and prevent uneven binding.

Reagents	**Incubation Buffer**
(x1) Tris Buffered Saline (TBS)-TX	50mls
Low-fat milk (Marvel)/BSA/fish skin gelatine	5g/5g/1.5ml

Table 2.8. Incubation buffer

Post overnight incubation with the primary antibody the membranes were washed in (x1) TBS-TX, three times, and 10 minutes each time. The washes were done at room temperature on an agitator. This step ensured that all excess unbound antibodies were washed off.

The membranes were then incubated with the appropriate secondary antibody (diluted in incubation buffer) for 1 hour at room temperature on an agitator.

The membranes were then washed in TBS-TX, four times, and 12-15 minutes each time, to get rid of any unbound secondary antibody.

Antibody	Immunoglobulin Subtype	Supplier and Code	Specificity of 1° Antibody	Source of 1° antibody	Working conc. of 1° Antibody	2° Antibody/ Supplier/ Code/ Working conc.
Anti-Rabbit Notch1	IgG	Abcam (Ab8925)	Reacts with mouse Notch1	Rabbit	1:400	Anti-Rabbit IgG-HRP conjugated/ 1:10000 (Sigma,A6154)
Anti-Rabbit Notch2	IgG	Abcam (Ab8926)	Reacts with mouse Notch2	Rabbit	1:500	Anti-Rabbit IgG-HRP conjugated/1:10000 (Sigma,A6154)
Goat anti Jaggde 1	IgG	Abcam	Reacts Mouse Jagged1	Goat	1:100	Anti-Goat IgG F HRP conjugated/1:10000 (Sigma, A5420)
Anti-Beta actin	IgG1	Sigma		Mouse	1:3000	Anti-Mouse IgG F HRP conjugated/1:10000 (Sigma, A4416)
Anti MMP1	IgG	Millipore Clone 4E7.2 (Mab13439)	Reacts with Human and non-human MMP1	Human	1:300	Anti-Mouse IgG F HRP conjugated/1:10000 (Sigma, A4416)

Table 2.9 List of primary antibodies used for western blotting

2.5.10 Detection and Band Density Analysis

Membranes then were washed three times, 10 minutes each, with 1x TBS before being covered with Enhanced Chemiluminescence (ECL – Pierce: #32106) substrate for 2 minutes. ECL was then drained from the membrane, which was then wrapped in cling film after which it was taped into a film cassette. The membrane was then exposed to HyperfilmTM for 10 minutes for the primary antibody and for three minutes with β-actin, the hyperfilm was then developed using Kodak-D19 developer and fixed for five minutes in Kodak Unifix fixative.

Band density of western blots was analyzed by Image J software. Target protein marker band intensity was measured relative to controls. All bands were standardized against β- actin loading controls either co-probed on the same membrane or generated by strip and re-probed of the same membrane. Where applicable the mean and standard error of the mean (SEM) were generated and plotted with significance assayed by two-tailed student t-Test.

2.6 Molecular Biology Technique

2.6.1 Total RNA isolation

All techniques were carried out under RNase free conditions using aerosol resistant filtered pipette tips. All solutions were autoclaved and stored in glass bottles, which had been baked at 140°C for 2 hours. The workspace was wiped clean with RNase Zap (Ambion Cat# AM9780). RNA was extracted using the RNeasy Mini Kit from Qiagen, (Cat#74104). This is a ready to use kit by which the purification of total RNA from skin samples was carried out as follows:

25-30mg of intact skin tissue was snap frozen and stored in RNAlater overnight. Fat and muscle was removed by scraping. Weighed tissue was transferred from RNAlater to a 2ml cryovial and incubated on ice until ready to grind. Tissue was disrupted and lysate was homogenized in 600 µl of RLT buffer plus 10 µl/ml Beta-Mercaptoethanol (BME,sigma),for 1-3 minutes. Ground tissue was incubated at room temperature till all samples were homogenized. 1160 µl RNAse free water was added to each sample

plus 40 µl of proteinase K (10mgs/ml stock). Samples were mixed by inversing and incubated at 55°C for 10 minutes. Lysate was transferred to a 2ml eppendorf and centrifuged for 2 min at $13.4x10^3$g. The supernatant was carefully removed by pipetting and transferred to a 50ml conical tube. 900 µl of 100% ethanol was added to the lysate, and mixed well by pipetting. Up to 700 µl of each sample, was transferred to an RNeasy Mini spin column placed in a 2 ml collection tube, the lid of each tube was closed the, and centrifuged for 15 s at $13.4x10^3$g and the flow-through was discarded. 350 µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at $13.4x10^3$g, flow-through was discarded. DNase digestion was performed on each column (kit from QIAGEN, # 79254), 10 µl of DNase plus 70 µl of RRD solution was added per column. Samples were incubated at room temperature for 15 minutes.

350 µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at $13.4x10^3$g, flow-through was discarded. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 2 min at $13.4x10^3$g, flow-through was discarded. RNeasy spin column was placed in a new 1.5 ml collection tube (supplied in QIAGEN kit). 30–50 µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at $13.4x10^3$g to elute the RNA.

2.6.2 Quantification and purity of RNA

The concentration and purity of RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, UK). Briefly, 1 µl of sample was loaded onto the optical pedestal, the absorbance at 260 nm and 280 nm was measured, and the concentration of RNA was calculated. The A_{260}:A_{280} ratio was used to assess RNA purity, with a value of 1.7-2.0 taken to indicate that the sample was sufficiently pure.

2.6.3 Assessment of RNA quality

To assess the quality of extracted RNA, 2µl of each sample were electrophoresed on a 2.0% gel, containing Ethidium bromide (0.1µg/ml) in 1x Tris Acetate-EDTA (TAE). Before electrophoresis, 2µl of loading buffer (0.25% bromophenol blue, 40% glycerol, made up in sterilised water was added to each sample. 2 sharp and clear 28s and 18s rRNA bands were expected to be seen. The intensity of the 28S rRNA band should be approximately twice as the intensity of the 18S rRNA band (Figure 2.3). This 2:1 ratio (28S: 18S) is a good indication that the RNA is completely intact.

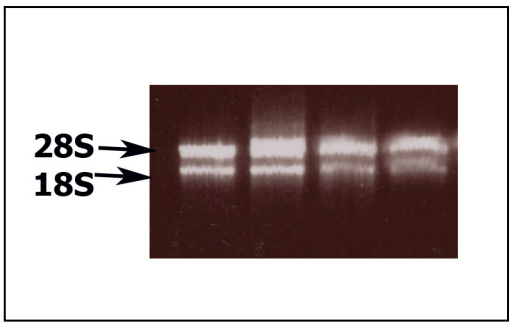


Figure 2. 3. Intact RNA.

Two µg intact total RNA was run a 2% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible.

2.6.4 cDNA synthesis

Total RNA was reverse transcribed using a Superscript III first-strand synthesis kit (Invitrogen) and quantitative PCR was performed under standard conditions with an

ABI 7500 fast real-time PCR machine. A summery of cDNA synthesis procedure is shown in figure 2.4.

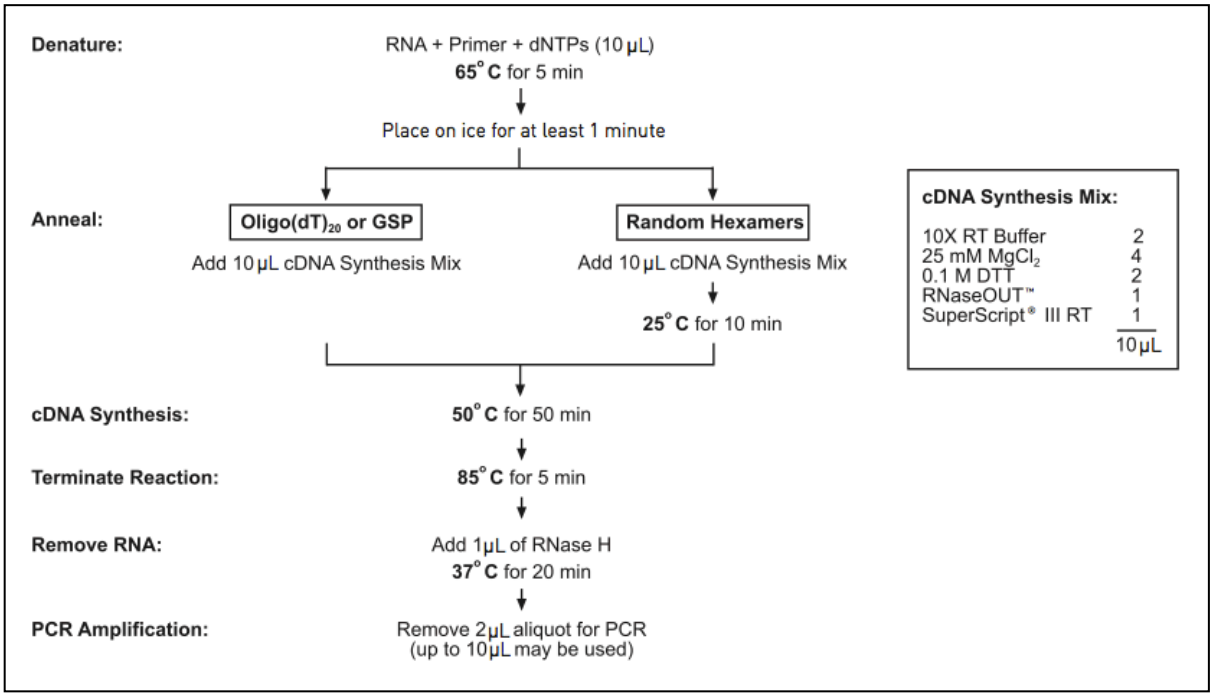


Figure 2.4 Summery of cDNA synthesis, using a Superscript III first-strand synthesis kit (Invitrogen)

2.6.5 Q-PCR

In real time Quantitative Polymerase Chain Reaction (Q-PCR), aim is to amplify and quantify the nucleic acid sequences throughout the PCR reaction. In this method a thermocycler is applied to achieve the quantified measurements of molecules that give a florescent signal a result of probe hydrolysis (TaqMan, Applied Biosystems); probe hybridisation (LightCycler, Roche); hairpin probe hybridisation (LUX, Invitrogen); hairpin-loop hybridisation (Molecular Beacons, Sigma) or binding to double stranded DNA (SYBR Green) (VanGuilder et al., 2008). In a real time q-PCR reaction, during the exponential amplification phase the fluorescence signal is measured. This measurement generates a value known as cycle threshold (CT), which represents the amplification cycle at which the fluorescent signal exceeds a threshold above the background fluorescence (baseline). Consequently, the higher the C_T value, the lower the gene expression. Absolute quantification (calculating the number of copies of a specific RNA) or relative quantification (comparing the gene expression in two or more samples) are two main methods that q-PCR data can be presented by.

2.6.6 qPCR primer validation

Prior to performing qPCR on the biological samples of interest, it is important to assess the efficiency of the primers. For each primer set, qPCR was performed on a cDNA dilution series (0.1 ng – 100ng diluted to 1000x dilution). Quantitative real-time PCR was carried out in a MicroAmp® optical 96 well reaction plates (Applied Biosystems). Twelve point five µl Fast SYBR ®Green Master Mix (Applied Biosystems, Nieuwekerk a/d Ijssel, NL), 5 µl cDNA, 5.5 µl nuclease-free water, 1 µl forward primer (1000 µmolar) and 1µl reverse primer (1000 µmolar) were added to each well. A negative control containing no cDNA was run for each primer pair that was used. MicroAmp® optical adhesive film (Applied Biosystems) was placed on the top of the 96 well reaction plate to reduce contamination and evaporation. The plate was centrifuged briefly to remove bubbles. Reactions were run in a 7500 Fast Real-Time PCR System (Applied Biosciences). The qPCR profile was as follows: 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds followed by 60°C for 30 seconds. Following this, a melt curve stage was performed in which the samples were heated to 95°C for 15 seconds, cooled to 60°C for 1 minute, slowly heated up to 95°C, and cooled to 65°C for 15 seconds. The baseline and threshold values were checked to

confirm the validity of the automatically generated parameters. In a qPCR reaction, the fluorescence signal is measured during the exponential amplification phase because it provides the most accurate data for quantification. Cycle threshold (C_T) values indicate the amplification cycle at which the fluorescent signal exceeds a threshold above the background signal (baseline). Thus, the lower the C_T value, the higher the gene expression. A standard curve was plotted where X-axis = log10 [cDNA] and Y axis = C_T values. Primers passed quality control testing if the gradient of the graph was between -3.3 +/- 0.3 as this indicates that the efficiency of the PCR reaction is 100% +/- 10% and hence allows for meaningful gene comparison. All primers used within this thesis were deemed suitable for qPCR analysis.

2.6.7 Quantitative polymerase chain reaction (QPCR)

QPCR to quantify Notch1, Notch2, Jagged1, TGFβ, TNFα, IL-22, IL-23α (p19), MIP1-α and SM22 was performed using a Rotor-Gene Q instrument (Qiagen), with a two-step rapid-cycling procedure as described by the manufacturer (Roter-Gene Probe Handbook, Qiagen).

Triplicate reactions (20ul) of each experimental sample were analysed using FAM probes for the gene of interest (TNFα, Mm00443258.m1;Notch1, Mm00435248_m1; TGFβ1, Mm01178819_m1; SM22α, Mm00441660_m1and IL23α (P19), Mm01160011.g1, Life Technologies) at a final concentration of 900 nM for each primer and 250nM for the probe and TaqMan Fast Universal PR master mix (ABI) at 1x. Reactions were subjected to an initial 3-minute denaturation step at 95^{o}C, followed by 45 cycles of 95^{o}C for 3 seconds and 60^{o}C for 10 seconds. Data were analysed using the Comparative Quantitation algorithm in the Rotor-Gene software, with calibrator samples for each run being compared in a common experiment. MIP1α and IL-22 were amplified using unlabeled primers (MIP1α: Forward 5'-GTTCTTCTCTGTACCATGAC-3'; Reverse 5'- CTCTTAGTCAGGAAAATGAC-3', final concentration 400 nM; IL-22: PPM05481A-200, Qiagen; Notch2: PPM051378_200,Qiagen, Jagged1: PPM26291A_200,Qiagen) in a reaction using SYBR-green master mix (Sigma). Reactions were subjected to an initial 3-minute denaturation step at 95^{o}C, followed by 45 cycles of 95^{o}C for 10 seconds, 60^{o}C for 10

seconds and 72^{o}C for 10 seconds. Reference genes (GAPDH and β-2 microglobulin primers) were used to quantify genes of interest.

3 Notch signaling activation during normal skin wound healing

3.1 Introduction

The mammalian epidermis serves as a barrier to protect the body against entry of toxins, chemical compounds and pathogen invasion, as well as providing mechanical resistance. The epidermis is essential for maintaining the self-renewing ability of the skin. Mitotically active cells located in the basal layer of epidermis constantly regenerate the epidermis. Following an injury, the remaining epithelial cells undergo a programmed repair mechanism to immediately close the defect (Hunt et al., 2000). This highly coordinated process involves a number of cellular functions including migration, proliferation and differentiation. Following injury, in the early inflammation phase neutrophils and macrophages infiltrate the wound site and clean the site of microbial contaminates. Epidermal proliferation, extracellular matrix deposition, granulation, vascularisation and wound contraction are described as signs of the proliferative phase. Following wound closure, excess cells are removed and the remodeling phase of the extracellular matrix begins. While many of the regulatory mechanisms governing skin wound healing have been studied before, the role of Notch signalling, a critical pathway during development, has not been entirely determined (Fuchs and Horsley, 2008, Hunt et al., 2000).

The Notch signaling pathway is a highly conserved and cell-autonomous network that regulates cell fate decisions during development and has critical functions in the skin (Artavanis-Tsakonas et al., 1999, Greenwald, 1998, Ambler and Watt, 2010, Demehri et al., 2009a). Notch proteins are membrane bound receptors with corresponding membrane bound ligands, Delta and Jagged. Binding of ligands results in proteolytic cleavages of Notch receptor; first in an extracellular domain and subsequently in the transmembrane domain. This releases the Notch intracellular domain (NICD), which translocates to the nucleus where it regulates transcription of target genes, such as Hairy/Enhancer of Split (Hes) (Bray, 2006, Schroeter et al., 1998).

Involvement of Notch signalling in the regulation of tissue homeostasis in adults recently has been implicated by numerous of studies. Although the importance of Notch signalling in several tissues wound healing has been highlighted by numerous

studies the involvement of Notch signalling and skin wound healing is still not clearly explained.

Wound healing involves an initial inflammatory response and subsequent changes in keratinocytes, fibroblasts and vascular endothelial cells that close the wound and regenerate the skin tissue (Hunt et al., 1999, Hunt et al., 2000). Previous studies have demonstrated that Notch is involved in lymphocyte expansion regulation and immune function (Kared et al., 2006). Another study indicates the involvement of Notch signaling in brain injury, showing that Notch is involved in the regulation of neural cell responses to injury (Arumugam et al., 2006).

The essential role of endothelial Notch1 during the formation of new blood vessels, angiogenesis, proliferation and migration of epithelial cells during angiogenesis, in normal tissues and wounds, has also been highlighted in previous studies (Limbourg et al., 2005, Paris et al., 2005, Sainson et al., 2005). Notch has also been shown to affect the behavior of keratinocytes, fibroblasts and platelets, additional cell types that play important roles in wound healing (Cereseto and Tsai, 2000, Rangarajan et al., 2001).

There are four mammalian Notch receptors (Notch1 - Notch4); all of which have previously been shown to be expressed in the human epidermis (Nickoloff et al., 2002). Despite the presence of all four Notch receptors, Notch1 and Notch2 have been shown to be the principal receptors involved in maintaining hair and skin epithelia by regulating cell differentiation (Pan et al., 2004). However, some studies suggest Notch2 also plays a non-redundant role in embryonic development and tissue maintenance (Pan et al., 2004).

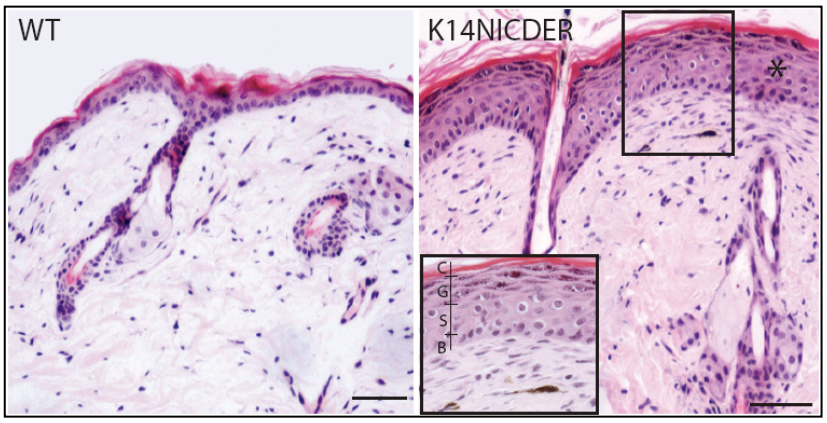
In normal human skin, the Notch receptors and ligands are abundantly expressed in keratinocytes within the epidermis. Notch1 is primarily confined to keratinocytes in the lower and mid level epidermal layers. Notch2 distribution is limited to keratinocytes in the basal layer (Blanpain et al., 2006).

In previous studies, deletion of Notch receptors and ligands and the effects on epidermal homeostasis and differentiation were examined. These reported data that presented an epidermal hyperproliferation in mice with postnatal ablation of the Notch1 gene, suggesting that Notch1 signaling is required for basal keratinocytes to withdraw cell cycling and to promote terminal differentiation into postnatal epidermis (Moriyama et al., 2008). Thus, ablation of Notch1 gene caused significant alternation in epidermis normal growth as well as a disruption of the well-defined border between

basal and upper differentiating layers. While deletion of Notch2 alone does not have any reported effects on the epidermis (Krebs et al., 2000, Pan et al., 2004).
Interestingly, combined loss of Notch1 and Notch2 was demonstrated by another study, which results in more severe epidermal phenotypes than loss of Notch1 alone (Krebs et al., 2000). This reported evidence suggests a significant role for both Notch1 and Notch2 in contribuing to the control of proliferation and differentiation in IFE in mice.
A previous study using topically-applied pan-Notch activators and inhibitors suggested that Notch might be involved in wound healing, however, the mechanistic details and site of action of Notch activation in skin injury have not been covered in previous studies (Chigurupati et al., 2007).

Previous studies have employed 4-hydroxy-tamoxifen-inducible transgene mice (K14NICDER), and showed that forced ectopic epidermal Notch1 activity resulted in extensive, epidermal proliferation (Figure 3.1 a) and severe inflammation (Figure 3.1 b) (Ambler and Watt, 2007, Ambler and Watt, 2010, Estrach et al., 2006).
This evidence of increasing the main phenotypic hallmarks of skin wound healing following forced ectopic epidermal Notch1 activity, led attention to investigate the role of Notch1 and Notch2 in wound healing. Mechanistic details and/or the site of action of Notch activation in skin injury have not been previously studied. In addition, controllable Notch1 activation, by using the 4-hydroxy-tamoxifen-inducible transgene mice (K14NICDER) model, allowed this study to determine the essential role of epidermal Notch1 and Notch2 in the wound healing process.
To begin exploring the level of Notch activation in response to physical injury during wound healing in normal and wounded skin from C57BL/6 mice, Notch1 and Notch2 activity was analysed at 1, 4 and 7 days post wounding. It has been previously presented that in 4OHT-treated K14NICDER back skin, there was a massive infiltrate of CD3- and CD4-positive cells, with few CD8-positive T cells (Figure 3.1b) (Ambler and Watt, 2007, Ambler and Watt, 2010, Estrach et al., 2006).

A)



B)

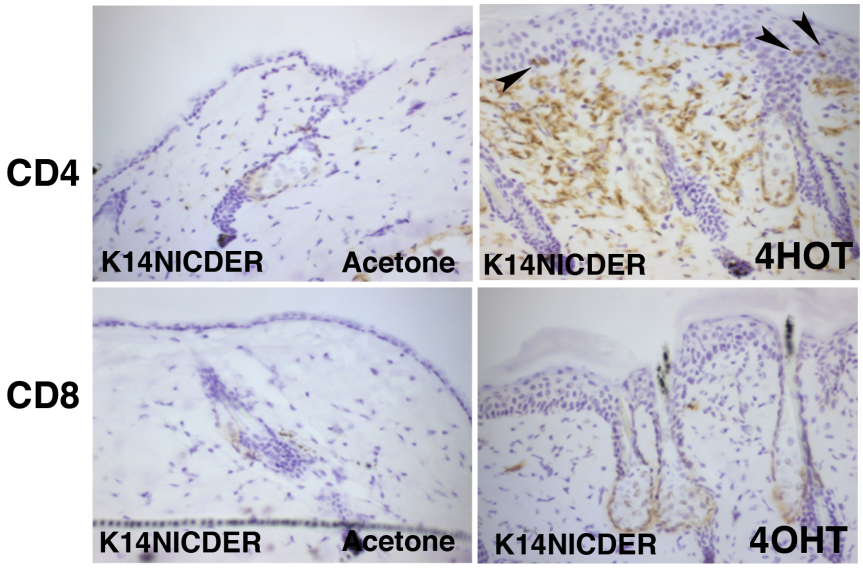


Figure 3.1. Characterisation of K14NICDER transgenic mice.

(**A**)Haematoxylin and Eosin-stained back skin sections of 4OHT-treated wild-type (WT) and K14NICDER littermates. Inset shows a higher magnification view of epidermis and underlying dermis. Epidermal layers are indicated. B, basal; S, spinous; G, granular; C, cornified. (B).**Notch-induced skin inflammations.** Back skin sections of 4OHT-treated or Acetone-treated K14NICDER stained with antibodies to CD4 and CD8 (brown). Arrows indicate T cells in epidermis and dermis (Ambler and Watt, 2010).

Evidence of the involvement of Notch signalling in wound healing and also increasing the main phenotypic hallmarks of skin wound healing by forced ectopic epidermal Notch1 activity drew attention to the need to determine how Notch activity in epithelium interacts with the wound healing process.

Therefore it was hypothesised that epidermal Notch signalling may be active in skin as a response to wound healing. In this chapter, Notch activation during wound healing was examined. Notch1 and Notch2 were both rapidly activated in skin, predominantly within epidermal keratinocytes after injury with different timings, suggesting epidermal Notch1 and Notch2 might play different roles in skin wound healing.

It has also been reported that Jagged 1 acts as a ligand for all Notch receptors. In developing skin, Jagged 1 is detected both in the epidermis and dermis (Powell et al., 1998) whereas in adult skin, Jagged 1 is primarily expressed in the bulb of anagen follicles (Estrach et al., 2006). Research has previously reported the Notch activity via Jagged1in mice. Ambler et al., 2010, demonstrated that in 4OHT-treated K14NICDER skin Jagged 1 was upregulated in the interfollicular epidermis. The increase in Jagged 1 protein was also confirmed by western blotting of total skin lysates (Ambler and Watt, 2010). In addition, a previous report also showed that *Jag1* is positively regulated by Notch signalling (Ross and Kadesch, 2004).

Thus there is clear evidence of an obligate relationship between Jagged1 and Notch activity in maintaining the skin epithelium. There is a feedback loop between Notch activity and Jagged1; Notch activity induces Jagged1 gene expression in epidermal keratinocytes and it has been demonstrated previously that Jagged1 expression in the epidermis is required for Notch1 function (Ambler, 2010). This chapter aims to consider how the Notch signal is transmitted through specific receptors, Notch1 and Notch2 in wound healing.

Previous research, examined the role of jagged 1 in the Notch-induced skin phenotype, by using crossed K14NICDER, K14CreER and $Jag1^{flox/flox}$ strains of mice (Ambler, 2010). This work demonstrated that in these mice, 4OHT treatment results in deletion of *Jag1* in the same cells in which Notch is activated. K14NICDER/K14CreER/$Jag1^{flox/flox}$ (triple) transgenic mice were compared with untreated K14NICDER/K14CreER/$Jag1^{flox/flox}$ mice and littermates that were wild type for Notch. Therefore this work confirmed that epidermal jagged 1 is required for

epidermal Notch- induced dermal jagged 1 expression and the accumulation of dermal cells (Ambler, 2010). Thus to test if jagged-1 expression is dependent on Notch1 and Notch2 activity, K14NICDER/K14CreER/*jag1*$^{flox/flox}$ triple mice lacking functional jagged1 were employed.

3.2 Experimental plan

To begin exploring the level of Notch activation in response to physical injury during wound healing in normal and wounded skin from C57 BL/6 mice, I analyzed Notch1 and Notch2 activity at 1,4 and 7 days post wounding.

Experimental procedures were performed under a UK government Home Office licence (Licence number: 60/3941), in accordance with the UK Animal (Scientific Procedures) Act, 1986. For the wounding experiment seven-week-old male, C57BL/6 mice were used. The generation and characterisation of transgenic mice (K14NICDER (also known as K14N$^{ICD\Delta OP}$ER), K14CreER, *Jag1*$^{flox/flox}$, in which epidermal Notch1 can be induced under the control of 4OHT treatment has been described previously, see section2.1). To activate the NICDER and CreER transgenes, seven-week-old mice were treated topically with 2 mg 4OHT (Sigma) dissolved in acetone (Estrach et al., 2006). Thereafter, 4OHT or acetone was applied topically three times per week for 21 days.

3.2.1 Dermal Wounds and Quantification of Healing

Seven-week-old C57BL/6 were anesthetized using 2 - 2.5% vaporized inhaled isoflurane and the dorsal skin cleansed with (iodine povidone solution – 10%), two full- thickness dermal wounds were created in the skin on the back of the mouse using a 4-mm biopsy punch. A number of mice in each group were euthanized on days 1, 4 and 7 post wounding and skin tissue samples from the wound site were collected for histological and biochemical analyses (Figure 3.2). Animals from each genotype/treatment group (n = 3) were evaluated daily for 7 days following wounding. Wound size was measured and digital photographs of the injury site were taken after injury up to 7 days post wounding.

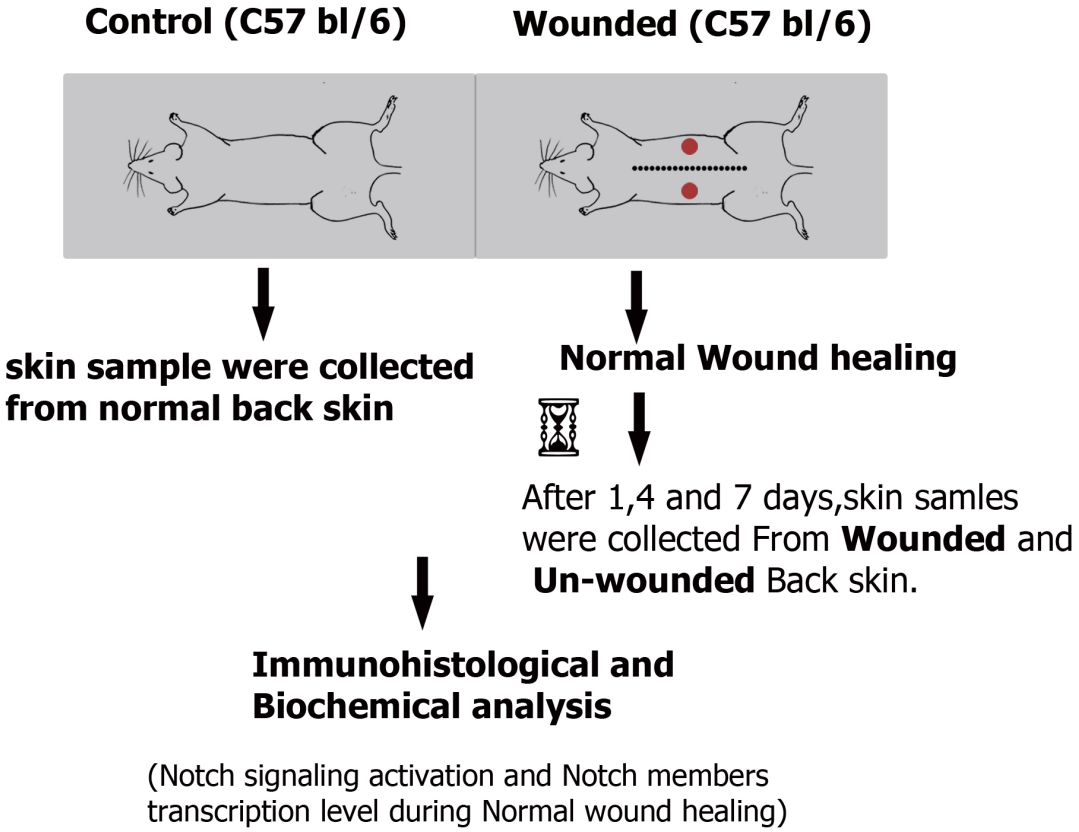


Figure 3.2 Experimental plan1.

Investigate the level of Notch1 and Notch2 activation during wound healing and determine the relation between the activity of Notch1 and Notch2 at different stages of normal skin wound healing.

3.2.2 Epidermal Notch activation via a 4OHT-inducible transgene

K14NICDER, K14CreER, *Jag1*$^{flox/flox}$ and C57BL/6 mice were treated with vehicle (acetone alone) or 2mg of 4OHT (Sigma) dissolved in acetone, applied directly to the normal back skin three times per week, for 21 days. A number of animals in each group were euthanized between 3 and 14 days post treatment and back skin tissue samples were collected from all of the mice for histological and biochemical analyses (Figure 3.3).

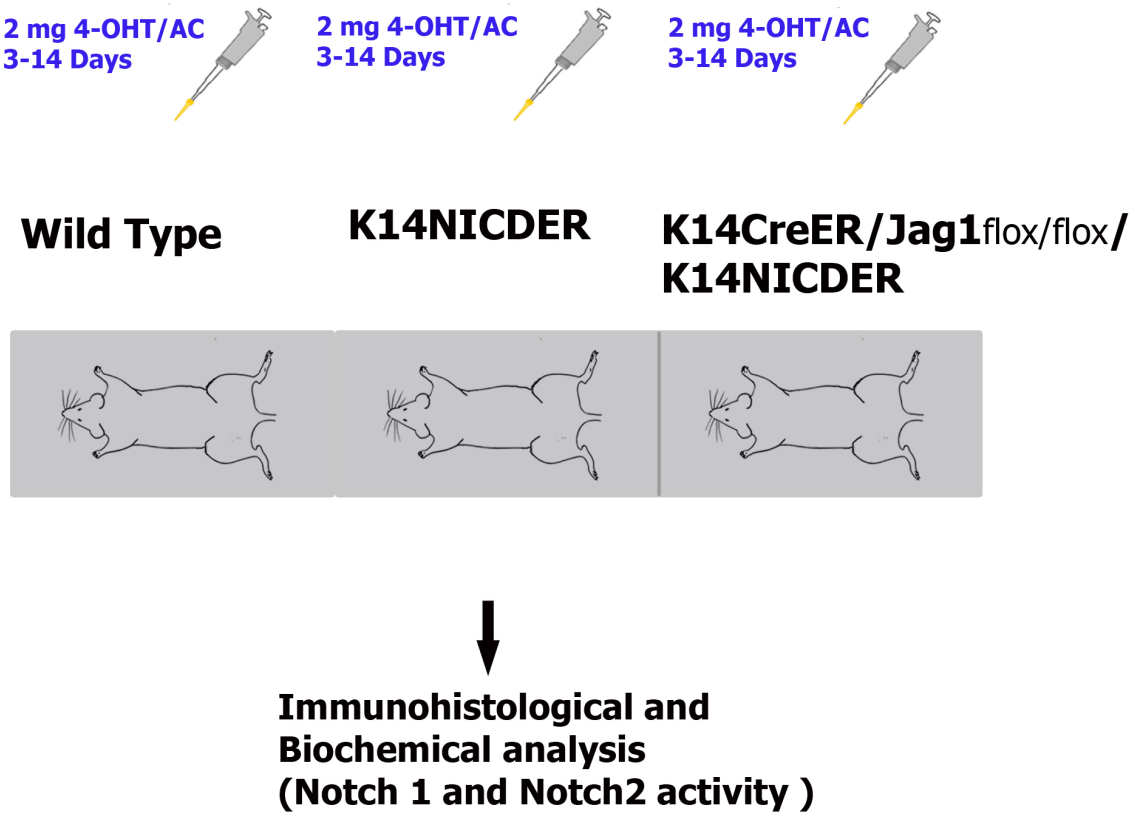


Figure 3.3. Experimental plan 2

Investigate the level of Notch1 and Notch2 activation in response to forced epidermal Notch activity via the K14NICDER transgene, and whether this activation is caused by a jagged-1 dependent Notch1 forced activity.

3.3 Results

3.3.1 Histological analyses of normal Skin Wound Healing

To address whether the Notch signalling pathway is activated during normal wound healing, 4 mm cutaneous wounds were performed in male, C57 BL/6 mice and tissue collected for molecular analysis. Paraffin embedded sections of wounded and un-wounded site of skin tissue were prepared as described previously, section 2.3.1, and stained with haematoxylin and eosin (H&E) to examine general histological characteristics (Figure 3.4). The stratified squamous un-wounded epithelium adjacent to the wound site (Figure 3.4, normal skin) showed normal characteristics of un-wounded epithelium at all time points (A, D, G). The structure of all skin layers (epidermis, dermis and hypodermis) is all well organised. By 1-day post wounding, the wound area is filled by a blood clot (C). In the transitional site, that is immediately next to the migrating epithelial tongue (B), an increase in the thickness of the spinous layer has initiated, as a result of the migration of the epithelium behind the leading edge. By day 4 post wounding, an expansion in fibroblast migration and a rapid proliferation of epithelial cells to the wound bed are detected, which initiates the proliferation and the formation of extracellular matrix. The newly formed neoepithelial layer from the marginal edges of the wound, termed granulation tissue due to its appearance, is evident and h as thickened (approximately 100 microns) (F). A healed wound is demonstrated by a thickened epidermal layer within the newly healed lesion and transitional site that is not seen in uninjured skin. By 7 days post wounding, although the epidermal thickness (50-60 microns) had not yet been restored to the normal level, wound is completely filled with granulation tissue (I). The wound is completely covered with a neoepidermis. Increasing the epithelial thickness, granulation tissue formation and reduction in the size of the wound are all parameters of normal wound healing process and are all observed in this wound healing model organism. Quantification of epithelial thickness, at different stages of wound healing in H&E staining sections is shown in Figure 3.5. This quantification shows a statistically significant increase in thickness of the wounded epithelia, 4 days post wounding when compared to the normal skin adjacent (P value is < 0.0001). Comparing the wounded epithelial thickness 1 days post wounding to the normal skin

adjacent showed nearly 50% less than it was on 4 dpw, however thickness at 1dpw is still significant (P value=0.003).

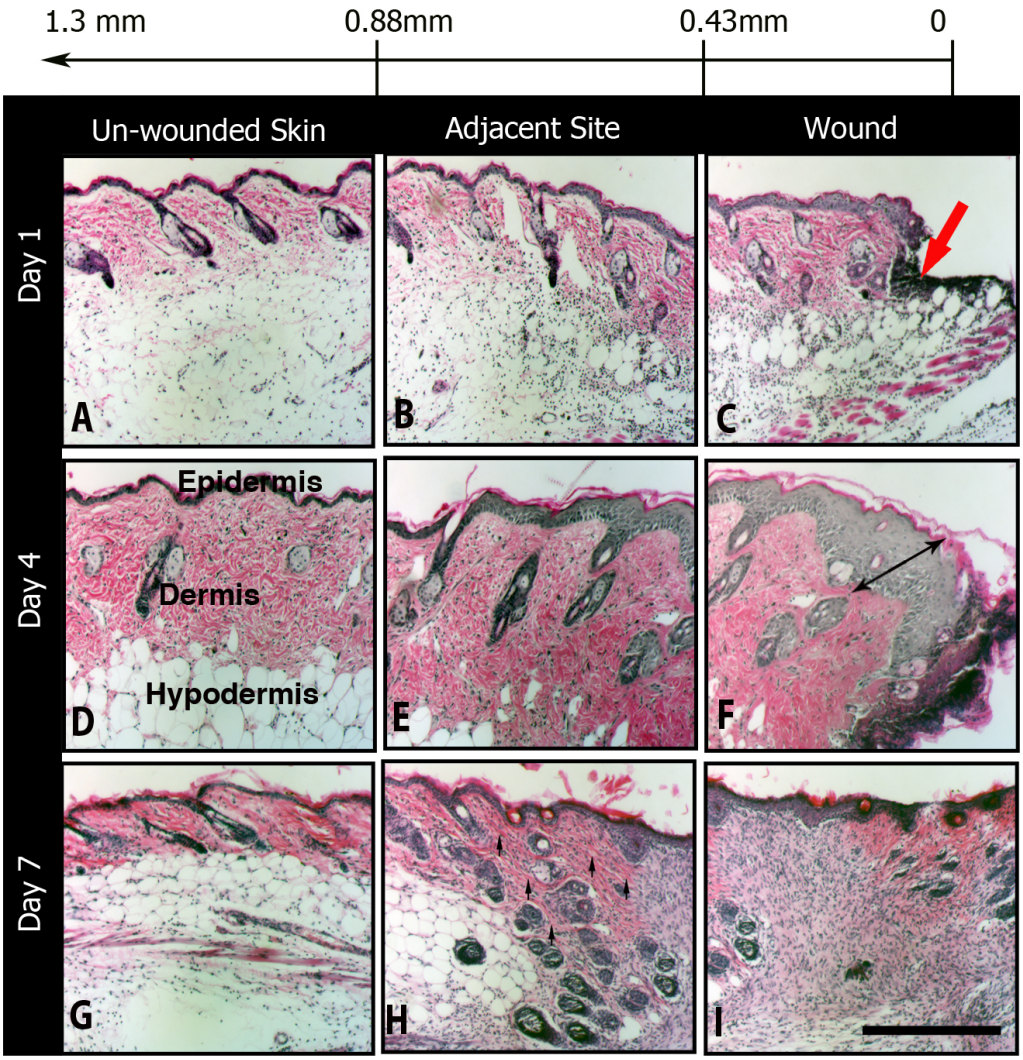


Figure 3.4. Representative histological features of cutaneous wound healing in mice.

Full-thickness, 4 mm, wounds were created in seven-week-old C57BL/6 mice. (A-C Upper panel, D-F middle panel and G-I lower panel) Hemotoxylin and eosin stained back skin section of tissue removed 1, 4 and 7 days post wounding1dpw, respectively (panels A, D and G) displaying the histological characteristic of the unwounded epithelium, located around 0.88 mm from the wound site (un-wounded adjacent). This is followed by growth in the thickness of the spinous layer (adjacent) located 0.44 mm from the wound site (panel B, E and H), and existence of migration epithelial cells

that form an epithelial tongue at the wound edge (C, F and I). n=3. 2 biopsies from each). Red arrow in panel C indicating blood clot. Black arrow in panel F shows the epidermal thickness. Black arrows in panel H indicating myofibroblasts. Scale bar, 200 microns.

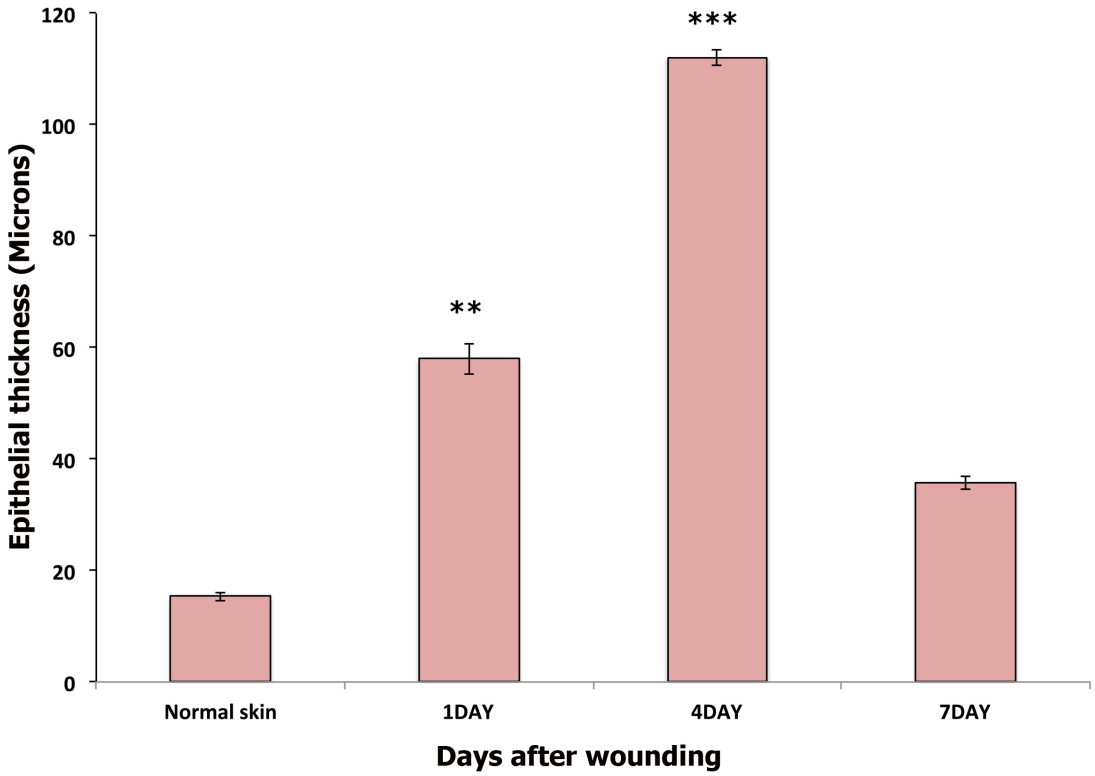


Figure 3.5. Epithelial thickness at wound site.

The thickness of the epithelium was measured in histological slides at wound site on day 1, 4 and 7 post-wounding and compared to the normal skin from unwounded animals. Measurements were done in triplicates from 3 different time points and two wounds per each mouse (n=3). Data shown as mean ± SEM; **P value <0.005, ***P value <0.0005(compared to normal skin). Student's t-test.

3.3.2 The Notch signalling pathway is activated in wound healing

The Notch pathway is a key cell-autonomous signalling pathway that directs cell fate choices, yet also controls pleiotropic functions in the skin. A previous study using topically-applied Notch pathway activators and inhibitors suggested that Notch might be involved in wound healing and previous work showed that forced, ectopic epidermal Notch activity resulted in extensive, epidermal proliferation and severe inflammation –two phenotypic hallmarks of skin wound healing (Ambler, 2010). Thus, I analysed Notch1 and Notch2 activity at 1,4 and 7 days post wounding. An inactive form of the Notch protein is synthesised in the cytoplasm and translocated into the endoplasmic reticulum, before it reaches the plasma membrane due to a proteolytic cleavage (S1), it is made into a ligand-accessible form. Cleavage results in a C-terminal fragment N (TM) and a N-terminal fragment N (EC). After ligand binding the yield of two photolytic cleavages (TACE and NEXT) is the released intracellular domain (NICD) from the membrane (Kopan and Ilagan, 2009). Immunohistochemical staining of wound samples in this investigation exhibited an increase in the levels of activated Notch1 and Notch2 in cells adjacent to the wound site at 4 days post wounding. These data demonstrated that at day 4, Notch1 was primarily active in basal and suprabasal epidermal keratinocytes and Notch 2 in both the epidermal sheet and underlying dermal stroma (Figure 3.6). To validate the data, levels of activated Notch1 and Notch2 proteins at 1, 4 and 7 days post wounding were quantified by Western Blotting. These data demonstrated that wounding caused in an increase in Notch1 and Notch2 activity with peak Notch1 activity detected 4 days post wounding (16x)(Figure 3.7) and Notch2 activity 1 day post wounding (2.5x) (Figure 3.8). Cleaved Notch1 (Val1744) antibody detects endogenous levels of the Notch1 intracellular domain (NICD) only when released from the membrane by cleavage between Gly1753 and Val1754 (equivalent to Gly1743/Val1744 of murine Notch1). This antibody does not recognize full-length Notch1, or Notch1 cleaved at other positions.

These results demonstrated that although the Notch pathway is active throughout wound healing, maximal activation of Notch1 and Notch2 occurs at different time points and in different cells suggesting separate roles during the healing programme.

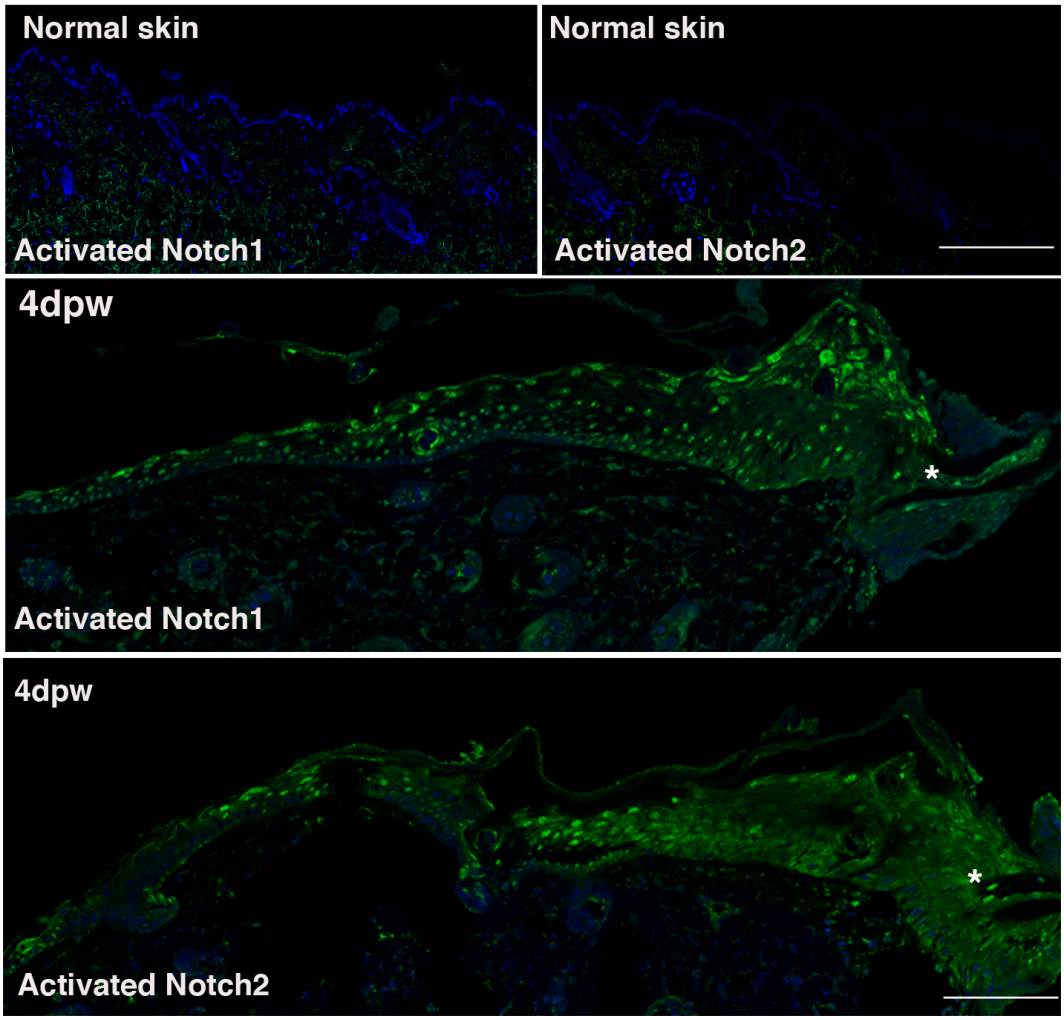


Figure 3.6. Notch1 and Notch2 are activated in response to an injury in skin.

Representative immunohistochemistry of a cross-section of back skin tissue from punch-wounded wild type mice at, 4 days post wounding (4dpw) and normal skin (control). Antibodies specific for activated Notch1 and Notch2 reacted with cells adjacent to wound 4dpw. Note activated Notch1 is detectable in epidermal cells while activated Notch2 is detectable in both epidermal and dermal cells. White stars indicate the center of the wound. Scale bar equals 100 microns.

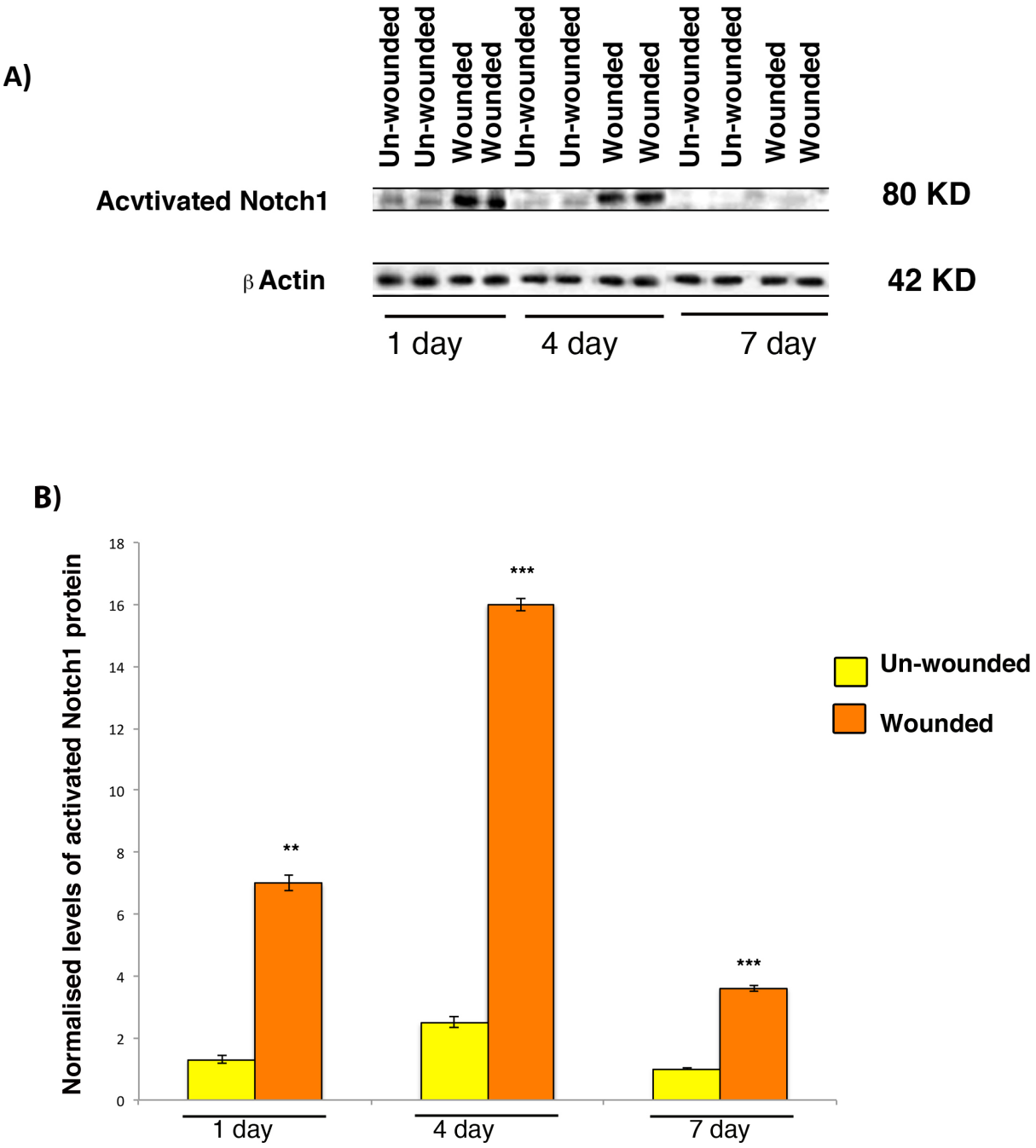


Figure 3.7 Quantified levels of activation Notch1 during skin wound healing.

(A) Representative immunoblot, showing quantified level of cleaved, activated Notch1 (80 KDa) and β-actin (42KDa) in lysates (20 mg/lane) in skin tissue samples from three independent punch-wounded (2 wounds on each mouse) wild type mice, collected at the indicated post wounding time points. (B) Quantified levels of cleaved,

activated Notch1 protein by antibody probes in western immunoblotting assay. As loading controls, Notch1 level was standardised to β-actin levels as a loading control and normalised to the average back skin protein levels (designated as 1) of Notch1 in uninjured littermate mice (n=3). “Un-wounded” back skin is taken from a punch-wounded mouse at a distal site (minimum 2 cm) from wound site. Student t-test. (Data shown as mean $\pm$ s.e.m of three individual experiments). **p value <0.005;***p value <0.0005.

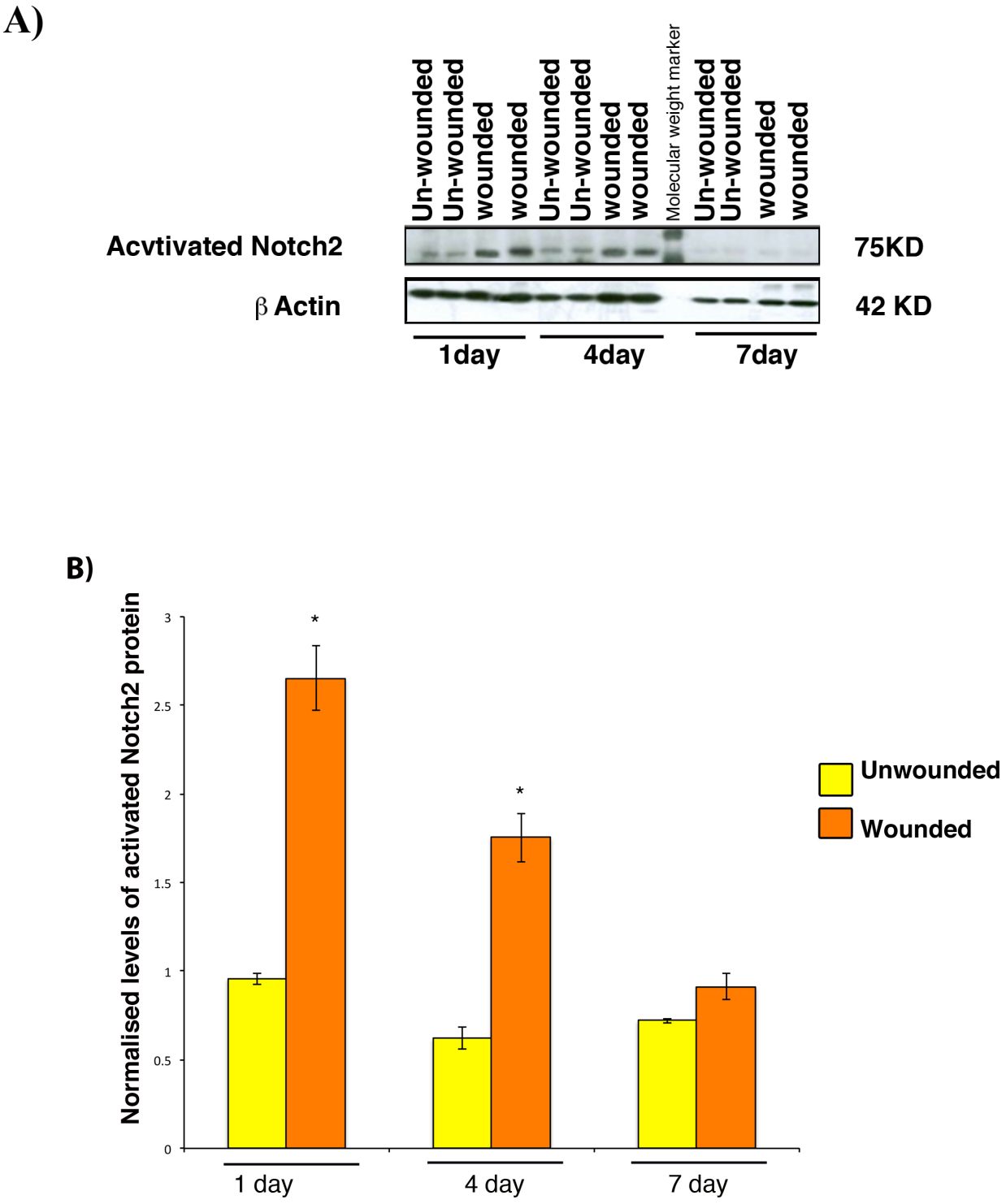


Figure 3.8 Quantified levels of activation Notch2 during skin wound healing.

(A) Representative immunoblot, showing quantified level of cleaved, activated Notch2 (75 KDa) and β-actin (42KDa) in lysates (20 mg/lane) in skin tissue samples from three independent punch-wounded (2 wounds on each mouse) wild type mice,

collected at the indicated post wounding time points. (B) Quantified levels of cleaved, activated Notch1 protein by antibody probes in western immunoblotting assay. As loading controls, Notch1 level was standardised to β-actin levels as a loading control and normalised to the average back skin protein levels (designated as 1) of Notch2 in uninjured littermate mice (n=3). "Un-wounded" back skin is taken from a punch-wounded mouse at a distal site (minimum 2 cm) from wound site. (Data shown as mean ± s.e.m of three individual experiments). *p value <0.05.

It has been reported that epidermal Notch1 activation resulted in up regulation of jagged1 in both the epidermis and dermis. It also has been demonstrated that jagged1 has a key function in non-cell autonomous Notch signalling in skin (Ambler, 2010). In this project, the level of full-length jagged1 was quantified via Western Blotting. Data verified a significant up regulation (5.6x) of full-length jagged1 by day 7 after wounding compared to the level of jagged1 in control-uninjured mice. However, the level of Jagged1 from day 1 to day 4 was increased ($p<0.05$) compared to controls, (Figure 3.9). These data also shows a significantly ($p<0.0005$) higher level of jagged1 on day 7 post wounding in compare with day 4 in wounded skin. Furthermore, the localisation of this ligand in the wounded area of skin after 1, 4 and 7 days post injury was confirmed by immunohistochemistry (Figure 3.10).

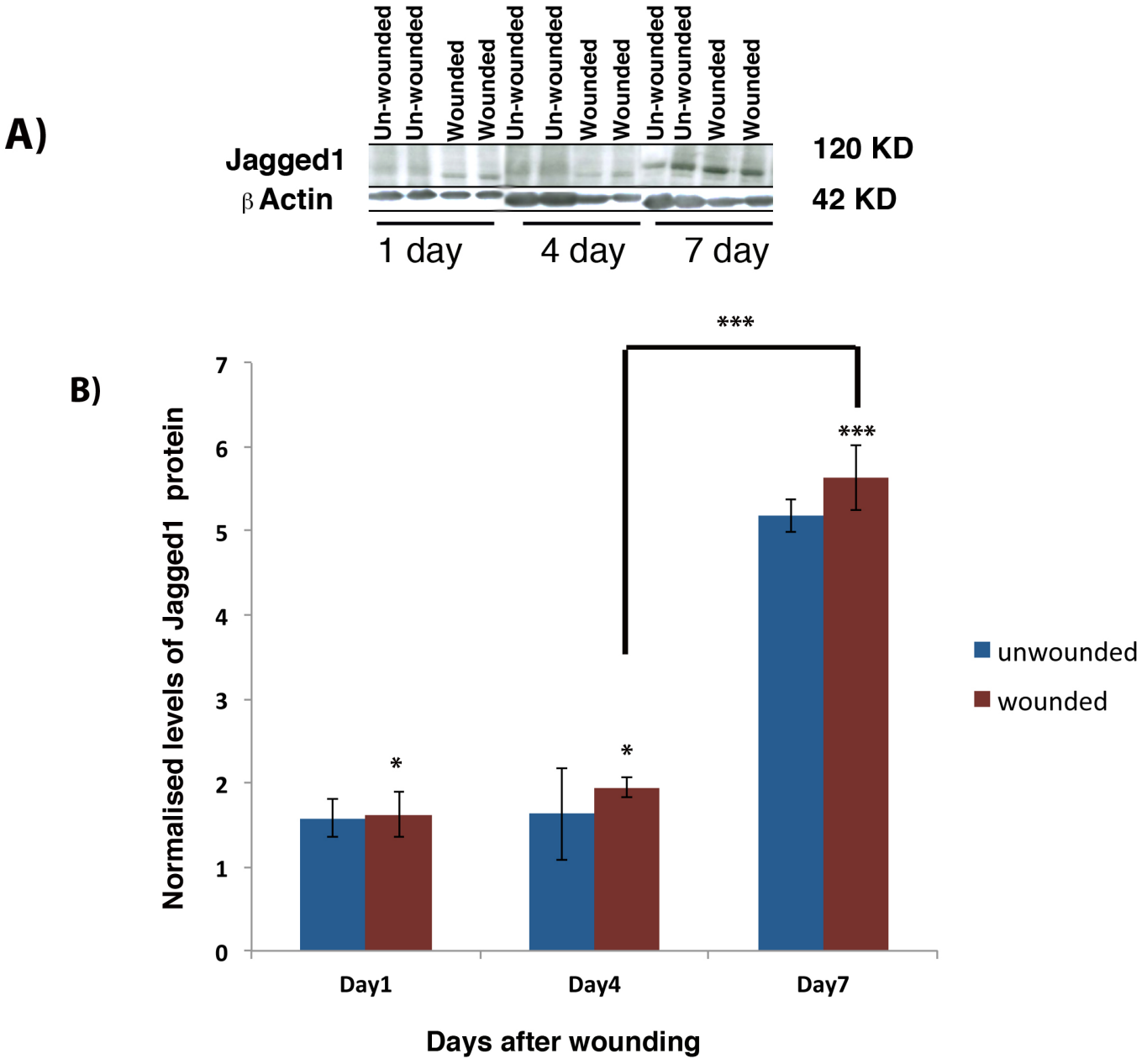


Figure 3.9 Quantified levels of full-length Jagged1 during skin wound healing.

(A) Representative immunoblot, showing quantified level of Jagged1 (120 KDa) and β-actin (42KDa) in lysates (20 mg/lane) in skin tissue samples from three independent punch-wounded (2 wounds on each mouse) wild type mice, collected at the indicated post wounding time points. (B) Quantified levels of full-length Jagged1 protein by antibody probes in western immunoblotting assay. As loading controls, Jagged1 level was standardised to β-actin levels as a loading control and normalised to the average back skin protein levels (designated as 1) of Jagged1 in uninjured littermate mice

(n=3). “Un-wounded” back skin is taken from a punch-wounded mouse at a distal site (minimum 2 cm) from wound site. (Data shown as mean ± s.e.m of three individual experiments). *p value <0.05, ***p value <0.0005 (wounded skins are compared to the un-wounded at each time point).

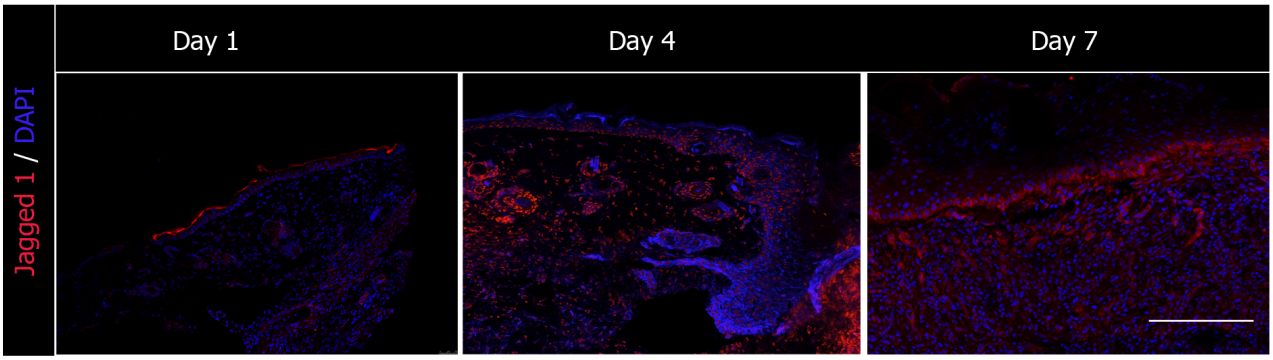


Figure 3.10. Jagged1 involved in skin wound healing.

Representative immunohistochemistry of a cross-section of back skin tissue from punch-wounded wild type mice at 1,4 and 7 days post wounding. Antibody specific for full-length jagged1 (red) reacted with cells in epithelium at 7dpw. Note the full-length jagged1(before being cleaved) is detected in cells within epidermal layer at 7dpw, which is delineated from the dermis by a white dotted line. Whereas this full-length protein is not detectable at 1 and 4dpw. Scale bar equal 100 microns.

3.3.3 Transcription of Notch family genes during wound healing

The results shown in section 3.3.2 suggest that Notch activity is increased during the early stages of the wound healing response. To further correlate skin epithelial Notch activity initiated by wounding in normal mice with expression and activity of Notch; I examined mRNA levels of *Notch1*, *Notch2*, and *Jagged1* during the process of wound healing. To determine how Notch signaling receptors (Notch1 and Notch2) and jagged1 ligand gene expression are during the wound healing process, I analyzed gene expression profile of wounded skin from C57BL/6 and littermate controls. However, by 1 day after injury, Notch1 and Notch2, mRNAs were increased (1.5x) in wounded skin as shown by real time qPCR assays comparing to littermate control (skin sample from un-wounded animal). Levels of Notch1 and Notch2 expression were higher at the early days (1-2) post wounding. There was no statistical difference between the expression level of Notch1 and Notch2 at day 4 and 7 compared to littermate controls) (Figure 3.11). Comparing the expression level of Jagged1 at applied time points demonstrated a statistically significant difference between day 4 and 7, compared to the littermate controls. There was no statistical difference observed between the level of Jagged1 expression on day 1 post wounding compared to control littermates (Figure 3.12).

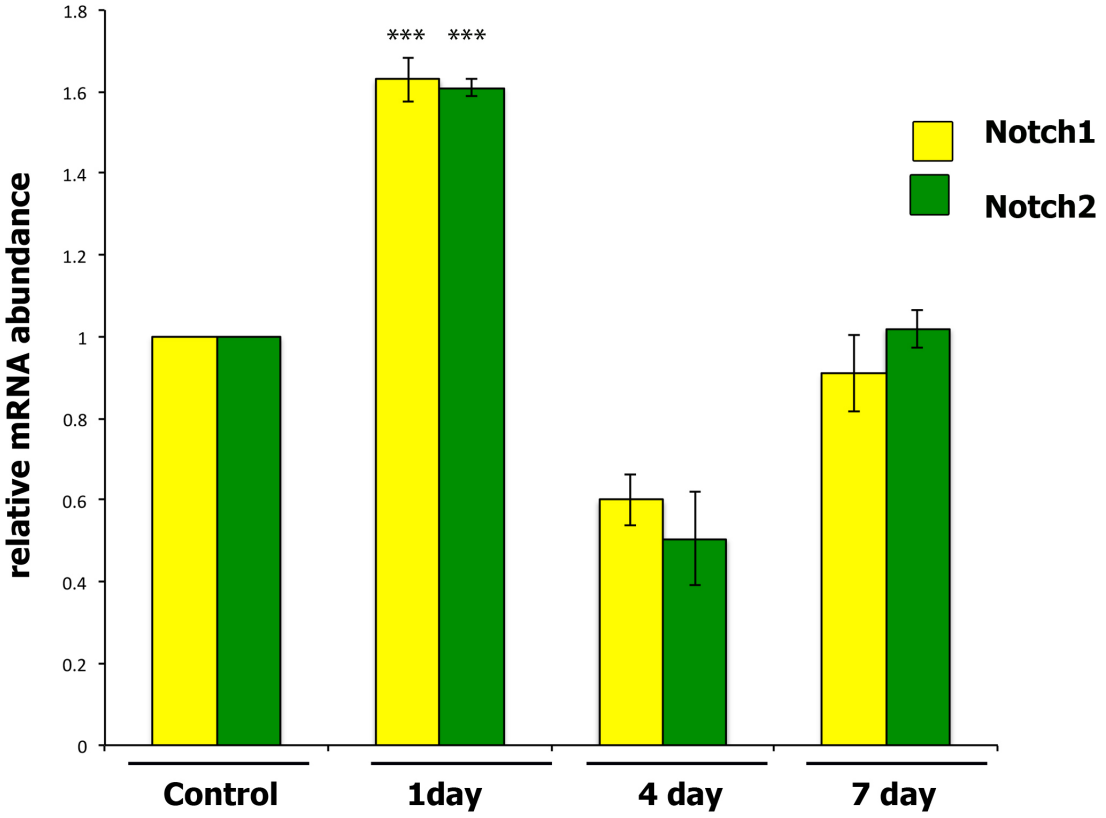


Figure 3.11. Relative Notch1 and Notch2 expression detected by qPCR.

Quantitative polymerase chain reaction of cDNA from punch-wounded wild type mice (n=3), collected at the indicated post wounding time points. Results (mean± s.e.m) are expressed relative to Un-wounded mice (=1) (n=3 at day1) for each TAQman probe. ***p value <0.0005.

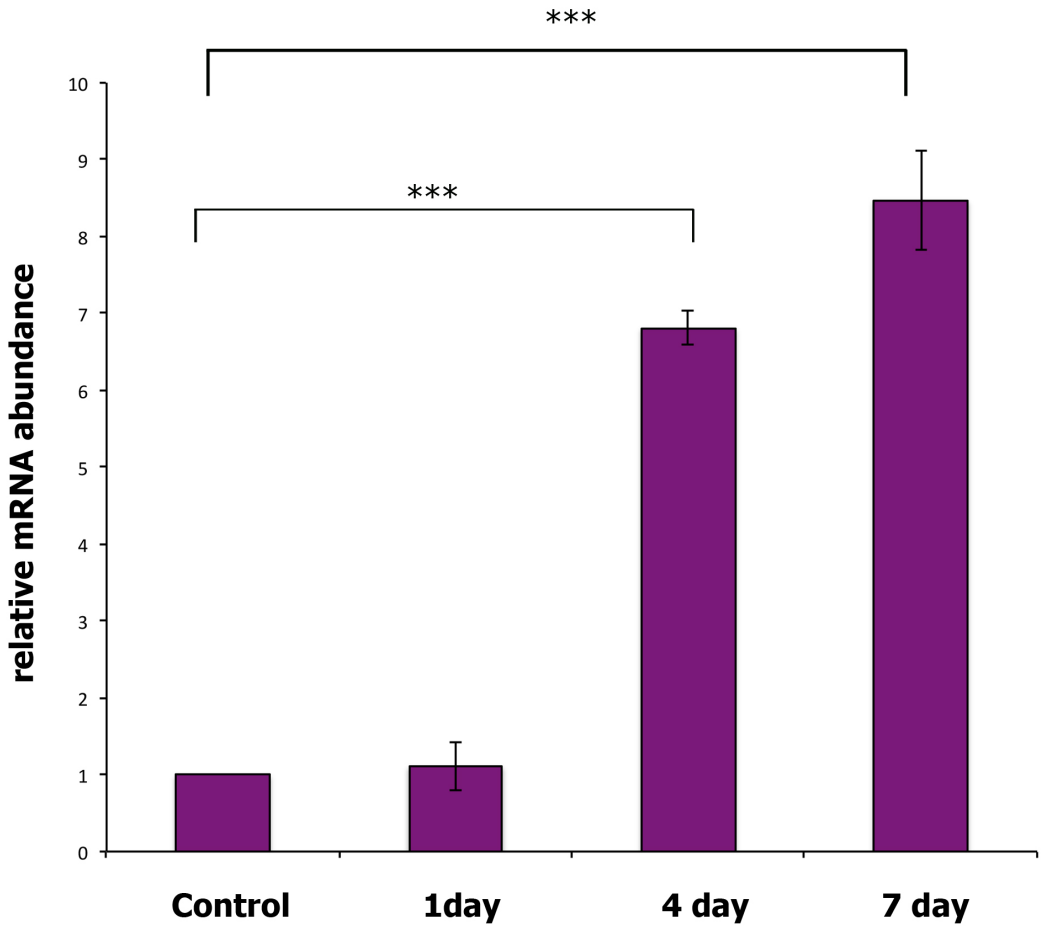


Figure 3.12. Relative Jagged1 mRNA levels detected by qPCR.

Quantitative polymerase chain reaction of cDNA from punch-wounded wild type mice (n=3), collected at the indicated post wounding time points. Results (mean+ s.e.m) are expressed relative to Un-wounded mice (=1) for each TAQman probe. ***p value <0.0005.

3.3.4 Notch2 activation in response to forced epidermal Notch activity via the K14NICDER transgene

During normal wound healing, activation of Notch1 and Notch2 are detected. It has been reported that jagged1 acts as a ligand for all of the family of Notch receptors (Blanpain et al., 2006). As Jagged1 acts as a ligand for all Notch receptors and Notch2 is activated during normal skin wound healing, this investigation determined if Notch2 was activated in a Jagged1-dependent mechanism in 4HOT-treated NICDER transgenic skin.

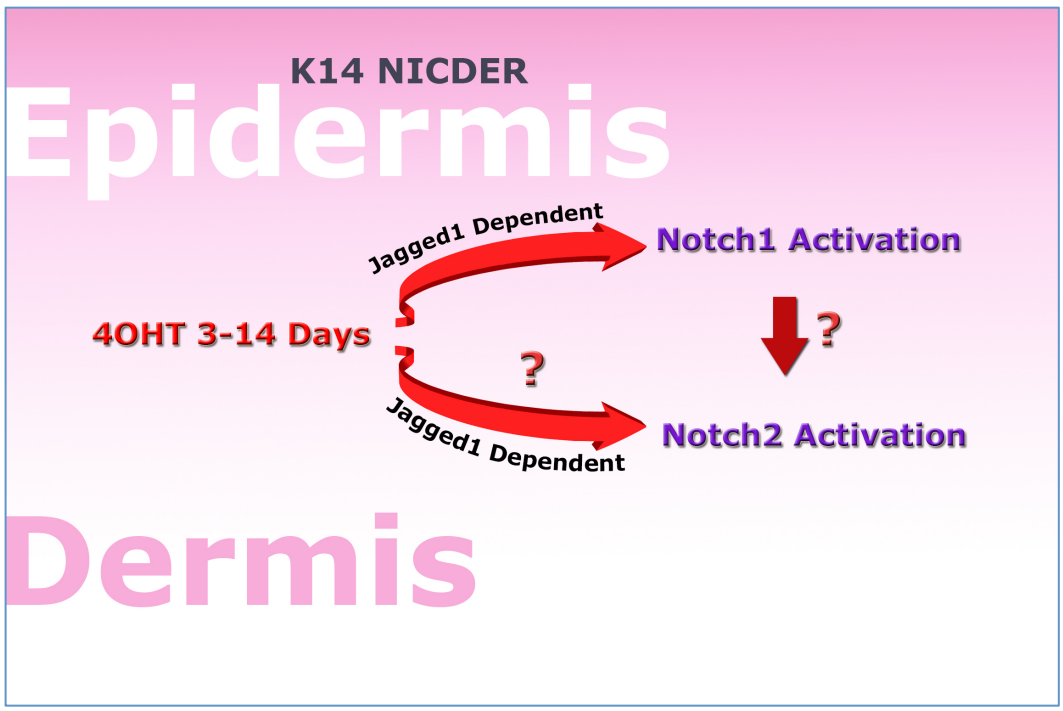


Figure 3.13 in absence of physical damage if Notch2 was activated in a jagged1-dependent mechanism in K14NICDER transgenic mice

For this reason Notch activation was forced in K14NICDER transgenic mice, where a 4-hydroxy-amoxifen (4OHT)-inducible, Notch1 intercellular domain (NICD) is controlled by the keratin 14 promoter. Back skin of K14NICDER transgenic or littermate control mice was treated with 4OHT for 3-14 days before analysis. A 4.9-fold increase in Notch2 activity was detected in K14NICDER skin 4OHT-treated for

3 days compared to control, (Figure 3.14) but epidermal and dermal Notch2 activity was undetectable in K14NICDER/K14CreER/*ja1*$^{flox/flox}$ triple mice lacking functional jagged1 (Figure 3.15). Therefore these data presented that the increase of Notch2 activation in 4OHT-treated k14NICDER transgenic skin after 3 days, strongly suggests that Notch2 may have also been activated in a jagged1-depended mechanism.

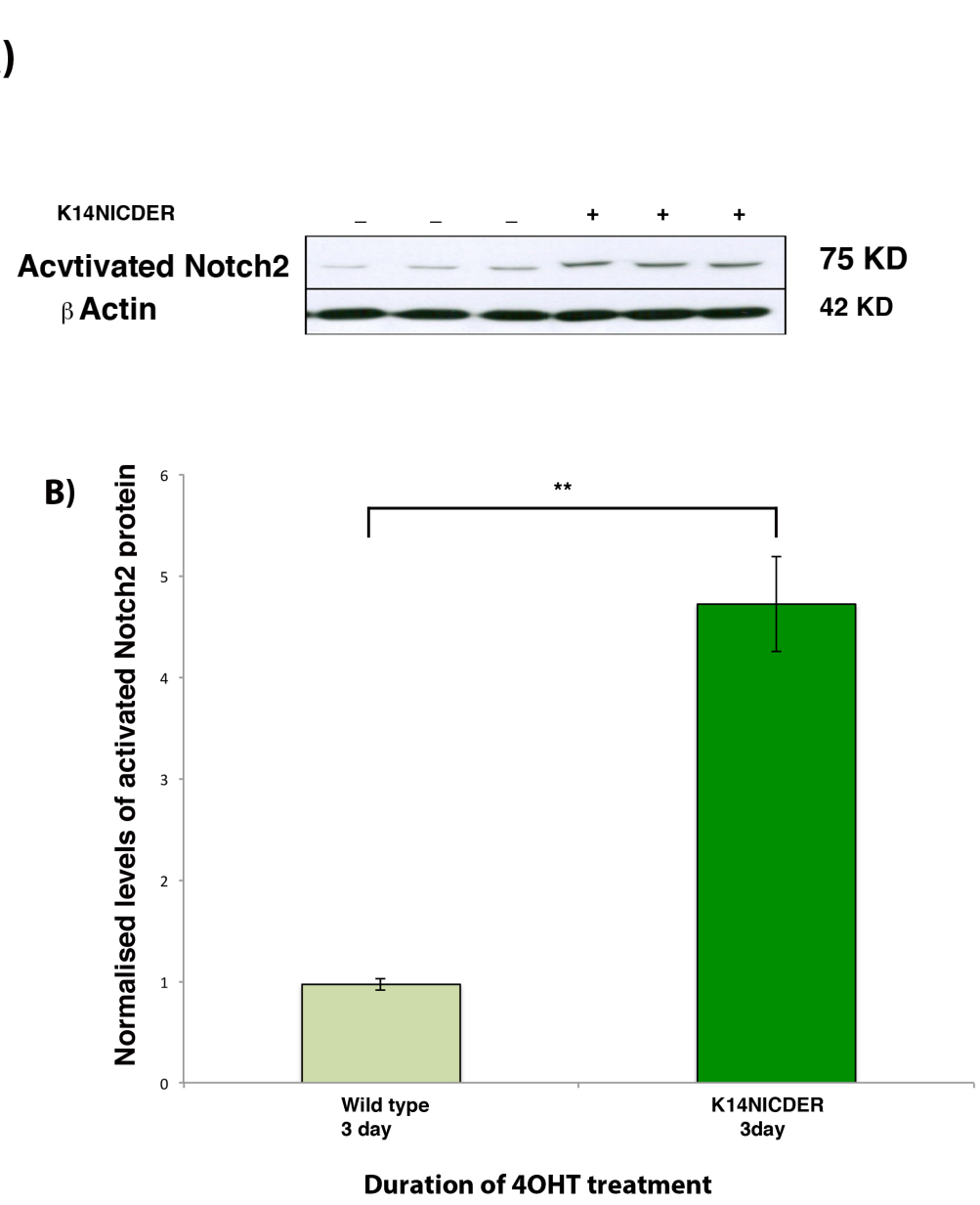


Figure 3.14 Forced epidermal Notch1 activity via the K14NICDER transgene, results in activation of Notch2.

(A) Representative immunoblot, showing quantified level of cleaved, activated Notch2 (75 KDa) and β-actin (42KDa) in lysates (20 mg/lane) in skin tissue samples from K14NICDER and littermate control mice, 4OHT-treated for 3 days (n=3) collected at the indicated post wounding time points. (B) Shows the quantitative analyses of immunoblot. The level of activated Notch2 was normalised to β-actin level. **p value <0.005 .

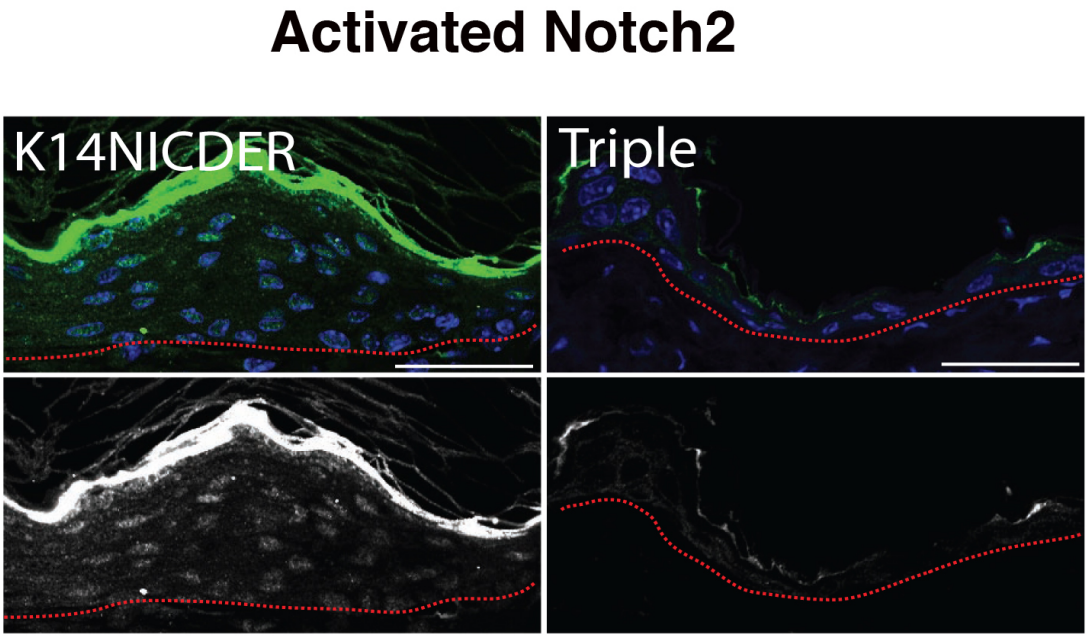


Figure 3.15. Notch2 activation in K14NICDER is a Jagged-1 dependent mechanism.

In K14NICDER /Jag1$^{flox/flox}$/K14NICDER triple transgenic mice lacking functional jagged1 cells reactive for activated Notch2 were not detected. Scale bar equals 100 microns.

3.4 Discussion

The critical involvement of Notch signalling pathway in cell fate determination during embryogenesis and also in postnatal life is well established. Data from ongoing studies indicate that Notch signaling orchestrates the process of epidermal differentiation and proliferation through the sequential activity of different Notch ligands, receptors and downstream pathways (Artavanis-Tsakonas et al., 1995, Bray, 2006, Okuyama et al., 2008). In normal human skin, the Notch receptors and ligands are abundantly expressed in keratinocytes within the epidermis (Artavanis-Tsakonas et al., 1995, Blanpain et al., 2006).

It has been suggested that Notch signaling might be involved in wound healing by a previous study that had used topically applied pan-Notch activator and inhibitor (Chigurupai et al., 2007). The effect of Notch signalling on skin wound healing, demonstrated a significant enhancement in wound healing in mice treated with a Notch ligand, Jagged peptide, and a delayed wound repair rate in response to Notch inhibitors (Chigurupati et al., 2007). However, the site of action and mechanism remain less clear. Recently it has demonstrated forced activation of epidermal Notch signaling promotes dermal accumulation of immune cells via epidermal production of TNFα and epidermal proliferation, resembling the remarks of early and middle stages of wound healing (Ambler and Watt 2010; Estrach et al., 2006). These results suggest a potential link between epidermal Notch signaling and skin wound healing.

The initial inflammatory response to wounding is typically the release of pro-inflammatory signals from local damaged tissue, following which the inflammatory cells infiltrate to the site of injury. An initial inflammatory response and subsequent changes in epithelial and endothelial cells, in order to close the wound, are all involved during the healing process to regenerate the skin tissue. A previous study demonstrated that forced ectopic epidermal Notch activity caused epidermal proliferation and severe inflammation (Ambler and Watt, 2010). The consequence of an initial inflammation is able to influence the re-epithelialisation and connective tissue contraction that are all hallmarks of skin wound healing. Hence the activation of Notch1 and Notch2 at day 1, day 4 & day 7-post wounding were analysed. These findings suggest that activated Notch1 and Notch2 levels were highest in the inflammatory and proliferative phases of the wound response. This project also confirmed that by seven days post wounding, when the wound has closed, both

Notch1 (Figure3.7) and Notch2 (Figure 3.8) activity returned to the level of unwounded skin. Recent investigations have shown the inhibition of wound closure and pro-inflammatory factors in the wound site as consequences of blocking Notch1 and Notch2 (Ambler et al, unpublished data), thus it can be concluded that Notch1 and Notch2 play a key role in regulating the wound response.

These findings suggest an increase in Notch1 and Notch2 activity in response to an injury at the initial phases of healing process. Maximum Notch1 activation was spotted on 4 days post wounding, whereas peak Notch2 activity detected within the initial inflammation stage, 1 day post wounding (Figure 3.8). An inflammatory related function for Notch2 is proposed by these data, suggesting that Notch2 as a possible coordinator element for the early events in the wound-healing cascade. These results are confirmed and further extended by ongoing work which has shown Notch activity can drive innate lymphoid cells (ILCs) recruitment (unpublished data, Ambler,et al).
However, all 4 Notch receptors are expressed in skin epithelia and considerable evidence in support of a well-maintained role for Notch receptors in interfollicular cell fate determination has developed, therefore it would be interesting to determine if other Notch receptors have a contributing role in wound healing (Okuyama et al., 2008, Watt et al., 2008). In this study the activation of Notch1 and Notch2 was considered. Notch1 and Notch2 receptors are abundantly expressed in keratinocytes within the epidermis in normal human skin. It has been reported that Notch1 and Notch2 are both required for embryo viability, are widely expressed in mammals, and are structurally conserved (Kraman and McCright, 2005).
There is not much evidence to confirm if these two receptors are functionally redundant or if they have unique capabilities related to differences in their structure. It has been reported that, the amino acid sequences of a large region of the Notch intracellular domain are not highly conserved and thus may be able to interact with distinct transcription factors and mediate the expression of different sets of genes (Kraman and McCright, 2005). These authors stated that even though the amino acid sequences of NICD region are only 37% identical, the C-terminal region of the Notch1 intracellular domain could functionally replace that of Notch2 *in vivo*.
Therefore, they concluded that, NICD, or the activation domain, is functionally conserved between Notch1 and Notch2. More evidence for non-redundant receptor function can be derived from tissues where both Notch1 and Notch2 are coexpressed,

but where deletion of one or the other results in a phenotype. In epidermal cells for instance, combined loss of Notch1 and Notch2 results in more severe epidermal phenotypes than loss of Notch1 alone (Krebs et al., 2000).

Previous studies demonstrated that, Notch1 and Notch2 ICDs are functionally redundant, although their activation is dependent on differential interaction with ligands or the effect of extracellular domain modifiers (Hicks et al., 2000)

Data presented demonstrate altered rate of Notch1 and Notch2 activity during the normal skin wound healing cascade. Strong nuclear expression of both active Notch1 and Notch2 throughout the epidermis in the inflammatory phase was apparent. However, active Notch2 was detected both in the epidermal sheet and underlying dermal stroma, with. Notch1 only located in basal and suprabasal epidermal keratinocytes. Thus, localisation specificity of cleaved Notch1 and Notch2 can be concluded and this provides striking evidence that the maximal activation of Notch1 and Notch2 occurs at different time points during wound healing, which can be correlated with functional specificity and contribute to skin wound healing of these receptors.

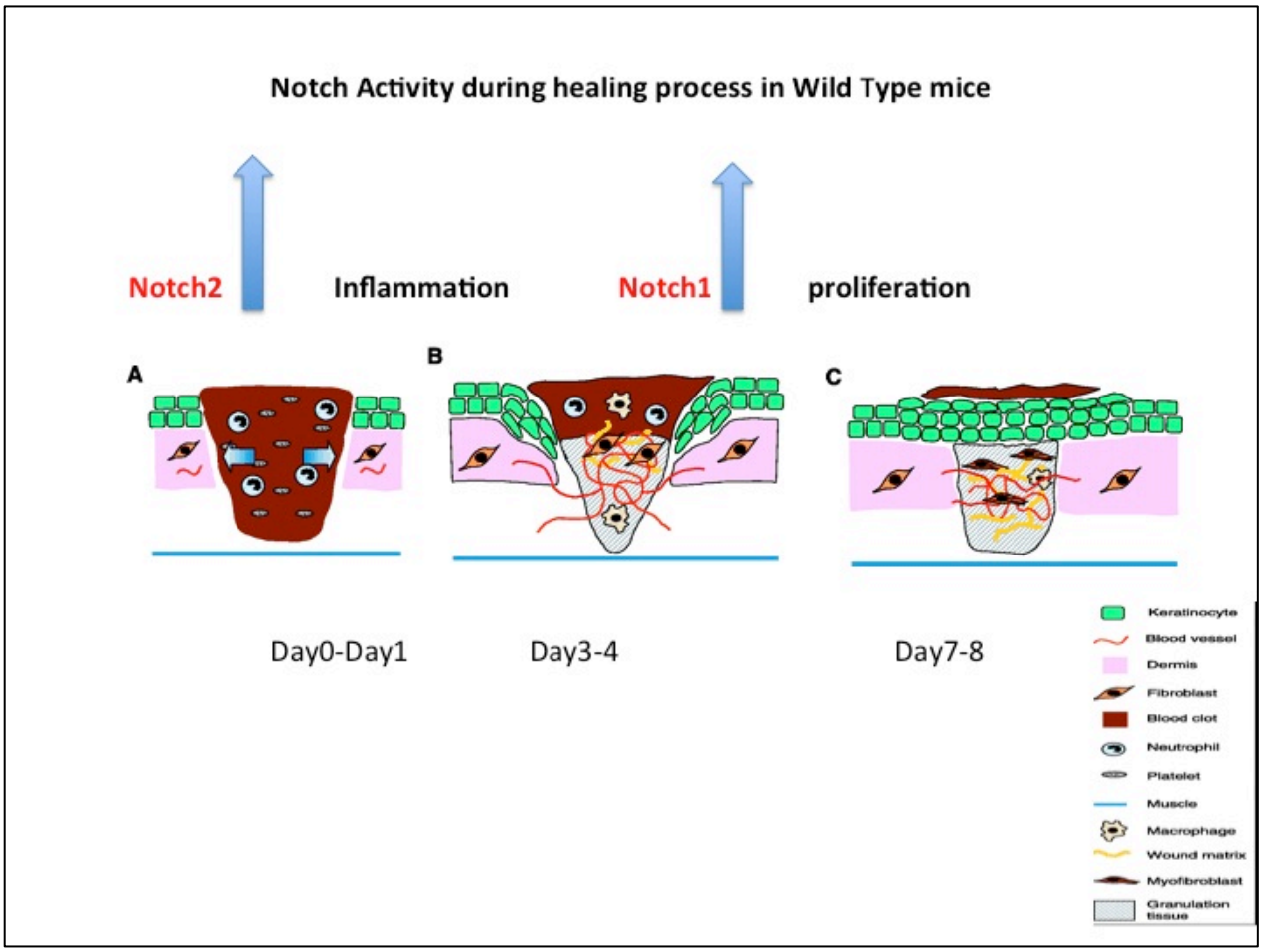


Figure 3.16: Notch signalling activation in the skin during the wound healing.

It has been reported that the consequence of epidermal Notch1 activation is an increase in expression of Jagged1 in the epidermis and dermis of K14NICDER mice (Ambler and Watt, 2010). While previous studies indicated a jagged1-dependent mechanism of Notch1 activation, these data also suggest that both Notch2 and Notch1 are activated during normal wound healing, it was also determined if Notch2 was activated shadowing the same mechanism in K14NICDER mice. These findings were also confirmed when Notch 2 activity was shown to be undetectable in K14NICDER/K14CreER/*jag1* $^{\text{flox flox}}$ triple mice lacking functional Jagged1.

Both the Notch receptors and their ligands are transmembrane proteins, thus signalling is restricted to adjacent cells. Two proteolytic events in the Notch receptor are the result of ligand binding which terminates with the translocation of Notch intercellular domain (NICD) to the nucleus. It has been confirmed that the activation of Notch signalling pathway is affected by post-translational and post-transcriptional modification, as well as trafficking of Notch ligands and receptors (Bray, 2006). To explain the structural modifications that the ligands and receptors in Notch pathway are prone to, different models have been proposed, summarised by Bray, 2006.Similar to other membrane-bound proteins, the surface level of Notch receptors and ligands are maintained appropriately through ubiquitylation-dependent endocytotic degradation in order to prevent over-activation of Notch signalling (Weinmaster and Fischer, 2011). The underlying mechanism is not completely understood, however, two models involving ligand recycling and pulling force have been proposed in order to explain how ligand endocytosis in signal sending cells promotes Notch activation. Thus, the surface level of Notch receptor and ligand and subsequent Notch activity are regulated by ubiquitylation-dependent constitutive endocytosis that induces protein degradation (Weinmaster and Fischer, 2011).

However, the determination of ligand cleavage and the functional consequences of ligand proteolysis on Notch signalling in *vivo* remain unclear. In this study a reduction in the level of Jagged1 was detected during the early stages of the wound healing. Structurally, the ligands share many characteristics with Notch itself and are prone to similar modifications including proteolytic processing (Klueg et al., 1998, Qi et al., 1999). However, the purpose of ligand cleavage remains unclear. One suggestion is that proteolytic processing of the ligand contributes to ligand

downregulation (Qi et al., 1999). Another suggestion is that cleaved or secreted ligands antagonize Notch signalling, because, under most circumstances, soluble ligand fragments inhibit receptor signaling. These reported works that indicated the ligand cleavage after binding of the ligands to the receptor in vivo, may describe the observed downregulation of full-length Jagged1 in our data.

In conclusion, these data indicate that skin injury rapidly activated Notch signalling in the epidermis. Notch1 and Notch2 activation in the early stages of healing process suggests that Notch is a key player in orchestrating the immunological and epidermal cell responses during the wound-healing cascade. A beneficial role for Notch signalling during inflammation and proliferation stages of wound healing has been also supported by previous research conducted in our laboratory (unpublished data). It has been demonstrated that differential Notch activation is a key stress/injury signal in skin epithelium driving induction of pro-inflammatory production that drive normal skin tissue repair. It will be of considerable interest to identify the consequences of forced epidermal activation of Notch during the healing cascade and also the role of Notch activity in cytokines and chemokines production during skin wound healing.

4 The timing and duration of Notch activity impacts wound closure and inflammatory cell recruitment.

4.1 Introduction

Wound healing is a dynamic process which comprises of four distinct, but overlapping phases: homeostasis, inflammation, proliferation and remodelling (Geer et al., 2004). In acute wounds, blood vessel injury releases platelets, which aggregate to form a fibrin-rich clot. Secreted adhesive proteins, growth factors, and cytokines at the injured site activate and recruit neutrophils, macrophages, and fibroblasts (Eming et al., 2007, Geer et al., 2004).

Numerous clinical and experimental studies confirm the essential role of inflammation during repair. The inflammation phase can be subdivided into two distinct components: early and late inflammation. The sequential entry and exit of inflammatory immune cells at appropriate timings are equally important in wound closure (Eming et al., 2007, Geer et al., 2004).

For instance, the transition from early to late inflammation depends on the replacement of neutrophils by lymphocytes and monocytes that have become activated (Eming et al., 2007). The macrophage has long been considered the key regulator for wound healing, they not only act as a predominant type of inflammatory cells (M1 macrophage) in the inflammation phase, but also are involved in the proliferation phase (M2 macrophage), as well as the remodelling phase, following activation by different pathways (Rodero and Khosrotehrani, 2010).

Infiltration of neutrophils in the inflammatory phase clears the wound of debris and contaminants, while secreting chemoattractants that stimulate monocytes movement into the wound (Eming et al., 2007, Geer et al., 2004). Once present, they bind to ECM receptors prompting their differentiation into macrophages which secrete a barrage of cytokines, including platelet- derived growth factor (PDGF), interleukins (ILs), tumour necrosis factor-alpha (TNF-α), and transforming growth factor-alpha and beta (TGF-α and -β) (Eming et al., 2007, Greaves et al., 2013).

4.2 Chronic wounds

Although numerous stimulating and inhibitory mediators engaged during diverse stages of wound healing have been identified, it is the balance between these mediators, which is crucial to achieve perfect tissue homeostasis and repair following injury (Fonder et al., 2008). Furthermore, a sufficiently regulated balance between a complex network of various leukocyte cell subsets and numerous pro- and anti-inflammatory mediators is essential to achieve proper tissue homeostasis during healing (Eming et al., 2007). Dysregulation of critical parameters in inflammatory response interactions results in pathological and chronic inflammatory disease states that impair the quality of healing.

A variety of causes have been identified that can delay wound healing including: chronic disease, diabetes, neurological defects, vascular insufficiency, nutritional deficiencies, age, and local wound-specific contributors; including bacterial biofilms and alterations in levels of various proteins (Fonder et al., 2008, Greaves et al., 2013). Chronic wounds occur at a high incidence in the general population. Annually in the United States, 2–3 million patients are at risk of developing diabetic ulcers, 600,000 patients develop chronic leg ulcers secondary to venous insufficiency, and 1–3 million patients suffer pressure ulcers secondary to immobility (Kuehn, 2007). In the United Kingdom alone, there are 24,000 hospital admissions per year with diabetic foot ulceration (Currie et al., 1998). The prevalence of chronic wounds varies in different age groups, being about 1% in the adult population but increasing to 3–5% in those over 65 years (Greaves et al., 2013) .

4.3 Mechanisms involved in chronic wound formation

It is a combination of systemic and localised stimuli that collectively overwhelm normal healing processes, which result in a hostile wound environment. The prolonged inflammation, which is associated with the proliferation and activation of macrophages and lymphocytes, and the production of an excess of inflammatory growth factors and cytokines, is one of the critical factors that promote hostile

conditions for wound healing (Mori et al., 2008). There are a number of common factors that promote adverse conditions for wound healing, these are summarised in figure 4.1.

Chronic wounds do not adequately complete the "normal" phases of wound healing (Greaves et al., 2013). However, in contrast to the short-lived stimulus that triggers the orderly steps of wound healing in the skin, the injurious stimulus caused by infections, autoimmune reactions, trauma, and other types of tissue injury, persist in chronic diseases, causing organ dysfunction and often organ failure (Mori et al., 2008). A state of chronic inflammation develops as many of the cells recruited to the wound in the proliferative phase of healing, adopt a pro-inflammatory secretory profile. Furthermore, inflammatory cells, particularly neutrophils and macrophages persist in the wound, creating a highly pro-oxidant, protease- rich environment with an abundance of pro-inflammatory cytokines such as interleukin-1 (IL- 1), interleukin-6 (IL-6), and TNF-α. The result is a hostile environment with down-regulation of protease inhibitors and direct damage to extracellular matrix (ECM), cellular components, and protective growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). Reactive oxygen species and proteases, such as matrix metalloproteinase (MMP) 1, 2, 3, 9, and 13, have also been described as deleterious influences (Figure 4.1.) (Greaves et al., 2013).

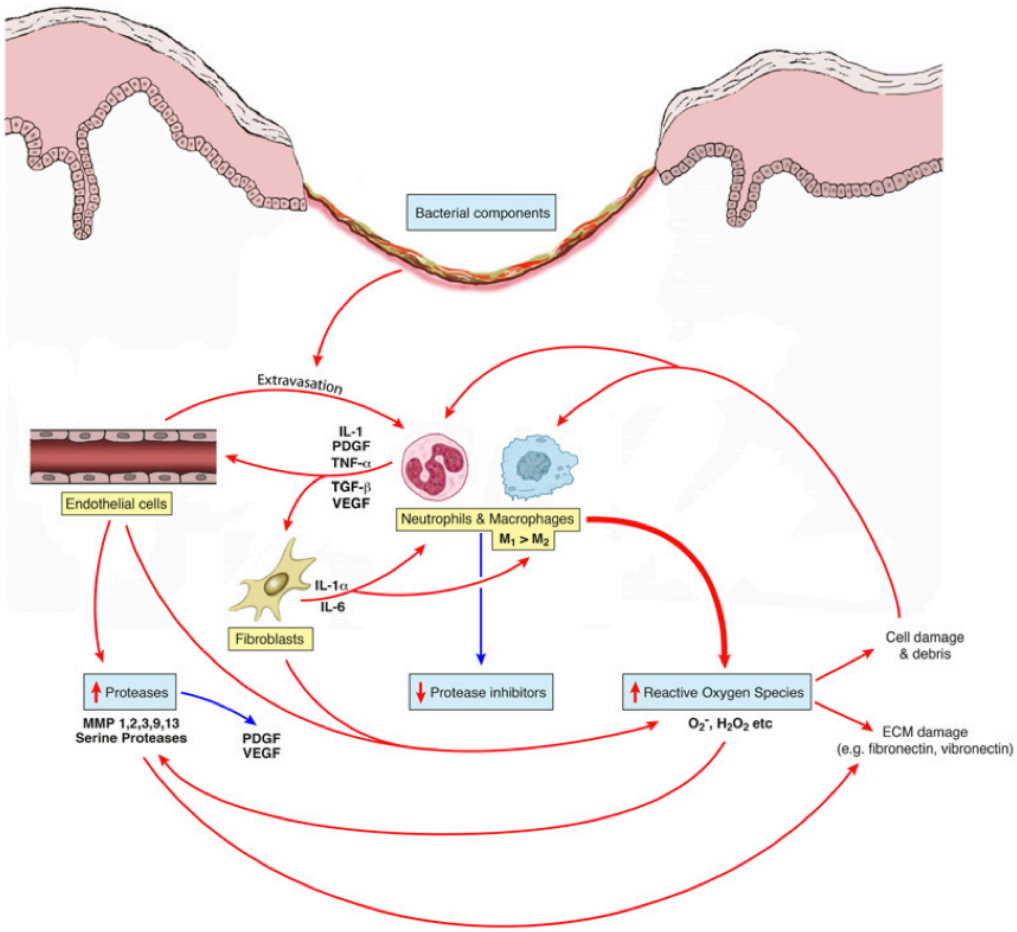


Figure 4.1 Mechanisms involved in the development and persistence of chronic wounds

(Greaves et al., 2013). Red lines indicate up-regulation and blue lines indicate down-regulation.

4.4 Notch signalling and it's interactions with inflammation

Beyond being a target of inflammation, recent studies demonstrated that Notch plays an active role in the development of inflammatory processes (Fung et al., 2007, Zhou et al., 2003). Notch is an important regulator of immune cell differentiation and activation. Inflammatory response related to Notch activation in different tissue types have also been reported. Numerous studies reported its role in T $CD4^+$, $CD8^+$

lymphocyte, regulatory T-cell, B-cell, natural killer (NK) and dendritic cell homeostasis (Kassner et al., 2010, Woollard and Geissmann, 2010).

In a recent study, Outtz *et al*., 2010, demonstrated that Notch1 partial deletion (Notch1$^{+/-}$ hemizygous mice) induced a decrease in macrophage recruitment to the site of vascular injury, associated with an impaired production of TNFα in the wound, compared to wild-type controls. Macrophages isolated from Notch1$^{+/-}$ subjects secreted less pro-inflammatory cytokines (TNF, IL6, IL12) and chemokines (CXCL10, CCL2) in response to (interferon gamma) IFNγ than Notch1$^{+/+}$ derived cells (Outtz et al., 2010).

Consistenty, constitutive activation of Notch1 in macrophages potentiates the induction of IRF-1 (interferon regulatory factor 1), SOCS1 (suppressor of cytokine signaling-1), ICAM1 (intercellular adhesion molecule-1) and MHC-II (major histocompatibility complex, class II) by IFNγ, while reducing NO (nitric oxide) production (Monsalve et al., 2006).

As an example, immunosuppressive effects by markedly reducing T-cell infiltration and proliferation were reported as a consequence of Notch blockade through Jag1-Fc or γ-secretase inhibitor treatment in large-vessel vasculitis (Piggott et al., 2011). It has also been reported that Notch signalling mediates IL10 production by T-cells via Dll4 presentation on plasmacytoid dendritic cells (pDCs) (Kassner et al., 2010).

Interestingly, Dll4 has been shown to reduce the production of the IL8 chemokine in response to ischemia or TNF, while selectively inducing the expression of inflammatory genes, such as iNOS (inducible nitric oxide synthase), pentraxin 3 and Id1 (Al Haj Zen et al., 2010, Fung et al., 2007). In support for a functional role of Notch pathway during the response to vascular injury, intimal hyperplasia after vascular injury is significantly decreased in *hey*2$^{-/-}$ mice. This effect correlates with reduced cellular proliferation and decreased chemotaxis and migration in response to platelet-derived growth factor (PDGF) (Sakata et al., 2004). Overall, Notch signalling in macrophages seems to favour pro-inflammatory responses by releasing more cytokines and chemokines, supposedly through Notch1 and Notch3 activation.

Furthermore, Notch activity in the epidermis has wide-ranging effects on both epidermal and dermal compartments (Estrach et al., 2006; Blanpain et al., 2006). There is increasing evidence to suggest that aberrant Notch signalling may contribute

directly to skin pathogenesis and altered expression of Notch receptors identified in Keloid disease (KD) (a fibroproliferative disorder). Syed and Bayat, 2012), showed the presence of a significant up-regulation of Notch receptors and ligand Jagged-1 (JAG-1) in KD compared to normal skin tissue samples (Syed and Bayat, 2012). Their study also demonstrated that activation/inhibition of JAG-1 and Notch signalling significantly altered the behaviour of primary keloid fibroblasts in invasion and angiogenesis. Their work demonstrated a potential role for the Notch signalling pathway in KD progression and targeting this pathway may provide a novel strategy for treatment of KD (Syed and Bayat, 2012).

Dermal changes including infiltration of the CD4- positive T cells, barrier dysfunction, epidermis thickening and epidermal hyperproliferation have been introduced as direct effects of forced epidermal Notch activity in un-injured skin (Ambler and Watt, 2010).

A number of growth factors produced in Notch-active keratinocytes were identified by microarray analysis as likely candidates for Notch-induced dermal inflammation. The gene expression profiling of the epidermis, dermis and whole skin from transgenic (K14NICDER) and wild type mice treated with 4OHT for 14 days was performed previously (Ambler and Watt, 2010). Examination of the microarray data for epidermal growth factors and cytokines demonstrated a more than ten-fold up-regulation in epidermal TNF α and a three-fold increase in TGFβ as a consequence of epidermal Notch activation in 4OHT-treated K14NICDER skin (Ambler and Watt, 2010). The link between the up-regulation of TNFα following Notch activation, and the inflammation has also been reported by another study (Incorvaia et al., 2008).

In chapter three the level of Notch1 and Notch2 activity in epithelial tissue as a response to injury was investigated. Data presented in chapter three demonstrated that wounding caused in an increase in Notch1 and Notch2 activity; with peak Notch1 activity detected 4 dpw (days post wounding) and Notch2 activity observed at 1 dpw. The detection of maximal levels of Notch activity in the early inflammation stages indicates a functional role for Notch in inflammatory events in the wound-healing cascade. Furthermore, a recent study investigating the role of Notch signalling in epithelial tissue repair has demonstrated that Notch1 signalling activation in epidermal keratinocytes following skin injury, recruits innate lymphoid cells (i.e. ILC3s) to the site of injury in a TNFα/ CCL20 -dependent mechanism which controls

macrophage/ monocyte recruitment via ILC3-dependent CCL3 (Ambler et al., unpublished). Also it found that Notch1 induces epidermal production of IL23, which facilitates ILC3s to produce IL22 for epithelial proliferation and skin repair (Ambler et al., unpublished). Using a 4OHT-treated K14NICDER mouse model, where Notch1 signalling can be controllably and specifically activated in epidermis, some key cytokine or chemokine candidates, including TNFα, and IL23, have been identified (Watt et al., 2008).

In summary, there is significant evidence to indicate the involvement of the Notch pathway in production of a number of chemokine and growth factors, via a pleiotropic function of Notch pathway in skin wound healing previously (Ambler and Watt, 2010, Demehri et al., 2009a, Lee et al., 2007). Prior research established Notch activation, via a 4-hydroxy-tamoxifen-inducible transgene, resulted in changes in skin architecture, induction of dermal inflammatory infiltration up-regulation of several growth factors and cytokines, including TNFα .

The basic mechanisms that occur in the poor healing associated with chronic inflammatory diseases are generally similar to the mechanisms of epidermal forced Notch activity that have been discussed earlier in chapter three of this thesis. Assuming the validity of these pathological effects of Notch activity, the question arises whether prolonged activation causes poor healing. Thus it can be hypothesised that persistent Notch activity may lead to chronic inflammation and poor wound healing.

It can also be suggested that prolonged epidermal Notch signalling might be involved in hindered skin wound healing by promoting a prompt inflammation reaction and mediating the interaction between keratinotyes and immune cells. Previous studies suggests a role of Notch signalling in promoting skin wound healing through use of a pan-Notch inhibitor (Chigurupati et al., 2007). However, the underlying mechanism is less clear, and the individual roles of Notch1 and Notch2 are unknown due to lack of appropriate inhibitory/stimulatory agents. Recent studies showed forced activation of epidermal Notch signalling promotes dermal accumulation of immune cells via epidermal production of TNF-α, TGF-β, and SM22α and stimulates epidermal proliferation via a mechanism that is less clear (Ambler and Watt, 2010, Estrach et al., 2006).

It is possible that prolonged Notch signalling may also be a consequence of the over-expression of IL-22 and IL23, which may result in a chronic inflammatory environment in wounds. Therefore, the correlation between prolonged Notch activity and inflammation using K14NICER models will be examined to investigate the expression level of IL-22 and IL-23 genes. The mechanism of how over-Notch activity, via TNF-α, regulates wound contraction, the ECM deposition and the accumulation of metalloproteinases in dermis will be determined during the healing process.

4.5 Experimental plan

To examine the influence of Notch activation, on the rate of wound closure, the K14NICDER mouse model was used. To active NICDER transgenes, seven-week-old K14NICDER and wild type mice were treated topically with 2 mg 4-hydroxy tamoxifen (4OHT, Sigma-Aldrich, USA) dissolved in acetone (Estrach et al., 2006). Mice were treated in one of the following treatment groups (Figure 4.2):

(1) 2 mg of 4OHT, three times a week for one week before wounding, followed with continuous treatment with 2 mg 4OHT three times a week, until 8 days post wounding

(2) 2 mg 4OHT, three times per week, before wounding for one week, followed with continuous treatment with acetone alone, until 8 days post wounding

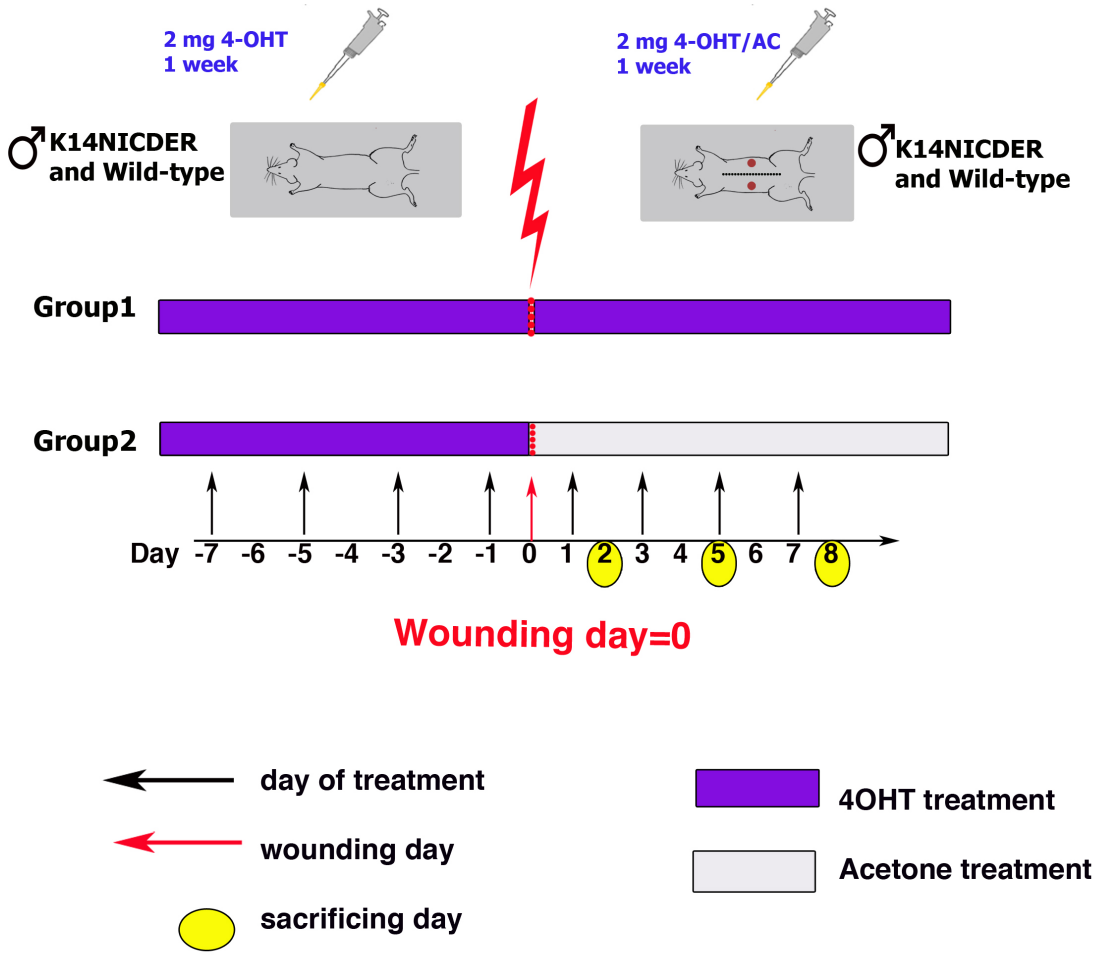


Figure 4.2. Experimental plan.

K14NICDER and wild type mice, 7-weeks old, were randomly assigned into two groups (n=18 mice per group). Each group of mice received 4OHT three times for one week. Wounding procedure was subjected to the mice back skin after a week of treatment. One group (Group1) received one more week of 4OHT injections after wounding and the other group of mice was exposed to acetone alone treatment after wounding (Group2). Back skin samples were collected on 2, 5 and 8 days after wounding from both groups for histological and molecular analysis.

4.6 Results

4.6.1 Persistent Notch signalling results in delayed cutaneous wound healing

To examine the effect of chronic Notch signalling on wound closure, punch-wounded mice were photographed and the wound diameter was measured daily. At five days post wounding, the wound size was larger in K14NICDER mice treated with 4OHT prior to punch wounding and during wound healing (Group 1) compared to non-transgenic wild type littermate controls, receiving the same treatment regime and K14NICDER that were not treated after wounding (Group 2) (Figure 4.3). Statistical analysis demonstrated that there was a significant difference in wound size (p value <0.0005) between the two experimental groups from day 4 to 7 post wounding (Figure 4.4). By 8 days post wounding, wound size was small in group 2 wild type and K14NICDER mice while in K14NICDER mice from group 1,wounds looked irritated and were larger compared to the wounds on K14NICDER and wild type mice in group 2 (Figure 4.4).

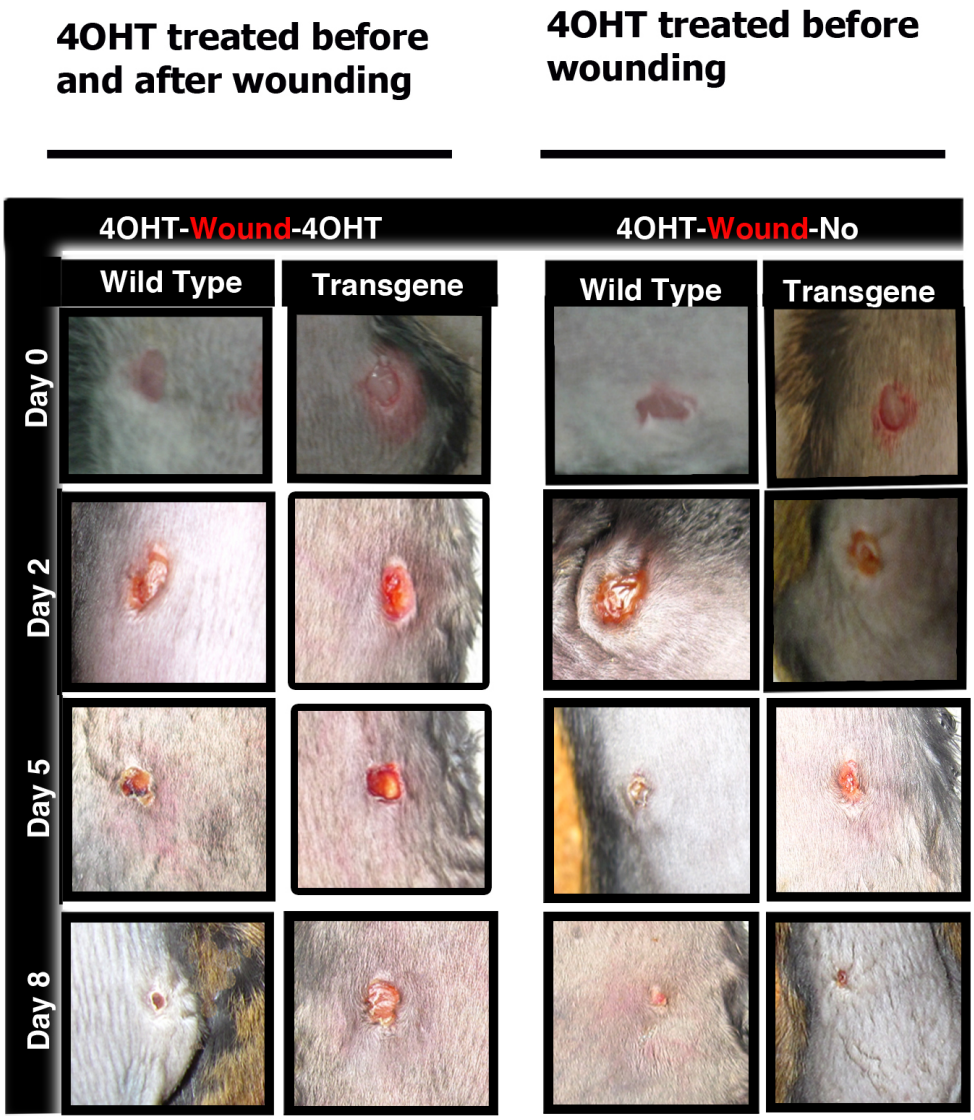


Figure 4.3. Full-thickness dermal wounds were created on back skin of K14NICDER mice and wild type mice

(n=3), Images of a representative mouse from each group taken on post-injury days 0, 2, 5, 8 and are shown.

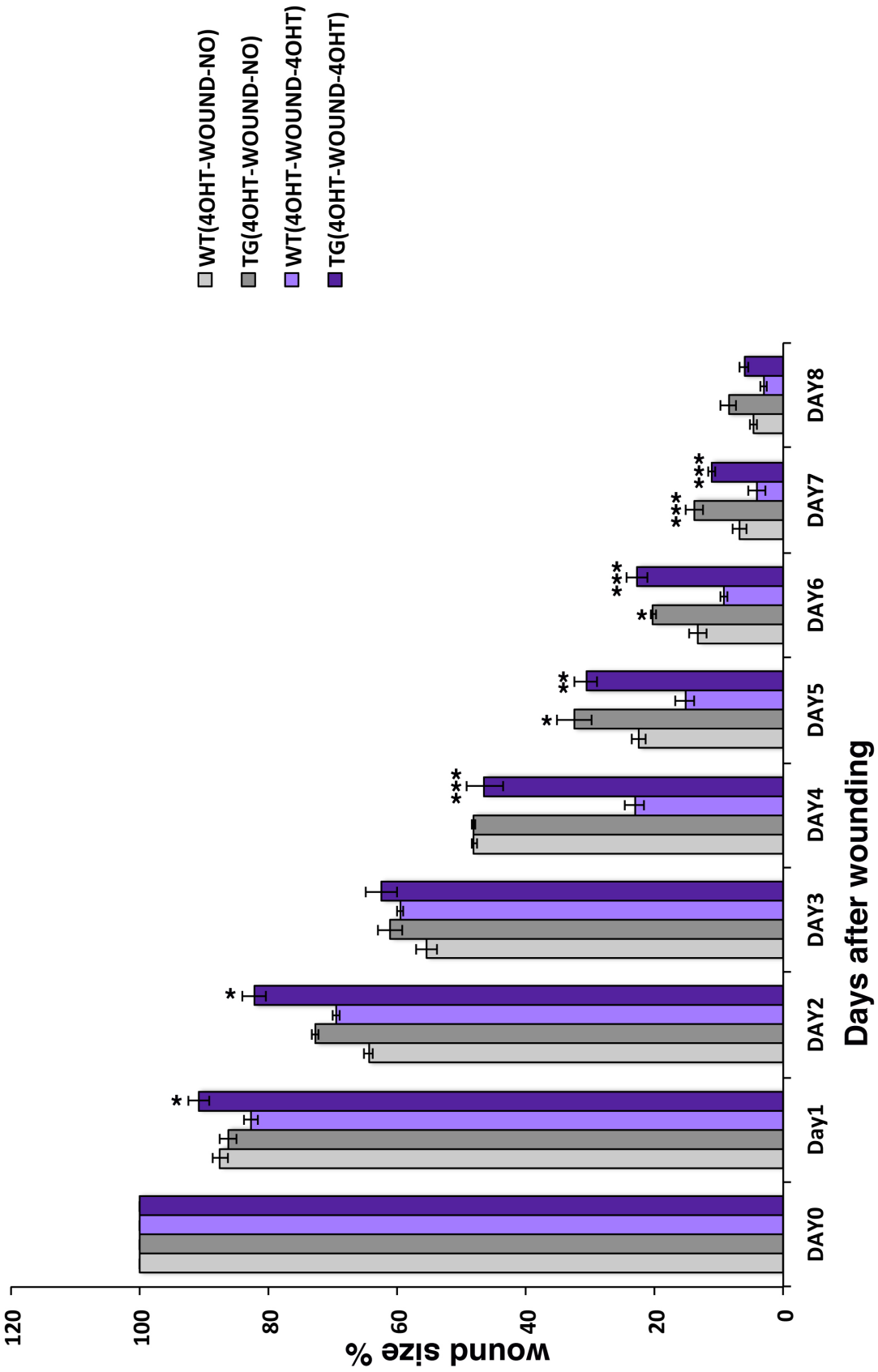


Figure 4.4. Wound sizes percentage at the indicated time points in K14NICDER and wild type mice. Values are the mean ± SEM, (n=3 mice) per condition. All comparisons calculated against wild type (4OHT-WOUND-NO), *p value<0.05, **p value <0.005; ***p value <0.0005. Abbreviations: WT, Wild type; TG, transgenic, 4OHT: 4hydroxy tamoxifen.

Detailed histological analysis of Haematoxylin & Eosin (H & E) sections showed delayed wound healing in K14NICDER animals treated with 4OHT, before and after wounding, at days 5 and 8 post-injury compared to non-transgenic control mice (Figure 4.5). On day 5, non-transgenic control mice presented a superficial layer in the wound, which was lined by a thin layer of damaged keratinocytes (Figure 4.5, top left panel). Within the damaged tissue of K14NICDER animals from group 1, a larger number of mononuclear cells were observed compared to the transitional site and normal skin. K14NICDER mice from both treatment groups on day 5 also exhibited large numbers of cellular infiltrates (cell density shown with white arrows in Figure 4.5, top right panel). On day 5, K14NICDER mice had fewer migrating fibroblasts in the superficial dermis of the wound site. Quantification of epithelial thickness at the wounded site (indicated by yellow arrows in Figure 4.5) of H & E stained sections from all experimental mice at day 5 post wounding, is shown in figure 4.6. A statistically significant increase in thickness of the wounded epithelia in wild type skin from both treatment groups at 5 days post wounding was apparent when compared to adjacent normal skin ($p<0.0005$). Comparing the thickness of epithelia from K14NICDER mice treated before and after wounding with normal adjacent skin did not show a statistically significant difference. The thickness of epithelia from K14NICDER treated mice, before and after wounding, was lower than that of K14NICDER mice treated immediately prior to wounding, but this comparison was found to be non-statistically significant ($p>0.05$, Figure 4.6).

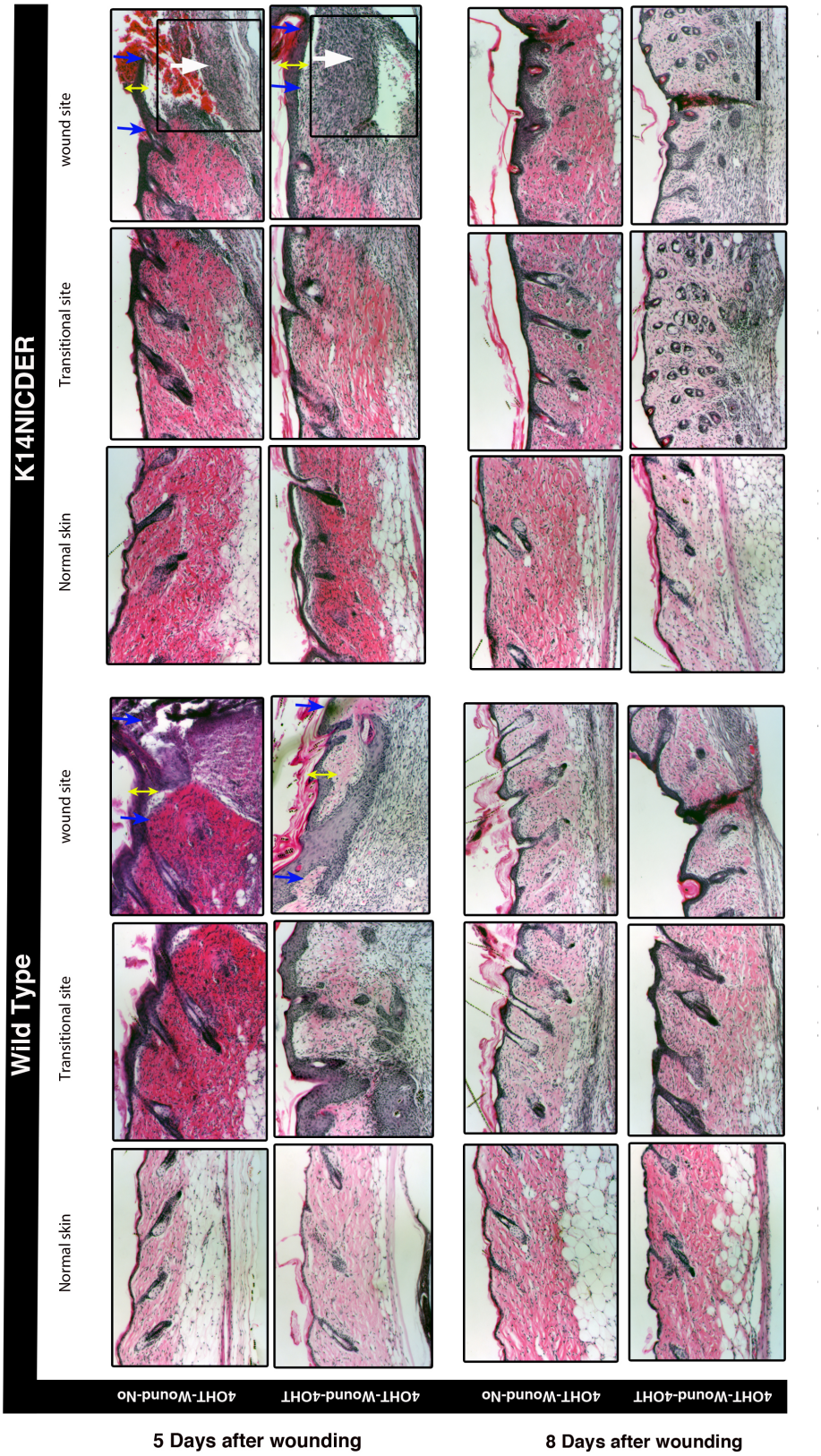


Figure 4.5. Histological features of wound healing in mice with increased Notch activity and wild type.

Images of skin tissue sections stained with H & E showing histological changes during the wound healing process in non-transgenic control mice and K14NICDER transgenic mice treated with 4OHT at post-injury days 5 and 8. K14NICDER transgenic treated with 4OHT mice exhibited delayed wound healing, compared to wild type acetone-treated mice. Scale bar = 200 microns.

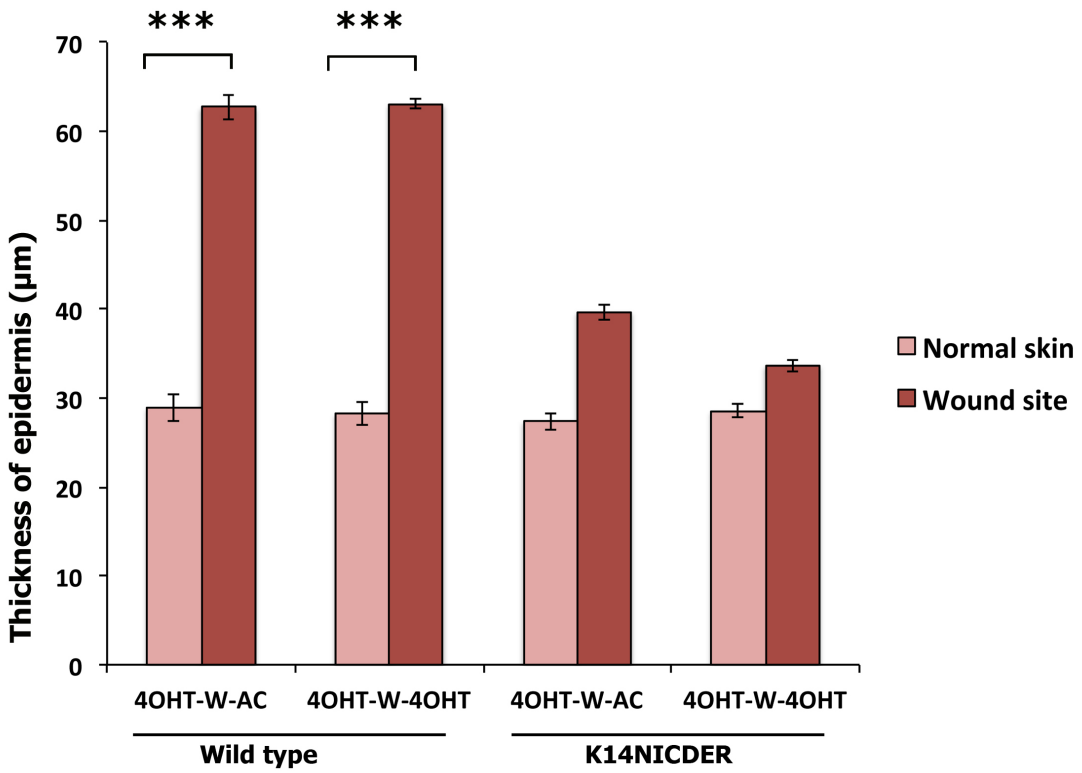


Figure 4.6 Epidermal thicknesses in control and 4OHT-treated wild-type and K14NICDER mice,

Measurements are on 5 days post wounding (5dpw), from 2 biopsies of each animal , (n=3 mice) per group. Mean ± SEM , ***p<0.0005. Abbreviations: 4OHT: 4hydroxy tamaxifen; W:wound; AC: acetone.

4.6.2 The proliferation of basal cells decreased with respect to over-activation of Notch signalling

As reported previously, phenotypic characteristics including: thickened, hyper proliferative epidermis, blistering and accumulation of dermal cells at the epidermal/dermal junction have been observed on the back skin of 4OHT treated K14NICDER mice (Ambler and Watt, 2010). To determine the effect of prolonged Notch activity in K14NICDER on the proliferation index, the number of Ki67-positive basal cells and the total number of nuclei were counted per 500 μm length from wound edge of the interfollicular epidermis. Ki67 is a nuclear protein, which is expressed in proliferating cells (Scholzen and Gerdes, 2000).

Immunolocalization of Ki67 in skin wound appeared to be localised to the basal layer of epidermis, with the level of cell proliferation altering following continuous activation of K14NICDER mouse back skin. The percentage Ki67 basal cell expression in wild type mice in both groups was 40 ±5 % of the total number of the cells within 200 μm length from the wound edge of the interfollicular epidermis at 5 days post wounding (Figue 4.7). In K14NICDER mice from group 1, the number of Ki67 positive cells near the wound site decreased ($p<0.01$) compared to the wild type. Cellular expression of Ki67 was not significantly decreased in transgene mice from group 2, however it was lower in these mice compared to the wild type animals within the same group (Figure 4.8).

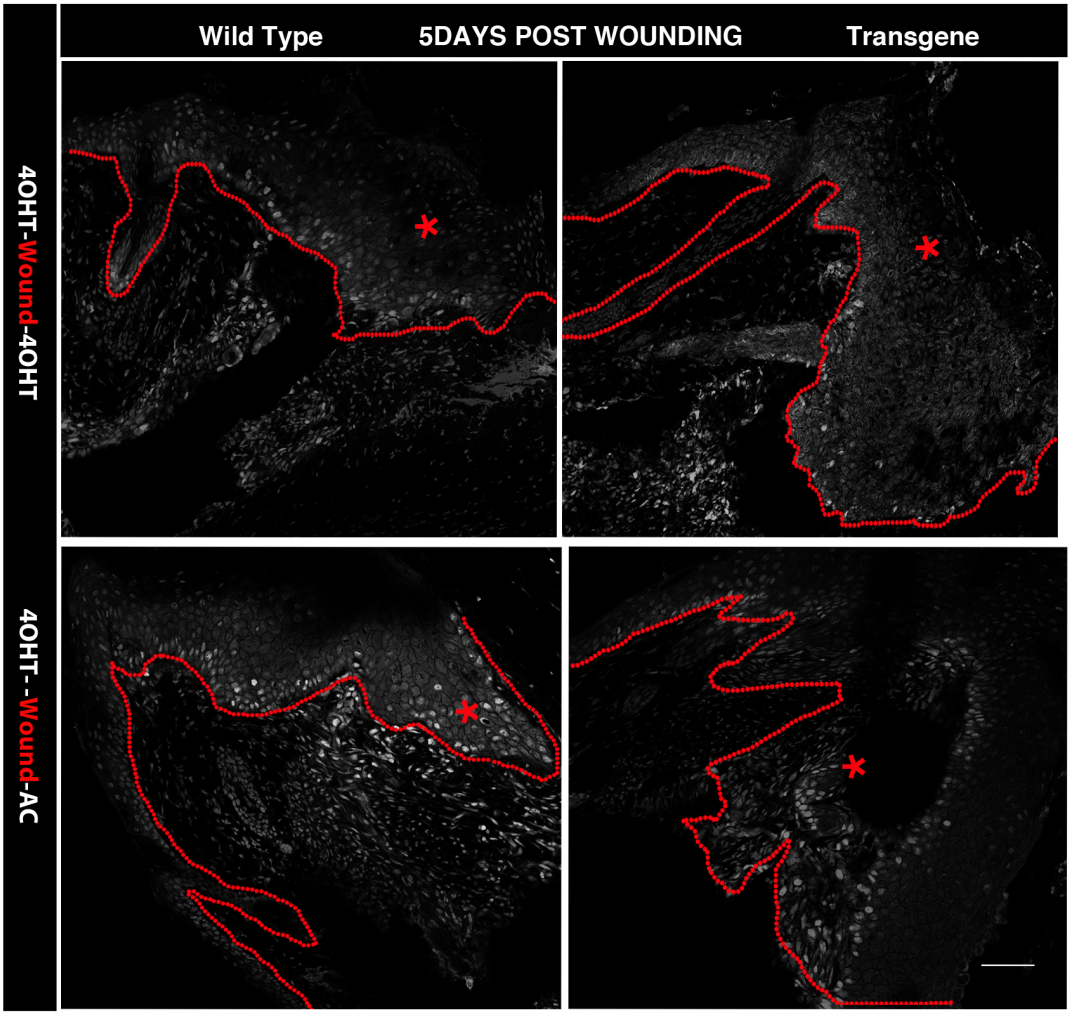


Figure 4.7 Activation of Notch signalling before wounding caused delayed wound healing coincident with a delay in epidermal proliferation.

4 mm full-thickness wounds were created in 6-week-old K14NICDER and wild type mice. Sections of wounded K14NICDER and wild type skin, treated with 4OHT before and after wounding, and non-treated after wounding, collected 5 days post wounding were stained with antibodies to Ki67 (White). Red, dashed line marks epidermal-dermal boundary; red asterisk marks wound site. Scale bar equal 200 microns.

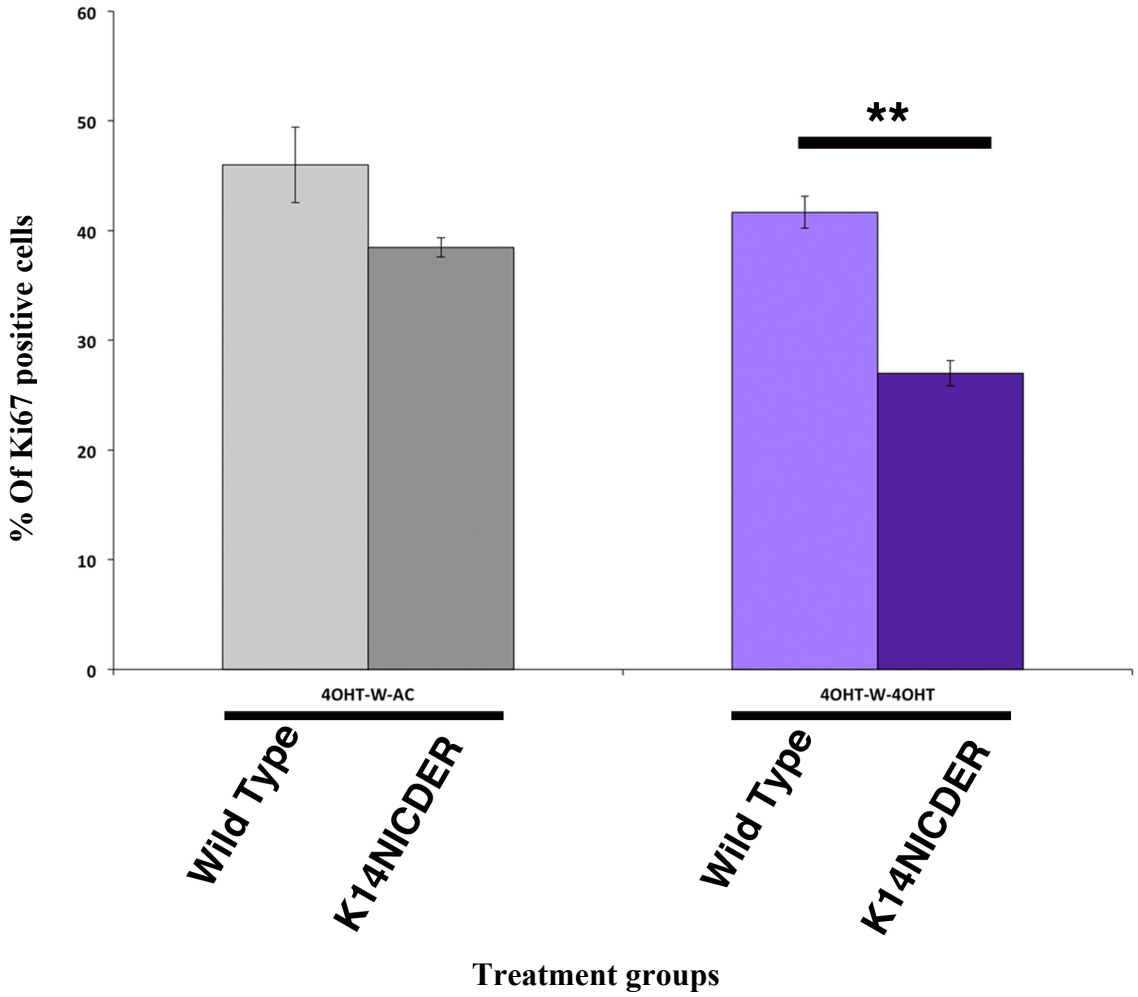


Figure 4.8. A graph to show percentage of total dermal and epidermal cells, which were Ki67 positive within 500 µm of wound site.

Comparing wild type animals to K14NICDER animals at five days post wounding Mean ± SEM, one section per mouse (n=3 mice), **p< 0.01 Student t-test. Abbreviations: 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

4.6.3 Additional macrophage infiltration as an effect of over-inflammation

Previous research has confirmed a role for Notch signalling in recruitment and function of macrophages at the site of inflammation via regulation of inflammatory cytokines (Outtz et al., 2010). A previous study has shown that forced, ectopic epidermal Notch activity resulted in phenotypic hallmarks of skin wound healing (Ambler and Watt, 2010). Data has shown that innate lymphoid cells (ILC) are recruited to skin wounds by a Notch/jagged1 dependent mechanism and these immune cells are involved in orchestrating immunological and epidermal cell responses during the wound healing program (Ambler et al. Unpublished data) . As macrophages play an important role in many pathological processes, understanding the link between Notch and macrophages during wound healing may lead to provide insights for novel wound therapeutic strategies. The role of Notch signalling in regulation of macrophage function during inflammation has been reported previously (Outtz et al., 2010). Both pharmacological inhibition of Notch and genetic analysis demonstrate that Notch1 regulates cytokine expression in macrophages . It has also been reported that Notch1 is important for the inflammatory response during wound healing in mice (Outtz et al., 2010).

This study hypothesised that additional macrophage infiltration as a consequence of prolonged Notch activity, may be the purpose of delayed wound closure in K14NICDER mice. To test this, dorsal wounds were created in K14NICDER and wild type mice from both groups, and wound tissue was harvested to assess macrophage infiltrate. Macrophages can be identified by specific expression of a number of proteins; the F4/80 antigen is expressed by a majority of mature macrophages and is the best marker for this population of cells. Immunolocalization of F4/80 in skin wound appeared to be localized to the basal layer of the epidermis and dermis, but the level F4/80 positive cells altered following prolonged Notch activity in K14NICDER mice (Figure 4.9). This investigation demonstrated that $F4/80^{+}$ cells were plentiful (20-25%) within the K14NICDER dermis in both experimental groups, with a highly statistically significant ($p<0.005$) infiltration of macrophages in K14NICDER mice compared to their wild type littermates, within

both treatment groups (Figure 4.10). Hence, similar to the chronic wound conditions, infiltration of more macrophages that persist in the wound, were observed as a result of continued Notch activity prior and after wounding. This condition may create a highly pro-oxidant, protease- rich environment for the delayed healing of K14NICDER wounded skin.

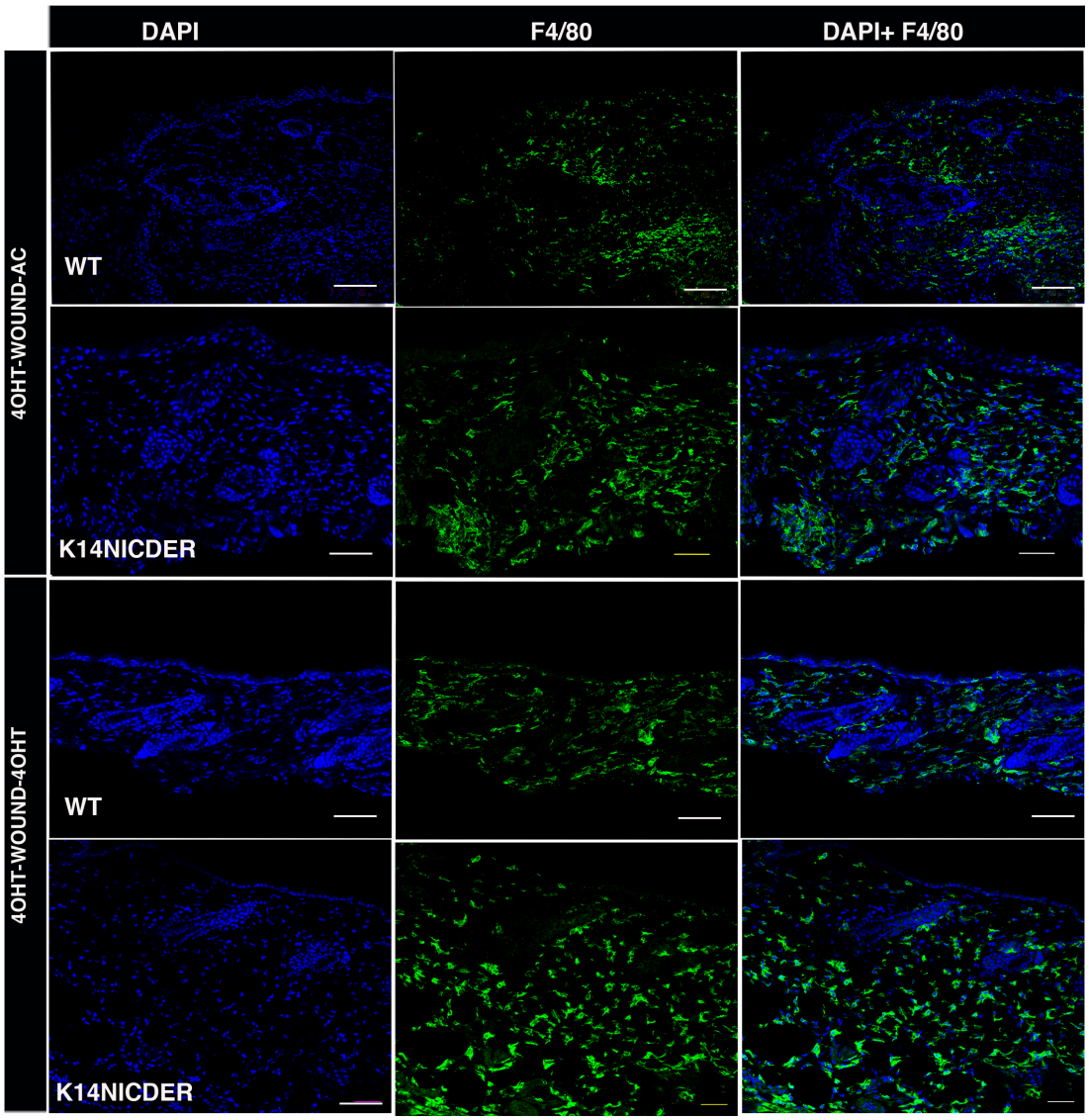


Figure 4.9. 5dpw back skin tissues were sectioned and stained with an antibody to F4/80 antigen (green) and (Blue) DAPI counterstained

Abbreviations: 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone. Scale bar, 50 microns.

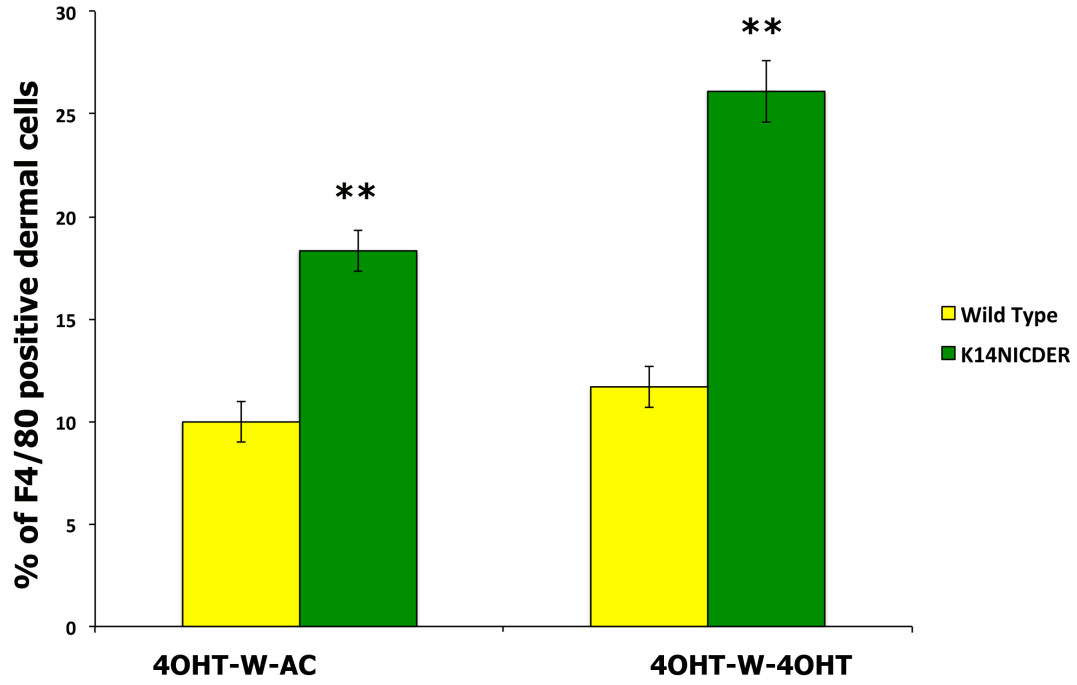


Figure 4.10. F4/80 positive dermal cells, as a percentage of total cells counted, in wild type animals compared to K14NICDER mice from both treatment groups 5 days post wounding

(4OHT before wounding only and 4OHT before and after wounding). Mean ± SEM, n= 3, **p<0.005. Student's t-test, comparing K14NICDER with Wild type in each treatment group.

4.6.4 Q-PCR analysis

q-PCR was completed to determine the differences in Notch-dependent, inflammatory and wound healing related gene expression, as well as to try to establish evidence of differentiation. Therefore, samples from both treatment groups were prepared for q-PCR and probed for a number of genes associated with the wound healing and Notch activity. Wounded skin tissue samples from all treatment groups at day 5 and 8 post wounding were collected. Quantitative RT-PCR of wounded skin samples of all experimental groups was applied and data analysed and compared with the littermate not wounded, un-treated control mice. In these experiments, the mRNA of each gene was normalised to GAPDH or β-2M mRNA.

4.6.4.1 Prolonged Notch activity induced Notch2, Notch1 and Jagged1, expression

Changes were observed in macrophage recruitment and function in K14NICDER mice, in response to epidermal Notch activity (Ambler and Watt, 2010), characterisation of Notch expression and signalling in K14NICDER wounds, in the context of prolonged epidermal Notch activity was required. Previously published studies used topically applied Notch pathway activators and inhibitors and showed that Notch activity increased expression of Notch1 and Jagged1 (Chigurupati et al., 2007). Wounded skin tissue samples from all treatment groups at day 5 and 8 post wounding were collected in this study. Messenger-RNA (m-RNA) was isolated from unwounded controls, wounded wild type or K14NICDER skin collected 5 and 8 dpw. Relative mRNA levels of Notch1, Notch2 and Jagged1 were quantified by q-RT-PCR. mRNA levels were normalised to unwounded control wild type mice. Notch1 transcripts were significantly higher in tissue extracts from K14NICDER mice from both treatment groups than in normal skin at day 5 post wounding (Figure 4.11). Transcribed levels of Notch1 in K14NICDER wounds with prolonged Notch activity showed a statistically increased ($p=0.0001$) comparing to the controls in this group. Similar to Notch1, Notch 2 transcripts were increased ($p<0.05$) in K14NICDER mice from group 1 (Figure 4.12). Increased expression of Jagged1 was also detected in

response to prolonged Notch activity in K14NICDER mice group1 ($p<0.05$) and also group 2 ($p<0.05$) than in normal skin at day 5 post wounding, shown in Figure 4.13. Transcript levels of Notch1, Notch2 and Jagged1 were induced in response to Notch activity over the healing process.

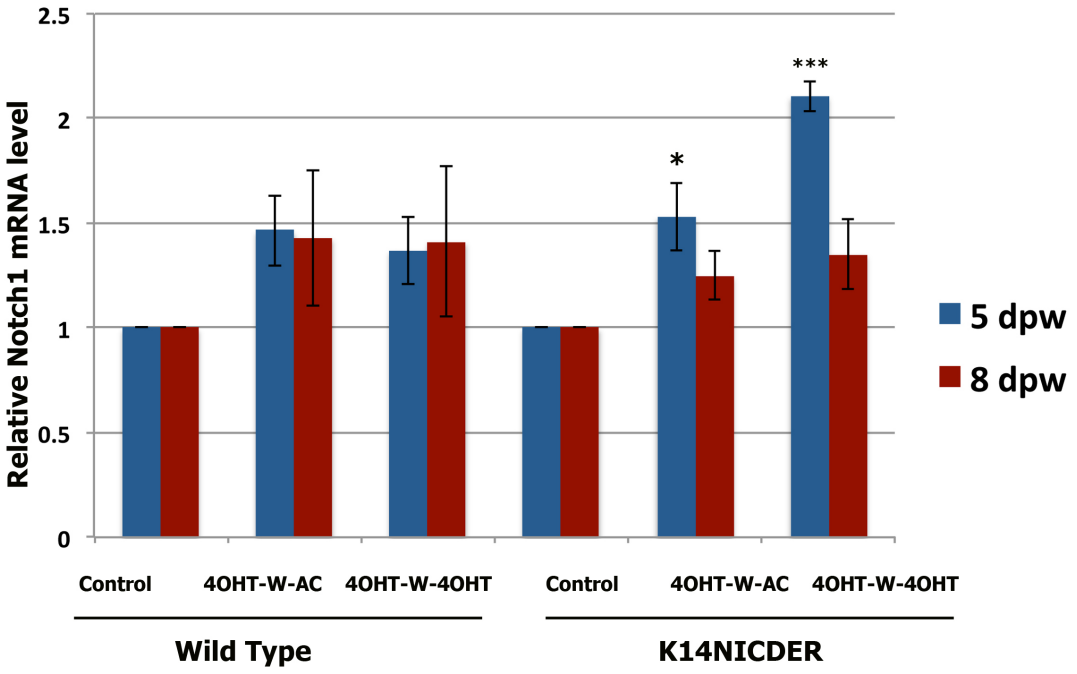


Figure 4.11. Relative Notch1 mRNA level

Messenger RNA isolated from unwounded controls or wounded wild type or K14NICDER skin was collected 5 and 8 days post wounding. Relative mRNA levels of Notch1 were quantified by q-RT-PCR. mRNA levels were normalised to unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats for each animal. Student's t-test. *$p<0.05$, ***$p<0.0005$. Comparison indicated the differences between each group to the corresponding control group. Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

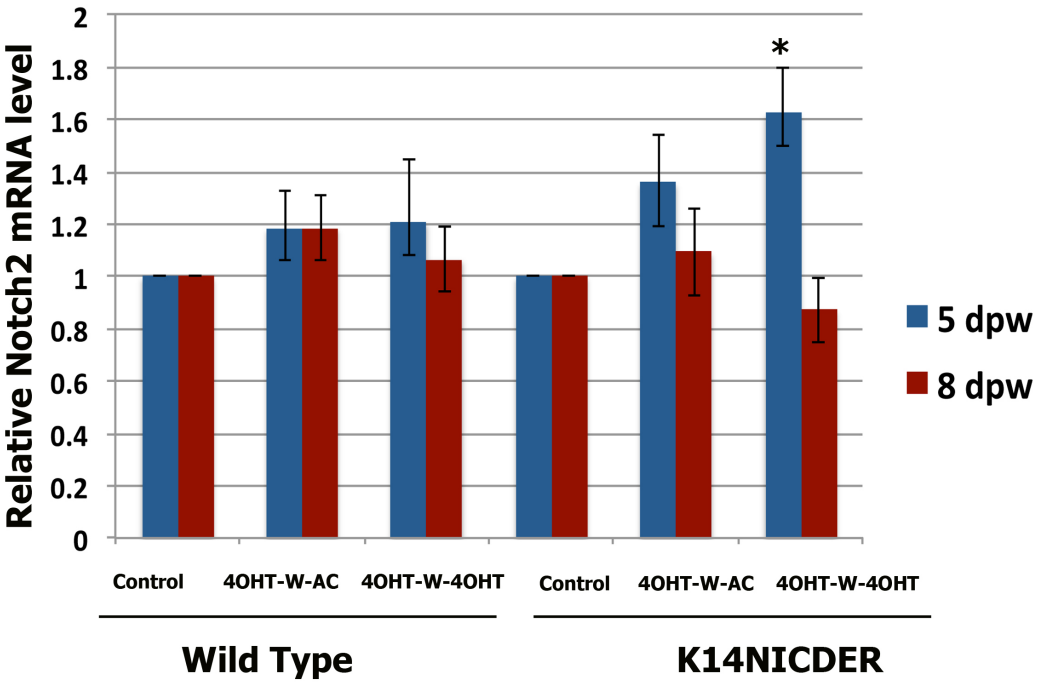


Figure 4.12. Relative Notch2 mRNA level

mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 days post wounding. Relative mRNA levels of Notch2 were quantified by q-RT-PCR. mRNA levels were normalised unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats. Student's t-test. *$p<0.05$. Comparison indicated the differences between each group to the corresponding control group Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

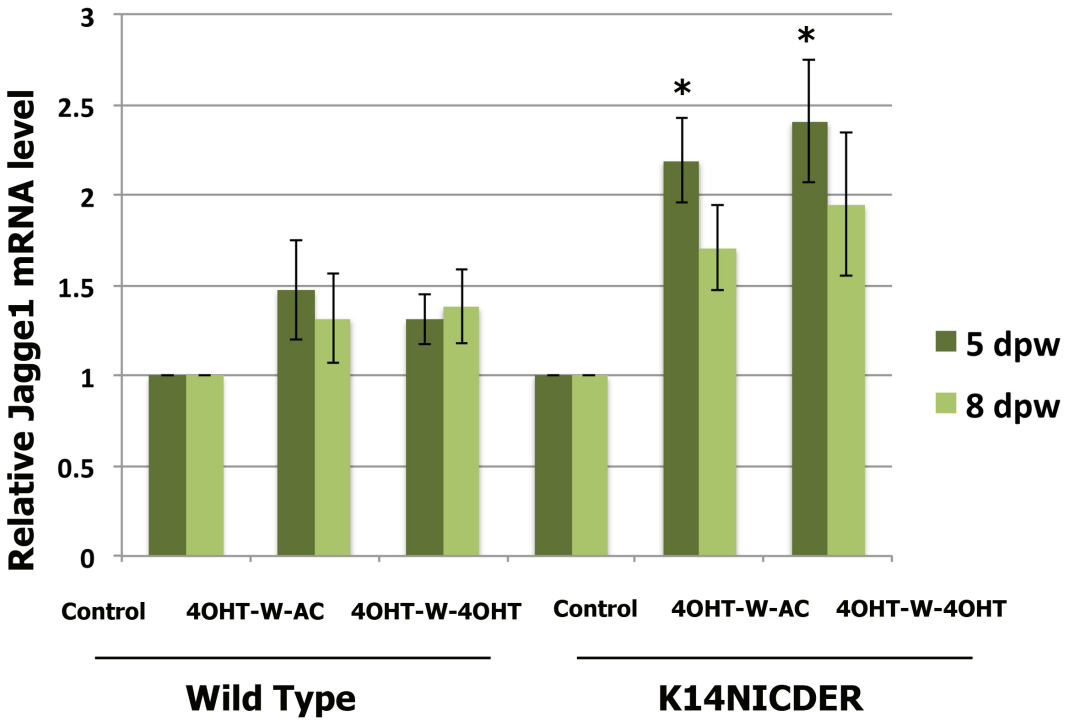


Figure 4.13. Relative Jagged1 mRNA level

mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 days post wounding. Relative mRNA levels of Jagged1 were quantified by q-RT-PCR. mRNA levels were normalised unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats. Student t-test .*$p<0.05$. Comparison indicated the differences between each group to the corresponding control group. Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W:wound; AC: acetone.

4.6.4.2 Prolonged epidermal Notch1 activation induces TGF-β1 and TNF-α expression in K14NICDER mice

Tumor necrosis factor-α (TNF-α) is an effective inflammatory cytokine expressed during the inflammatory phase of wound healing (Goldberg et al., 2007, Singer and Clark, 1999). Previous studies have confirmed the association of Notch pathway and TNFα up-regulation with epidermal barrier disruption and skin inflammation (Incorvaia et al., 2008). As reported previously, the up-regulation of TNFα and TGFβ are related to epidermal Jagged1-dependent Notch activation (Ambler and Watt, 2010). To gain insight into potential mechanisms by which persistent epidermal Notch activation during the healing cascade could induce the expression of factors such as TNFα and TGFβ, quantitative RT-PCR of wounded skin samples of all experimental groups was applied. These data demonstrated an extremely statistically significant ($p= 0.0002$) difference in the level of TGFβ expression at 5 days post wounding in K14NICDER mice with prolonged activated Notch, compared to the littermate controls (Figure 4.14). The level of TGFβ expression at 5 days post wounding in K14NICDER mice with activated Notch just before wounding, also demonstrated statistically significant ($p= 0.0022$) difference compared to the littermate controls. TNFα expression levels were also upregulated at 5 days post wounding in both groups of K14NICDER mice, compared to their corresponding controls ($p<0.0005$). Although the detected expression level of TNFα in wounded wild type skin tissue was statistically significant ($p<0.05$), comparing the K14NICDER with wild type mice from both groups on day 5 after wounding, K14NICDER mice still showed a higher level of TNFα transcribed level ($p<0.05$) (Figure 4.15). Presented data here not only confirmed the association of Notch pathway, inflammatory response with TNFα upregulation during the healing process, but also promoted increased transcript levels of TNFα and TGFβ in response to prolonged Notch activity over the healing process.

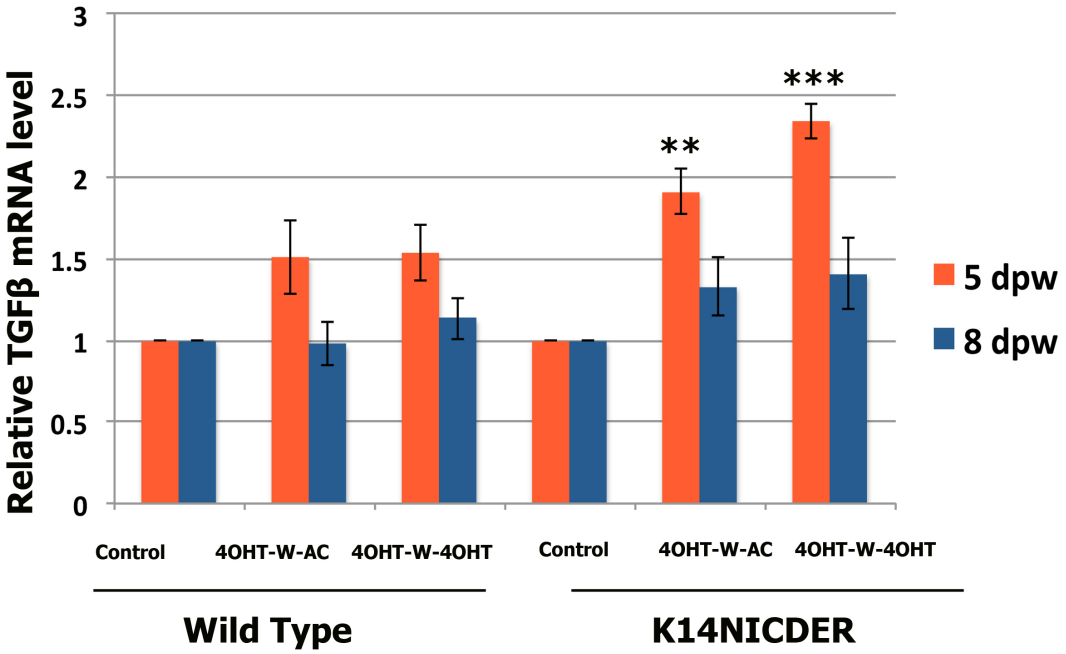


Figure 4.14. Relative TGFβ mRNA level

mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 days post wounding. Relative mRNA levels of TGFβ were quantified by q-RT-PCR. mRNA levels were normalised unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats. **p<0.005, ***p<0.0005. Student's t-test. Comparison indicates the difference between each group of mice at different treatment groups and their corresponding controls. Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

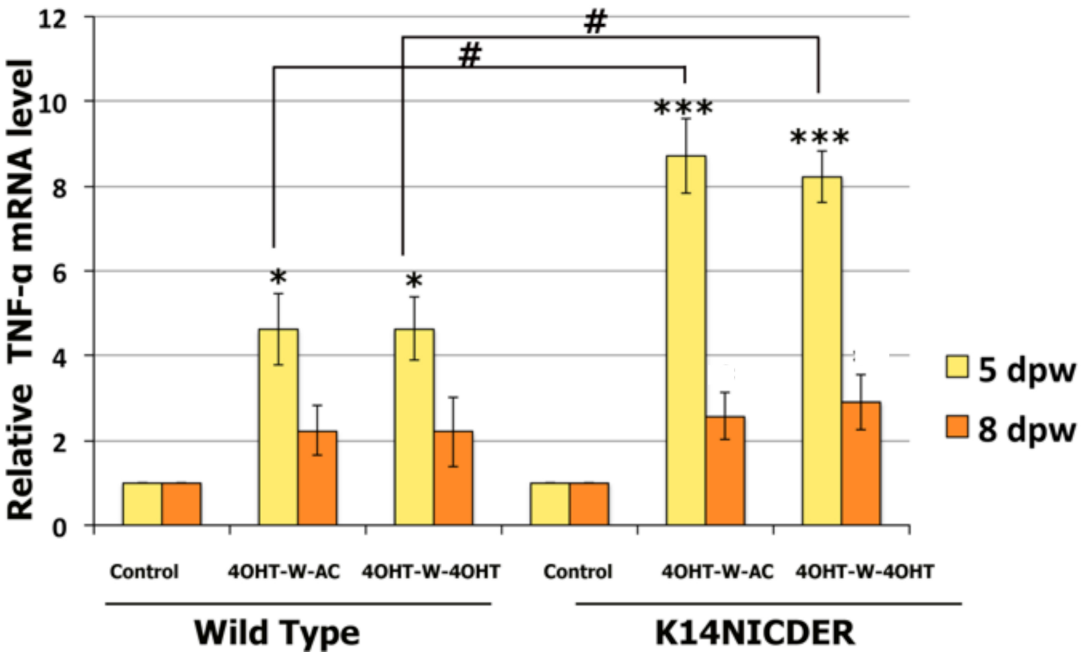


Figure 4.15. Relative TNF-α mRNA level

mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 days post wounding. Relative mRNA levels of TNF-α were quantified by q-RT-PCR. mRNA levels were normalised unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats. *p<0.05, ***p<0.0005. #p<0.05. Student's t-test. Comparison indicates the difference between each group of mice at different treatment groups and their corresponding controls. Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

4.6.4.3 Transcript levels of major cytokines implicated in wounding (IL22 and IL23) were induced in response to prolonged Notch activity over the healing process

Identification of crucial cytokines for wound healing regulation, such as TNF-α has been well established. Other cytokines including IL22 and IL23 were also found to contribute to this regulation and immune responses (Robinson and O'Garra, 2002). Previous research suggested that IL-22-deficient mice displayed major defects in the skin's dermal compartment after full-thickness wounding (McGee et al., 2013). The involvement of IL-22 signalling in wound healing has been further confirmed by demonstrating the activation of IL22 signalling in fibroblasts, using *in vitro* assays with primary fibroblasts, and that IL-22 directs extracellular matrix (ECM) gene expression and myofibroblast differentiation both *in vitro* and *in vivo*. These data defined that the IL22 receptor signalling pathway has a key role in skin wound healing by mediating interactions between immune cells and fibroblasts (McGee et al., 2013). Ambler et al., unpublished data, has recently provided evidence, which confirms that the control of wound closure occurs in part through activating the immune response, through a TNFα-dependent recruitment of IL22 producing ILCs (ILC22). This work has identified that Notch-regulated secreted factors including TNF-α and IL23, are up regulated in 4OHT-treated K14NICDER skin, which could facilitate immune cell infiltration into the dermis. These findings demonstrated that epidermal Notch activation induces TNF-α, which leads to IL22 production that drives normal skin tissue repair (Ambler et al, unpublished). As previously described, the link between IL23 and the stimulation of ILCs, leading to IL22 secretion (Sonnenberg et al., 2011) was reported. It can be hypothesised that a high transcript level of these cytokines via prolonged Notch activation, may explain the delay of wound closure by stimulation of more ILCs and over immune cell infiltration into the dermis. To test whether elongated epidermal Notch activation would affect the expression pattern of the major cytokines implicated in wound healing, the relative mRNA levels of IL22 and IL23 were quantified by q-RT-PCR. mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 dpw. Relative mRNA levels of IL22 and IL23 were quantified by q-RT-PCR. mRNA levels were normalised to unwounded control wild type or control transgenic animals. These data demonstrated that the samples from K14NICDER mice that had

an epidermally stimulated Notch activity during the healing process and K14NICDER mice that had Notch activity only prior to wounding, express high levels of IL22 and IL23 sequences comparing to controls. Increased transcribed levels of IL22 were induced as a response to prolonged Notch activation in K14NICDER mice ($p=0.0013$), likewise, in K14NICDER mice from group 2, where Notch was only activated before wounding, IL22 expression level increased (0.0012) at 5dpw compared to littermate control mice (Figure 4.16). IL23 relative mRNA levels in K14NICDER mice from group1 and group2 at 5dpw, showed a statistically significant increase ($p=0.0001$, $p=0042$; respectively) compared to littermate control mice (Figure 4.17).

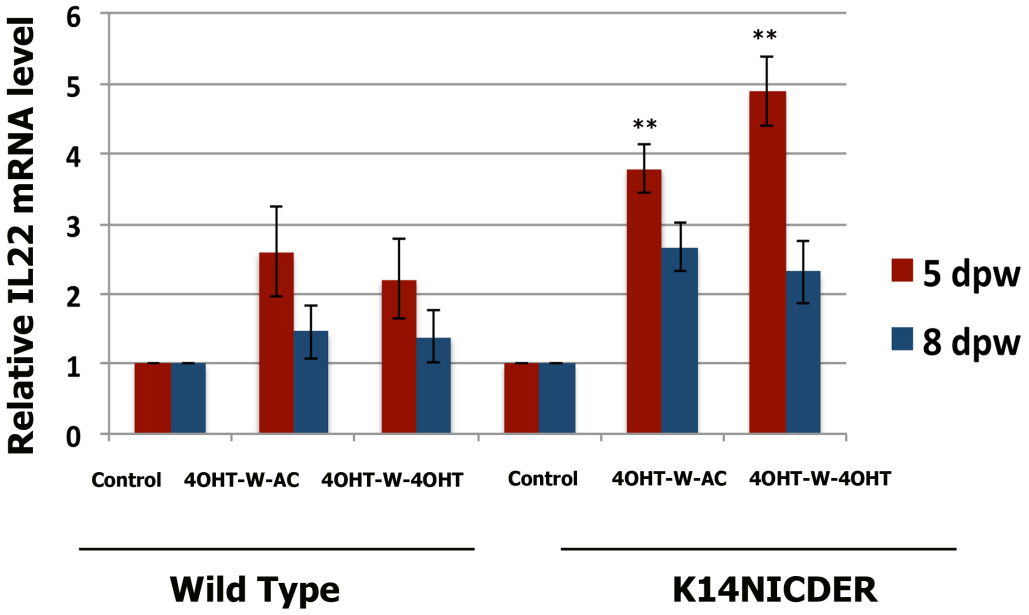


Figure 4.16. Relative IL22 mRNA level

mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 days post wounding. Relative mRNA levels of IL22 were quantified by q-RT-PCR. mRNA levels were normalised unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats. **$p<0.005$. Student's t-test. Comparison indicates the difference between each group of mice at different treatment groups and their corresponding controls. Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

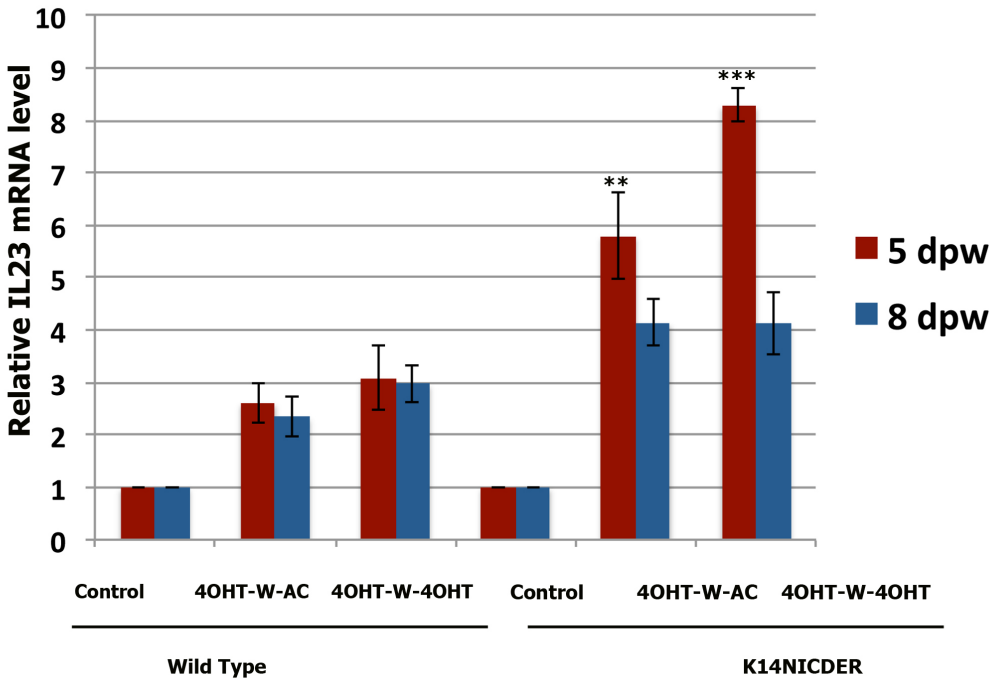


Figure 4.17. Relative IL23 mRNA level

mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 days post wounding. Relative mRNA levels of IL23 were quantified by q-RT-PCR. mRNA levels were normalised unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats. **p<0.005, ***p<0.0005. Student's t-test. Comparison indicates the difference between each group of mice at different treatment groups and their corresponding controls. Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

4.6.4.4 Negative effect of an excessive inflammatory response on Sm22-α expression

An efficient contraction of the granulation tissue that brings the wound margins together will be achieved when the mesenchymal cells of the granulation tissue differentiate into myofibroblasts. SM22 is a 22KDa protein expressed abundantly in the smooth muscle cells of vertebrates. Hence, induction of SM22 expression in fibroblasts is a critical step in wound healing. However, the link between inflammation and SM22 expression is still unclear; a recent study investigated whether SM22 disruption independently promoted arterial inflammation and characterised the underlying mechanisms of SM22 expression and inflammation. The authors concluded that disruption of SM22 expression promotes inflammation after artery injury via nuclear factor kappaB activation (Shen et al., 2010). The next question is to determine whether over-activation of Notch pathway during the inflammatory phase of healing suppresses the levels of SM22, at the mRNA level (Figure 4.18). In these experiments, quantitative real-time reverse transcriptase-PCR was used to determine the levels of expression of SM22. mRNA was isolated from unwounded controls, wounded wild type or K14NICDER mouse skin collected 5 and 8 dpw. Relative mRNA levels of Notch1, Notch2 and Jagged1 were quantified by q-RT-PCR. mRNA levels were normalized unwounded control wild type. These data showed that the relative SM22 mRNA level at 8dpw in wild type mice in both groups, was higher ($p<0.005$) compared to their corresponding controls (Figure 4.18).

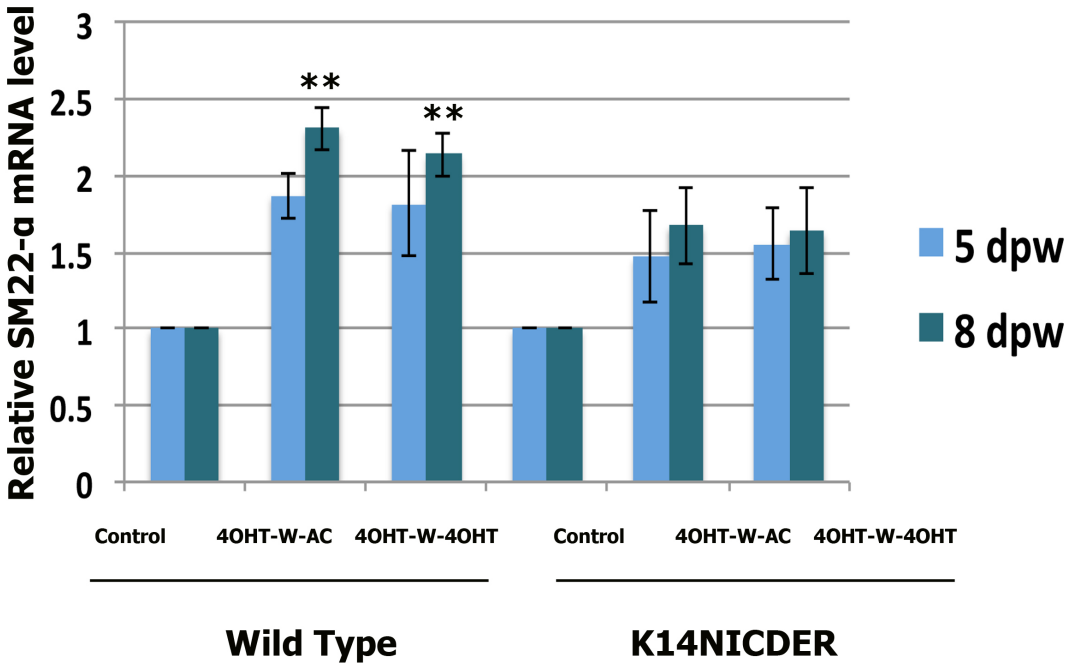


Figure 4.18. Relative SM22- α mRNA level

mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 days post wounding. Relative mRNA levels of SM22-α were quantified by q-RT-PCR. mRNA levels were normalised unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats. **p<0.005. Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

It was important to determine whether over-activation of epidermal Notch1 could induce SM22 expression that characterises myofibroblasts. Skin tissue samples from 4OHT-treated K14NICDER animals prior and post wounding, were collected at day 8 post wounding. The immunofluorescence, using monoclonal antibody–recognising mouse SM22, showed that Notch over activity decreased in dermal cells containing SM22 compared with the control group. Although previous studies reported a positive effect of TGFβ on SM22 expression in fibroblasts (Desmouliere et al., 1993), the incompatible activities of TNF-α against TGF-β-induced myofibroblast (fibroproliferative) phenotypic genes, for example, SM22, collagen type 1A, and fibronectin at the mRNA level, have been reported previously (Abraham et al., 2000, Goldberg et al., 2007, Verrecchia et al., 2002). The data in this investigation detected a diminution in the number of Sm22 positive cells after continued induction of Notch activity in K14NICDER wounded skin before and after wounding (Figure 4.18).

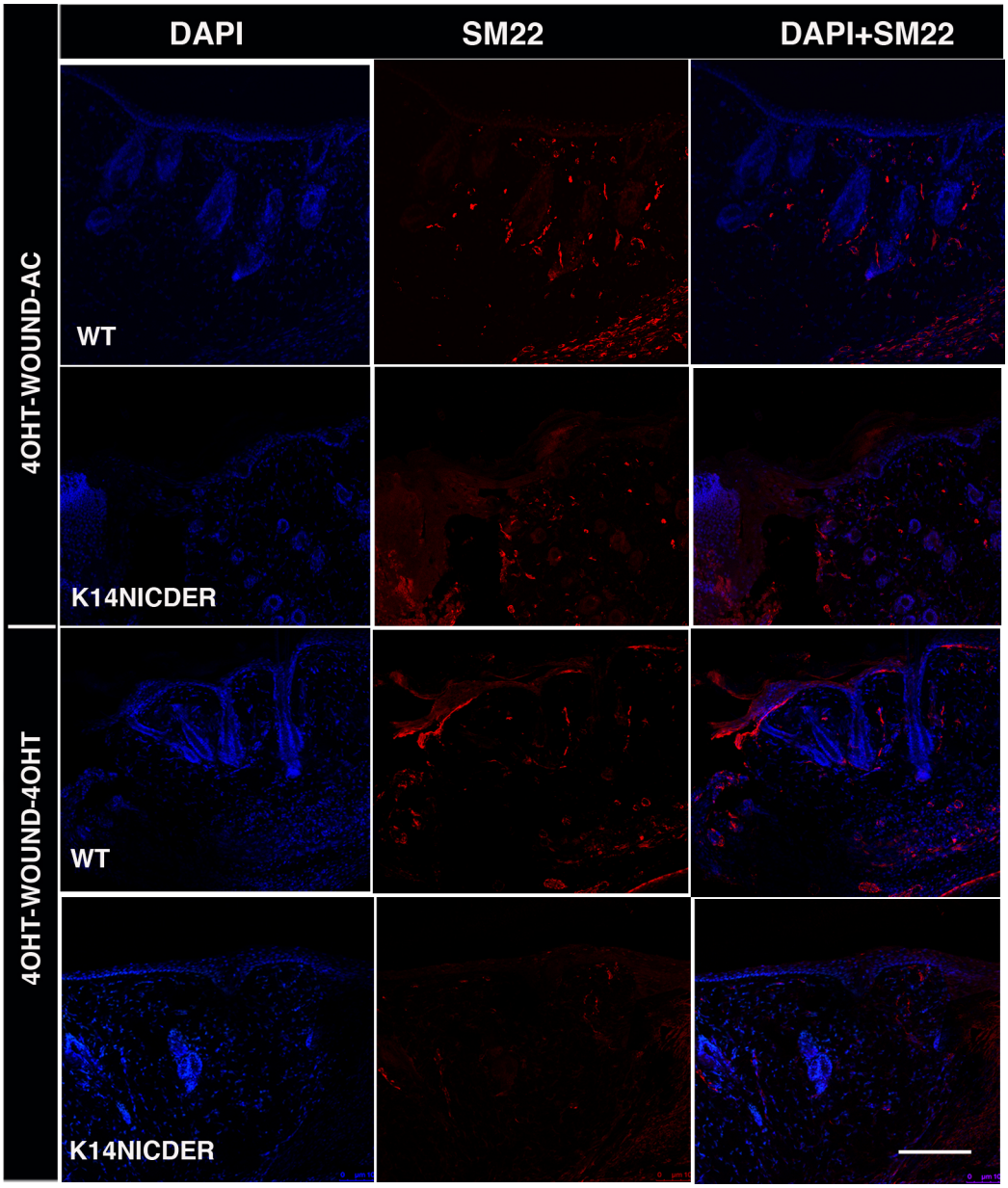


Figure. 4.19 back skin tissues were sectioned and stained with an antibody to SM22

8dpw back skin tissues were sectioned and stained with an antibody to SM22 antigen (Red) and DAPI (Blue) counterstained. Abbreviations; dpw, days post wounding; WT, wild type; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone. Scale bar 100 microns.

4.6.4.5 Increasing chemokines expression initiated by over-activation of Notch signalling

Although the functional capacity of the macrophage within the healing wound is well documented, little is known about the mechanisms of the monocyte recruitment into the wound bed. Among chemoattractants for monocytes that have been identified by previous studies, macrophage inflammatory protein1-α (Mip1-α) has been introduced as one of the most critical chemokines in mediating leukocyte infiltration into the site of injury. During the inflammatory phase of the normal healing process Mip1-α levels increase and as inflammation resolves and repair progresses, it decreases.

Data demonstrated in chapter 3, showed the activation of Notch1 and Notch2 in the wound site, with maximal Notch2 activity occurring 24 hours after wounding, during the acute inflammatory phase. To verify a conclusive function for chemokines, such Mip-1, throughout wound healing, and the relationship between this chemokine and prolonged inflammation, via over-activity of epidermal Notch, the expression pattern of Mip1-α in wounded K14NICDER and their corresponding wild-types and controls from both treatment groups were assessed. These data display statistically significant upregulation of Mip-1 at 5 and 8 days post wounding in K14NICDER mice with prolonged Notch activation (at 5dpw, $p<0.0005$), as well as in K14NICDER treated animals only prior to wounding (at 5dpw, $p=0.004$) (Figure 4.20).

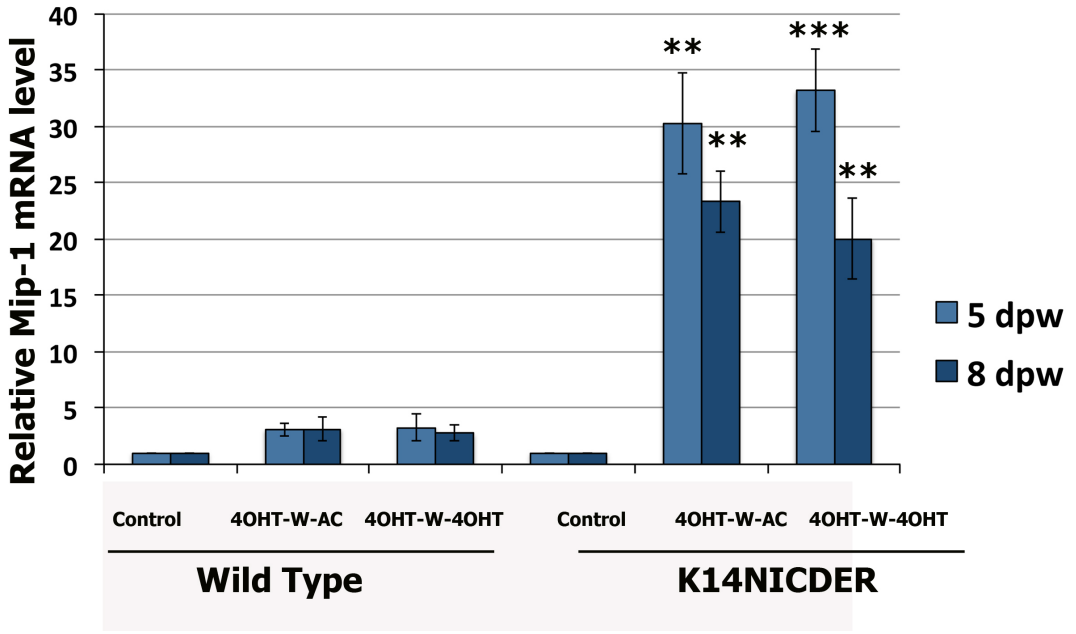


Figure 4.20. Relative Mip1 mRNA level

mRNA was isolated from unwounded controls or wounded wild type or K14NICDER skin collected 5 and 8 days post wounding. Relative mRNA levels of Mip-1 were quantified by q-RT-PCR. mRNA levels were normalised unwounded control wild type or transgenic mice. Mean ± SEM, n=3 mice per treatment groups from each genotype at each time point, 2 wounds per animal. Data shown are the average of three technical repeats. **p<0.005, ***p<0.0005. Student's t-test. Comparison indicates the difference between each group of mice at different treatment groups and their corresponding controls. Abbreviation: dpw; days post wounding; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

4.6.5 Accumulation of dermal MMP1 as a result of over-activation of Notch signalling

Chemokine and cytokine production during the inflammatory phase of the healing process is influenced by effector proteins, which include metalloproteinases (MMPs). Previous research demonstrated the inflammation regulatory functions of epithelial-derived MMPs, such as induction of leukocyte migration and chemokines activation during wound healing (Yong, 2005). Another study showed that up-regulation of MMPs, which are influenced by the inflammatory response, can alter many aspects of the wound healing process (Nguyen et al., 2006, Zhou et al., 2003). As delayed wound closure in K14NICDER mice treated before and after wounding was observed, metalloproteinase, such as MMP-1, within the wound may be responsible for this delay and overall impaired healing. The level of MMP1 protein accumulation within the wounded area of skin of K14NICDER animals, which were 4OHT, treated before and after wounding, at day 8 after wounding was investigated (Figure 4.21).

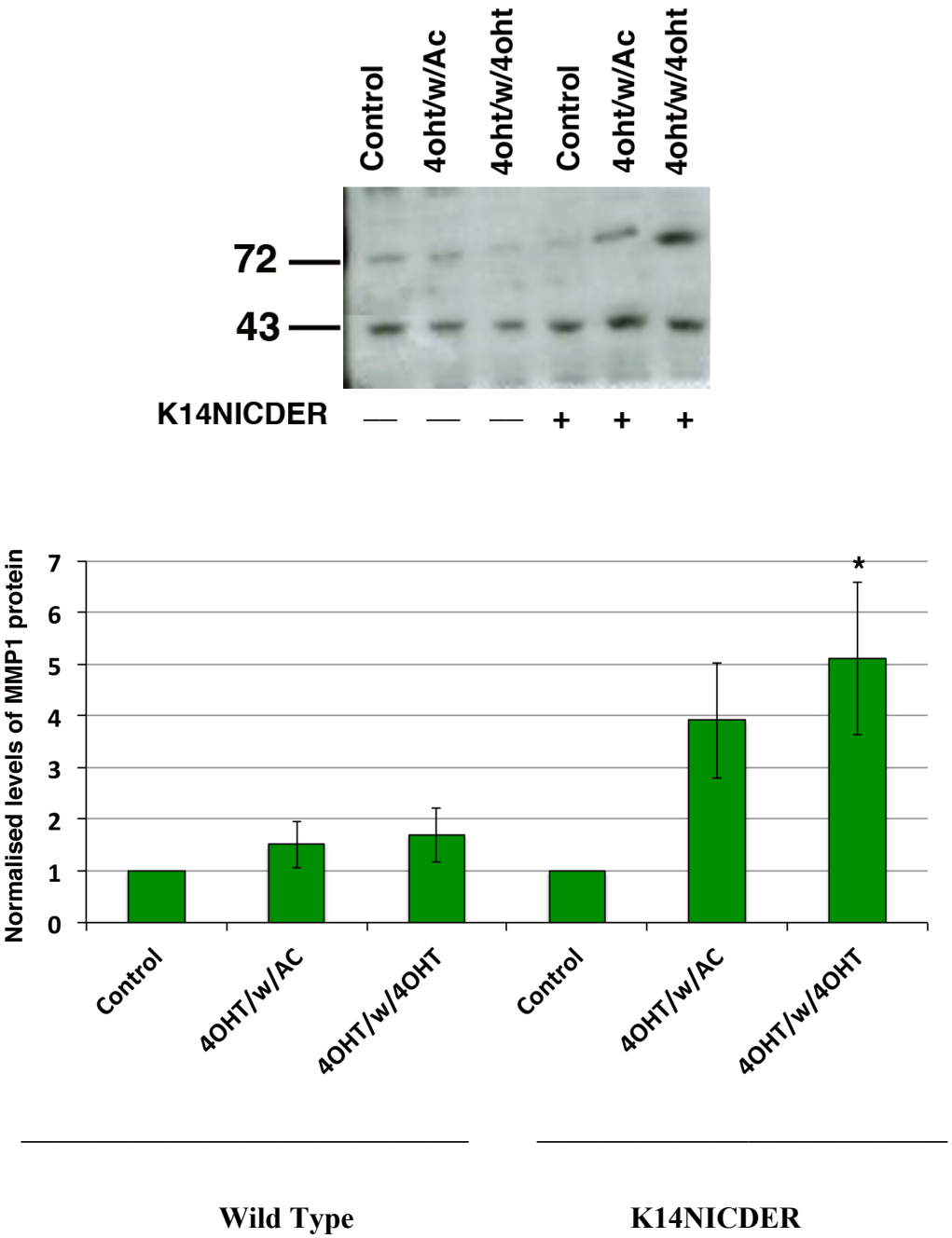


Figure 4.21. Quantification of MMP1 Levels.

(A) Representative immunoblot, showing MMP1(72 KDa) and β-actin (43KDa) in lysates (20 mg/lane) in skin tissue samples from punch-wounded wild type and K14NICDER mice, within indicated treatment groups, collected at 8dpw. Un-wounded back skin tissue taken from un-injured mice of each genotype as a control. (B) Quantitative analyses of immunoblot from in A). MMP1 levels were standardised to β-actin level and statistically compared to the average back skin protein level (defined as 1) of MMP1in Un-wounded littermate mice. Mean ± SEM, n=3..* p value <0.05. Abbreviations; 4OHT: 4hydroxy tamoxifen; W: wound; AC: acetone.

4.7 Discussion

This chapter explored the functional consequence of prolonged epidermal Notch activation in the context of inflammation during wound healing, using 4-mm punch biopsies on the dorsal skin of 7-week-old transgenic (K14NICDER) mice and non-transgenic litter- mates. Evidence demonstrated that persisted epidermal Notch activity during wounding in K14NICDER mice, results in postponed wound healing in these mice.

Normal wound healing goes through three overlapping phases: inflammation, proliferation, and remodelling (Geer et al., 2004). This project focused on time points during the inflammatory and early proliferative phases, where Notch activation is most pronounced (data presented in chapter three). Wound healing was delayed at post-injury day 4 to 8 within group 1 (Notch activated was prolonged) K14NICDER mice, and increased expression of pro-inflammatory cytokines was observed in the wounded mouse skin.

The results of persistent activity of epidermal Notch1 on wound healing are likely to be similar to that in chronic wounds as Notch may effect the function of multiple cell types that would be expected to play a role in inflammatory phase of wound healing. This is validated by a previous investigation that demonstrated that mice with myeloid-specific deletion of Notch1, display decreased macrophage recruitment and decreased TNF-α expression in wounds (Outtz et al., 2010). It was also reported that Notch signalling plays a role in regulating macrophage responses to inflammation. In a wound-healing assay, macrophage recruitment was decreased in *Notch1*$^{+/-}$ mice, and the wounds were characterised by decreased TNF-α expression (Outtz et al., 2010).

It is likely that the changes in accumulation of macrophages observed in K14NICDER wounds are due to Notch activity in epidermal cells, where Notch function within the epidermal immune response and in regulation of the macrophage response to inflammation has been well documented (Ambler and Watt, 2010, Outtz et al., 2010).

Macrophages have long been considered the key regulator for wound healing, since they not only act as a predominant type of inflammatory cells (M1 macrophage) in inflammation phase, but also are involved in proliferation phase (M2 macrophage) as

well as remodelling phase, following activation by different pathways (Rodero and Khosrotehrani, 2010). The abnormal macrophage activation found in the chronic wound may fail to lead to the inflammatory phase and hence transmit the wound straight into repair phase. Consequently, constitutive over-activation of Notch may result in excessive inflammation, which over-rides other positive effects of Notch in wound healing. Taken together, the genetic mouse model (K14NICDER) has established a specific role for Notch signalling in control of the inflammatory response during wound healing, but further study is needed to elaborate on how Notch1 and Notch2 in other cell types may contribute to wound healing and inflammation.

Furthermore, expression levels of cytokines that are under the control of Notch activation may also affect wound healing. Pro-inflammatory cytokines such as IL-1α, IL-1β, IL6 and TNFα, have been demonstrated to be principal mediators during the inflammation phase of skin wound healing and up-regulated at this stage of healing cascade (Hubner et al., 1996). Ambler et al., unpublished data, recently presented that the blocked Notch1 inhibited the wound closure, TNFα, IL23 and IL22 production. Hence the fundamental role of Notch signalling as a wound response regulator can be concluded. It has been reported that neutrophils and macrophages are major sources of these cytokines (Mori et al., 2004). TNF- α is a pleiotropic pro-inflammatory cytokine that is involved in the pathogenesis of a number of inflammatory diseases and has been specifically implicated in the tissue damage associated with chronic venous disease (Cowin et al., 2006). Previous studies demonstrated higher levels of TNF-α in wound fluid from non-healing ulcers compared with wound fluid from healing ulcers (Cowin et al., 2006, Murphy et al., 2002). Data presented in this investigation concurs with previous reports,with a higher level of TNFα in delayed wound closure skin samples from K14NICDER mice in-group 1 that received a prolonged Notch activity. These results can be supported by another study that illustrated a delayed in wound closure as a result of TNF-α deficiency that affected expansion of neutrophils, macrophage infiltration and reduced the rate of angiogenesis and collagen deposition at the wound site (Ishida, 2006). These studies provide unique insights into a better understanding of the effects of Notch on skin wound healing, which will facilitate the development of new therapies for impaired wound healing through modulating Notch signalling.

Previous research provided evidence that Notch plays a role in regulating the inflammatory response and wound healing. This study hypothesised that the constant epidermal Notch activity in K14NICDER mice would provide an over-inflammatory level of cytokine expression. Notch1 and Notch2 prolonged activation in the epidermis showed that the expression level of TNF-α and TGB-β largely boosted in K14NICDER mice. Increasing the level of IL23 and IL22 production as a result of prolonged Notch activation in wound healing was also detected. However, an enhanced induction of the immune response to the wound site, with the over recruitment of ILC and increased cytockines production may inhibit wound closure.

These results suggest that over-activation of epidermal Notch is able to inhibit a normal wound closure by increasing the pro-inflammatory cytokines present in the wound tissue. It is possibly promising that a reduced activation level of Notch may be able to inhibit the presence of TNF-α in chronic wounds. Detecting the influence of constant epidermal Notch activity during skin wound healing on pro-inflammatory cytokines expression, may deliver new therapeutic aspects for non-healing chronic wounds. For instance, a decrease in TNF-α expression by controlling Notch activity could result in a decline in chronic inflammation, and non-healing wounds may then be able to enter a healing phase resulting in improved healing.

In addition, the consequence of continuous epidermal Notch activation leads to excessive TGF-β1 expression. A previous study has confirmed an up-regulation of TGF-β in the skin as a result of inducible epidermal Notch activation (Ambler and Watt, 2010). TGF-β has been introduced as a pivotal mediator in wound healing, which contributes to the infiltration and activation of inflammatory cells (Ashcroft 1999). After injury, TGF-β is rapidly upregulated and secreted by keratinocytes, platelets, and macrophages (Singer and Clark, 1999). TGF-β is essential for initiating inflammation and granulation tissue formation (McCartney-Francis and Wahl, 1994). Additionally, TGFβ may be required for cell migration during wound repair (Gailit et al., 1994; Zambruno et al., 1995). Previous wounding experiments have shown an impaired wound healing in humans by reducing the TGF-β expression level (Schmid et al., 1993; Cowin et al., 2001; Jude et al., 2002). Furthermore, the application of exogenous TGF-β, in animal models of impaired wound healing in which TGF-β expression was reduced, improved the rate of healing (Pierce et al., 1989; Salomon et al., 1990; Beck et al., 1991, 1993). Correspondingly TGFβ has also been

demonstrated that directly induces the α-smooth muscle actin expression in fibroblasts by which controls and encourages wound contraction (Montesano and Orci, 1988; Desmouliere et al., 1993).

Considering that TGF-β is a potent chemotactic cytokine for virtually all leukocytes, as well as endothelial cells and fibroblasts (Wahl et al., 1987, 1993; Wahl, 1992, 1994), all of which are involved in the development of inflammation, the pro-inflammatory effect of TGF-β is likely to predominate in the skin. In supporting this notion, overexpression of TGF-β in basal keratinocytes and hair follicles initiates chronic skin inflammation, marked by epidermal hyperplasia, leukocyte infiltration, and angiogenesis (Liu et al., 2001; Li et al., 2004).

It has been demonstrated that transgenic mice constitutively overexpressing wild-type TGFβ in keratinocytes (K5. TGF-β wt) exhibited a significant delay in full-thickness wound healing compared to non-transgenic mice. This previous work suggested that the reason for the delayed wound healing was linked with profound inflammation throughout all stages of wound healing in K5. TGF-β wt mice. The authors suggested that excessive and prolonged TGFβ at the wound site does not benefit wound healing, which is partially owing to its pro-inflammatory effect. Likewise, presented data in this chapter demonstrated that the level of TGF-β reached a peak level in mice with the prolonged Notch activity, which coincide with the peak of the inflammation. Therefore, the reason of the delayed wound healing in K14NICDER mice can be linked with profound inflammation and rising the TGF-β expression level in response to over-activation of Notch.

Beside the fundamental roles of growth factors and pro-inflammatory cytokines during inflammation, the significance of other mediators that are involved in healing process know as chemokines are also highlighted (Lusier, 1998).

To verify a conclusive function for chemokines, such as Mip-1(macrophage inflammatory protein 1), throughout wound healing and particularly during the inflammatory phase, genetically modified mouse models with altered expression of these exclusive chemokines have been evaluated. A transgenic mouse model, which lacks the critical macrophage chemoattractant did not show an alternation with the total number of macrophages at wound site whereas the angiogenesis, collagen synthesis and re-epithelialisation in this model was significantly reduced and delayed

wound closure was reported (Low, 2001). This study described an unaffected healing process in animals lacking Mip1-α (Low2001). A previous study isolated bone marrow-derived macrophages (BMM) from mice and characterized Notch signalling in the context of stimulation. Outtz and colleagues stimulated BMM with LPS/IFN-γ, then collected culture supernatants and assessed cytokine levels. They demonstrated a Notch related increased levels of MIP-1α, IL-6, IL-12, MCP-1 and TNF-α in the supernatant of stimulated BMM. They also proved that the induction of these cytokines upon stimulation was diminished when Notch signalling was inhibited with (Outtz et al., 2010). The data presented here exhibited no changes at the level of Mip-1α expression during day 5 and 8 post wounding in wild-type littermates from both treatment groups. Surprisingly, the level of Mip-1shows a significant increase at 5 and 8 days after wounding in K14NICDER with prolonged Notch activation, as well as in K14NICDER mice treated just before wounding. This magnification of Mip-1α expression might be related with the activation and recruitment of macrophages caused by over-activation of Notch in these mice.

This investigation provides data that suggest that the delayed cutaneous wound healing in K14NICDER mice with an over activation of Notch, can be partially attributed to excessive inflammation throughout all stages of wound healing. Involvement of some proteases, such as matrix metalloproteinase (MMP 1, MMP2, MMP3, and MMP9) in cell migration during wound healing has been reported previously (Eming et al., 2007).

The expression and activity of all these MMP classes have also been shown to be up-regulated in chronic venous ulcers (Eming et al., 2007). It has been reported that pro-inflammatory cytokines are potent inducers of MMP expression in chronic wounds, and have been shown to down-regulate tissue inhibitors of metalloproteinase expression, thereby creating an environment with a relative excess of MMP activity. This investigation presented the accumulative level of MMP1 in K14NICDER in a response to prolonged Notch activity. Therefore, prolonged inflammation in K14NICDER wounds creates an environment with a relative excess of MMP1 activity. Indeed, components of the ECM (fibronectin and vibronectin) are downgraded or inactivated within chronic wounds (Grinnell et al., 1992, Grinnell and Zhu, 1996). This matrix degradation may explain the delay in wound closure seen in K14NICDER mice when Notch was over-activated. Previous research has shown that

in chronic wounds, protective growth factors such as PDGF and vascular endothelial growth factor are also targeted when there is excess protease activity (Mendez et al., 1998, Roth et al., 2006).

Normally, TNF-αis not present during the re-epithelialisation and ECM reorganisation phases of wound healing (Singer and Clark, 1999). Healing proceeds only after the inflammation has subsided. Under pathological conditions, such as in chronic wounds, the cascade of wound healing is disrupted and the wounds are locked into a state of inflammation characterised by abundant neutrophil infiltration with associated reactive inflammatory cytokines (TNF-α). Previous research has reported a very high level of TNF-α in chronic wounds fluid compared to healing wounds (Trengove et al., 2000).

The results from this study showed high levels of TNF-α in prolonged Notch activated mice and suppression of SM22. It has been reported that in the chronic wound healing process TNF-α- suppressed myofibroblast differentiation and cytoskeletal changes are required for normal matrix contraction (Goldberg et al., 2007). TNF-α mediates its antagonistic effects on TGF-β1 through the JNK pathway. Inhibition of Smad3 phosphorylation leads to a decrease in transcription of TGF-β1 response genes, that is, collagen 1A, fibronectin, and α-SMA (Goldberg et al., 2007). Therefore, chronic wounds with a high level of TNF-α would fail to contract in a normal fashion. Consequently, continued inflammation, mediated by TNF- α, as a result of prolonged Notch activation, might prevent normal matrix deposition and myofibroblast- dependent wound contraction in normal wound healing.

Conclusion: Although several studies have documented that a prolonged inflammatory phase in wounds impairs tissue repair (Fonder et al., 2008, Greaves et al., 2013), other reports have raised questions as to whether prolonged Notch activation at the wound site is necessary or conducive for wound healing. Due to the extra release of inflammatory cytokines and accumulation of further macrophages, elongated epidermal Notch activation at the wound site may be associated with tissue destruction that prevents the proliferation and remodelling that is necessary for wound resolution. Accordingly, the activation of Notch signalling plays an important role in whether they promote or prevent wound healing, and inflammation, the over-activation of Notch may do more harm than good in terms of over inflammatory

response at the wound site. Using K14NICDER mice, this study has shown that Notch1 and Notch2 are important for TNF-α expression and infiltration of inflammatory macrophages to wounds. These data suggest that Notch plays an important role in early stages of skin healing process, a finding that has implications in a variety of settings, including in wound inflammatory response.

Thus, although a great deal of work remains to be done, the ability of Notch signalling to regulate the activity of bioactive molecules, such as growth factors, cytokines and chemokines, proposes an fundamental role for Notch family genes in all phases of wound healing.

5 Different rate of healing on wounds located at different anatomic regions of the mouse body

5.1 Introduction

There are numerous factors that effect one or more phases of the wound healing process, these are classified into local and systemic factors (Guo and Dipietro, 2010). Local factors are those that directly influence the characteristics of the wound itself, while systemic factors are the overall health or disease state of the individual that affect the ability to heal. Local factors that influence wound healing include growth factors, tissue fluid (oedema), blood supply, oxygen and infection. Many of these factors are related, and the systemic factors act through the local factors to effect wound healing. Single or multiple factors may play a role in any one or more individual phases, contributing to the overall outcome of the healing process (Hess, 2012). Although different factors have been extensively researched for their roles in tissue repair, the role of the anatomical location has not been fully defined.

The anatomical position of wound is a critical factor in entire healing process; the quality and the duration of healing. Wounds located on mobile areas, such as around a joint, or located on areas that are under pressure, are more difficult to be managed during the healing process than other anatomical locations (Blume et al., 2008, Hess, 2012). Previous research focusing on the diabetic foot has shown that it is a particularly difficult location for wound healing, with the site of the foot ulceration particularly important in healing (Hess, 2012). Research has demonstrated, that while the overall healing rates of foot ulcers were similar at different locations, heel ulcers tended to heal more slowly (Falanga, 2005, Harding et al., 2002).

A number of anecdotal reports suggest that wound location in mice also impacts upon closure rates. In most wound healing studies, multiple wounds are created on each animal. A few studies emphasised in their experimental design the randomized assignment of differently sited skin wounds to each treatment (Falanga, 2005, Harding et al., 2002, Mendoza et al., 2012); however, many have not clearly stated

the factors which determine the anatomical location. Literature searches have not produced any studies, prior to this investigation, which directly compare wound healing on different sites on mouse back skin, although previous burn healing studies reported rapid healing on the posterior quarter to third of the animal (but without providing data) (Davis et al., 1996, Davis et al., 1990). Another study indicated no difference between the healing of cranial or caudal burns (Singer and McClain, 2003) whereas, Wang, et al.,(2009) examined healing in different sites on a porcine burn model and indicated that caudal burns healed significantly better than cranial burns, demonstrated by earlier closure of caudal wounds and less scar formation (Wang et al., 2009). This investigation examined the reason of different rate of healing on wounds located at different anatomic regions of the mouse body. Data presented in this chapter showed that lower body wounds healed significantly better than those in the upper posterior, or middle to posterior of the mouse back skin wounding model.

5.2 Experimental plan

Male C57BL/6 mice at 7 weeks of age (the resting stage in hair cycle) were employed. The back skin of the mice was shaved and animals anesthetised using 2 to 2.5% vaporised inhaled isoflurane. A line was drawn to indicate the distance between the tip of the nose and the caudal edge of the hair region of the body. This distance was measured and taken to be 100%. Lines of 75%, 50% and 10% from the caudal border of the hair region were drawn (Figure 5.1).

Dorsal skin was cleaned with Iodine/Povidone 10% solution and wiped with distilled water. Full-thickness dermal wounds were created in the skin of the back of the mice (Figure 5.1) using a 4-mm biopsy punch (Stiefel, USA) pressed into skin with twisting motion until both layers of skin were punched through. Wounds were made at different anatomic regions; upper posterior (A=75%), middle to posterior (B=50%) and posterior-most (caudal) (C=10%) (Figure 5.1). At the desired time points (1, 4 and 7 days after wounding) three mice wounded at each wound location were sacrificed. Skin tissue samples were excised at the wounds, including a few millimeters of the surrounding skin from all of animals for histological and biochemical analyses. For RNA extraction and protein lysates, the same amount of intact tissue was collected from each mouse. Digital photographs of the injury site were taken; wound size was measured and quantified.

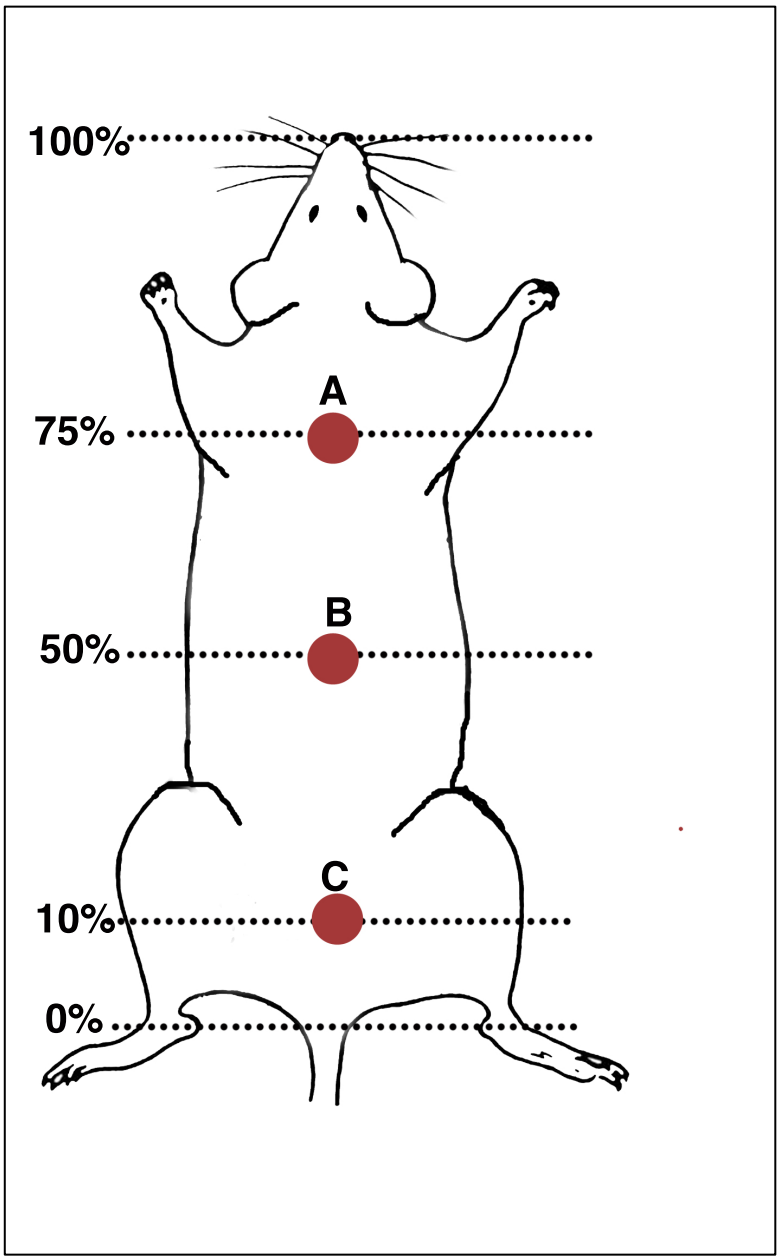


Figure 5.1 Diagram showing the relative position of injured sites.

Several percentage lines were drawn between the tip of the nose (100%) and the caudal edge of the hair region of the body (0%). 4 mm wounds were made at the mid line of the body (A, B, C).

5.3 Results

5.3.1 Anterior and mid-region wound healing was delayed compared with posterior wounds in mice back skin

As skin wound healing is a complex and dynamic process, consisting of various sequential phases, the wound repair was carefully analysed over different stages of healing. 4 mm wounds were created in the back skin of wild-type mice at different regions and the healing process was analysed at days 1, 4 and 7 post-injury (n=3 for each time point and each wound location). As wound healing of the middle and upper posterior region wounds were visibly delayed, to assess the role of anatomical region in wound healing *in vivo*, wound sizes were measured daily until complete epithelialisation. Macroscopically, wound sizes were larger in upper and middle sites (Figure 5.2 a). Daily measurements revealed a statistically significant difference in wound sizes between the three locations from day 2 ($p<0.0005$) to day 5 ($p<0.005$) post wounding (Figure 5.2 b).

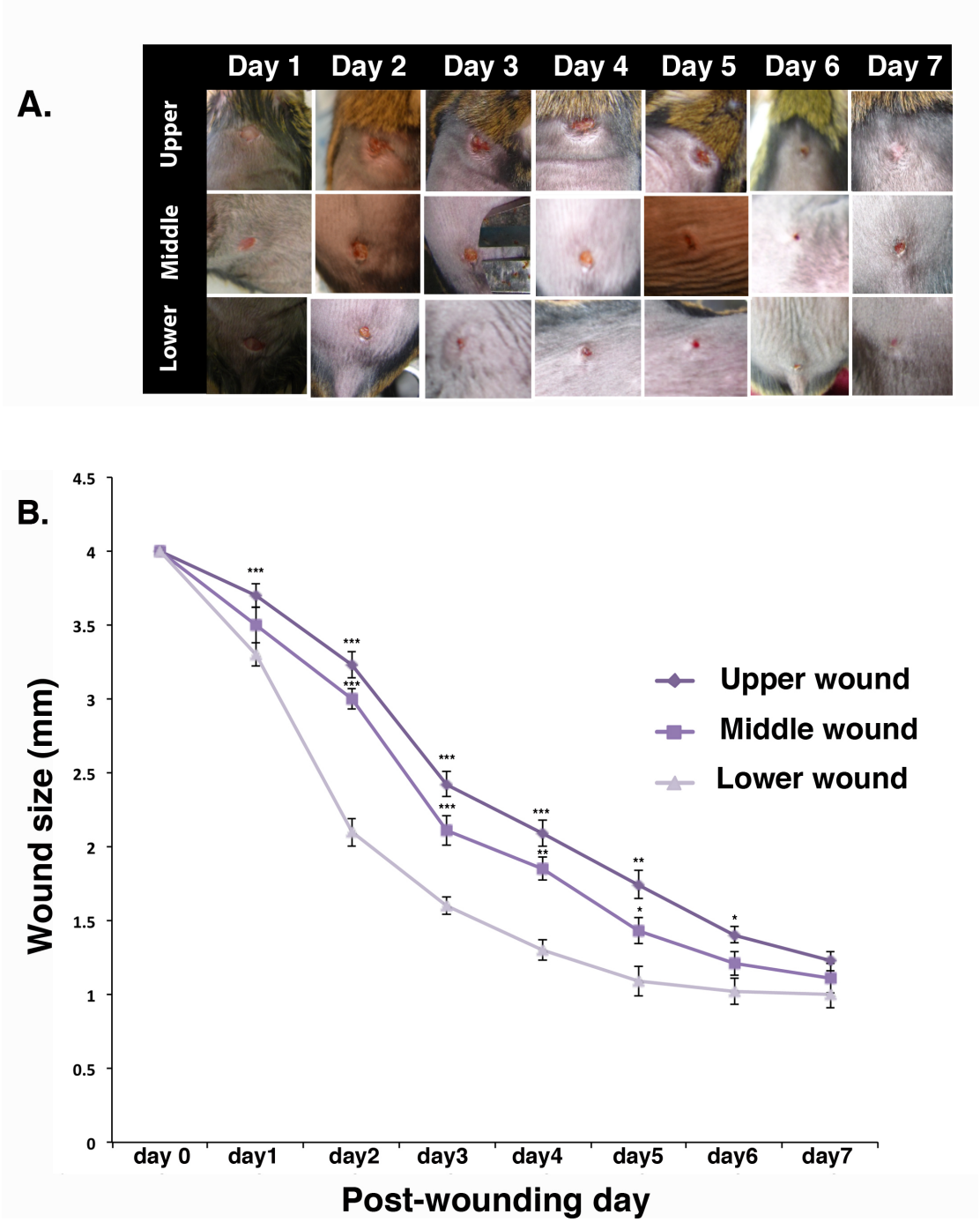


Figure 5.2. Daily measurements of wound size at different locations

Lower wounds heal faster than the middle and upper sites A) Representative appearance of wounds at day 1 to 7 at different regions. B) Maximum length of the wound in posterior, middle-posterior and upper-posterior regions post wounding (n=3, for each time point and location). Data are presented as mean ± SEM. *P<0.05, **P<0.005, ***P<0.0005. Student's t-test.

Figure 5.3 shows representative micrographs of wounded skin at days 4 and 7 post wounding, showing a larger wound gap in the upper and middle locations compared with the lower site of mouse back skin (posterior). These findings suggest that posterior wounds heal faster than the middle and upper sites of mouse back skin.

Histological analysis demonstrated keratinocyte migration into the wounded area and subsequent migration of epithelial leading edges in wounds from all considered locations at (Figure 5.3).

In conclusion, most posterior wounds demonstrated faster re-epithelialisation and completion in closure of wounds, compared with anterior and middle wounds by 4 dpw (Figure 5.3). Arrowheads signify the wound margins at 4 dpw, the distance between the migrating epidermal tip and the original wound edge was also measured. The length of the migrating epidermis of upper and middle wounds was greater than that of wounds located in the caudal region at day 4 post wounding, these measurements showed an statistically significant ($p<0.05$) difference between both upper and middle wounds compared with the lower ones (Figure 5.4).

Seven days after injury, the wounds on all locations exhibited near-complete wound closure. However, Van Gieson staining revealed elastic fibres had not yet regenerated in the wound bed of upper and middle body injuries, whereas dense elastic fibres were observed in lower body dermis adjacent to the wound (Figure 5.5 arrows). These data indicate that the absence of elastic fibres in anterior and middle regions of mouse back skin negatively affected wound closure in these anatomical locations.

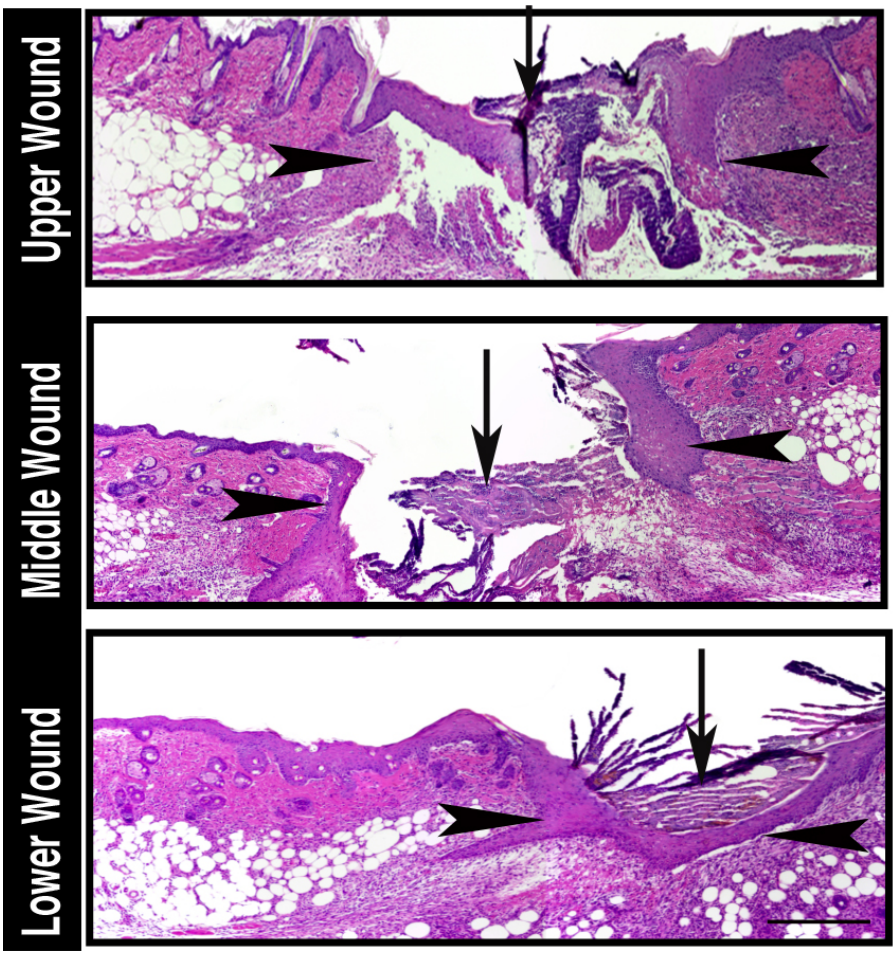


Figure 5.3. Representative micrographs of wounded skin at days 4 post wounding

Hematoxylin and eosin (H&E) staining of wounded skin at day 4 post wounding. Arrowheads demarcate the wound margins and arrows indicate the centre of the wound injury. Scale bar=200 microns.

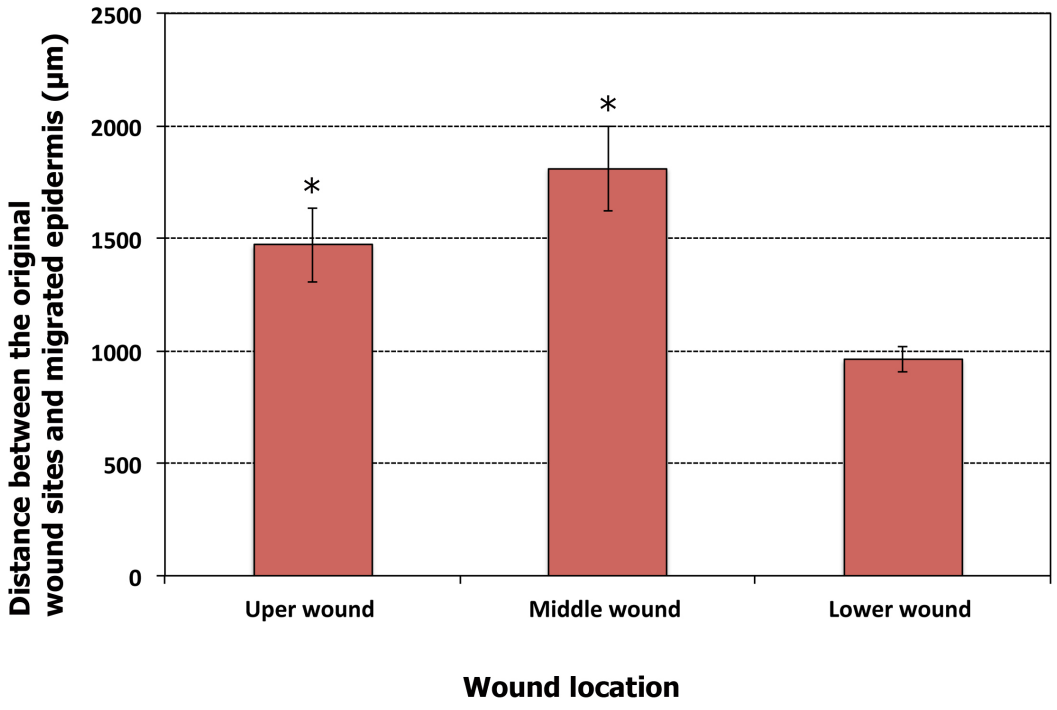


Figure 5.4 distances between the original wound sites and migrated epidermis

The distance from the original wound site to the migrating wound tip from different anatomical sites of mouse back skin at 4 dpw. (n=3 from each location). * p value<0.05, indicate a significant difference between upper and middle wounds, comparing with the lower wounds.

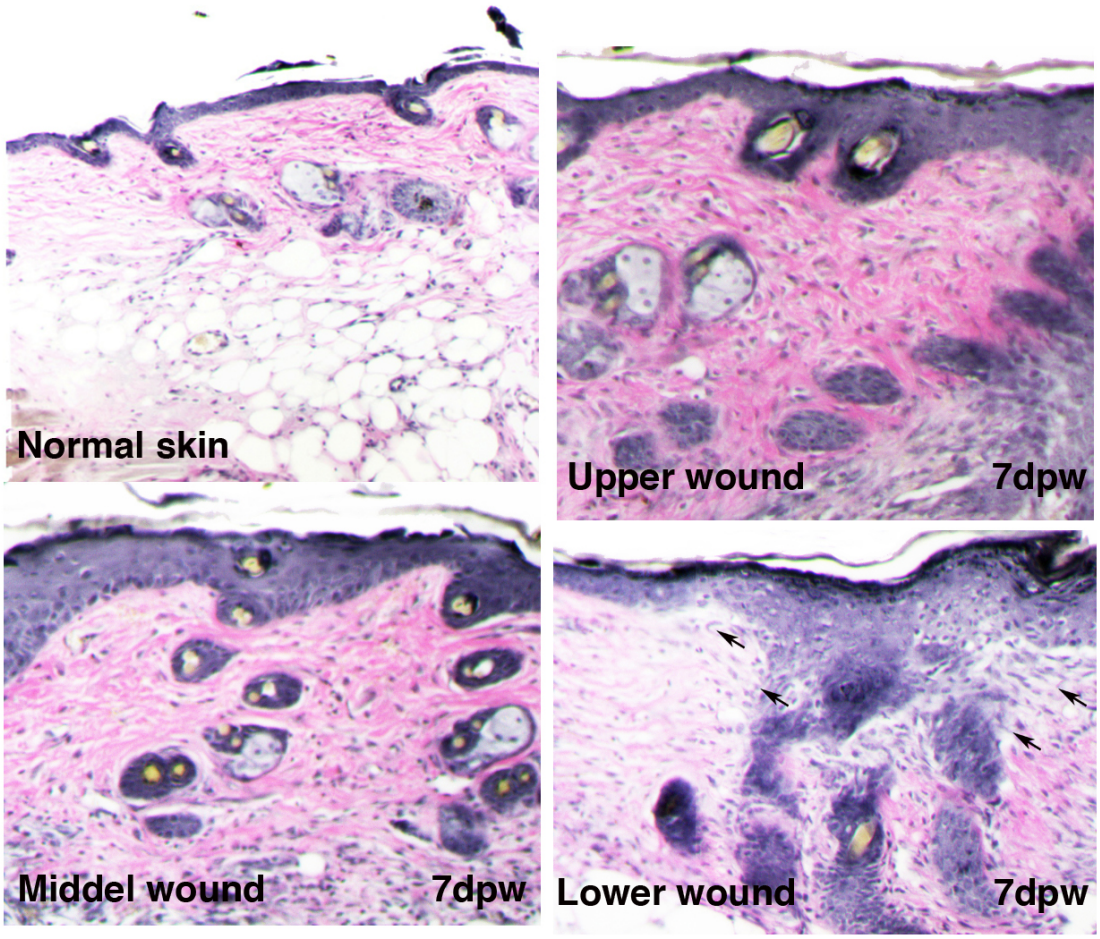


Figure 5.5. Verhoeff's Van Gieson staining for elastic fibres

Verhoeff's Van Gieson staining for elastic fibres shows a network of elastic fibres in the dermis adjacent to the wound in the lower body wounded skin after 7 days (arrows), which is not seen in the upper and middle body wounds.

5.3.2 The delayed re-epithelialisation in upper and middle body wounds may be a result of decreased keratinocyte proliferation

Location of the wound may be functional in the proliferation and migration of keratinocytes at the early stages of healing. The delayed re-epithelialisation in upper and middle wounds may occur as a result of decreased keratinocyte proliferation. The number of both epidermal and dermal Ki67-positive cells after wounding were compared to assess keratinocyte proliferation in wounds located at different regions (Figure 5.6). In lower body wounds, the number of epidermal and dermal Ki67-positive cells was increased ($p<0.005$), with a peak (50% increase) observed 4 days after wounding. However, the increase in Ki67-positive cells was delayed in upper and middle wounds at day 4 (32-36%). Lower wounds showed more Ki67- positive cells at day 4 post wounding, both within dermal and epidermal cells (Figure 5.7). These results suggest the existence of a posterior-anterior gradient in dermal and epidermal proliferation and wound closure in response to injury.

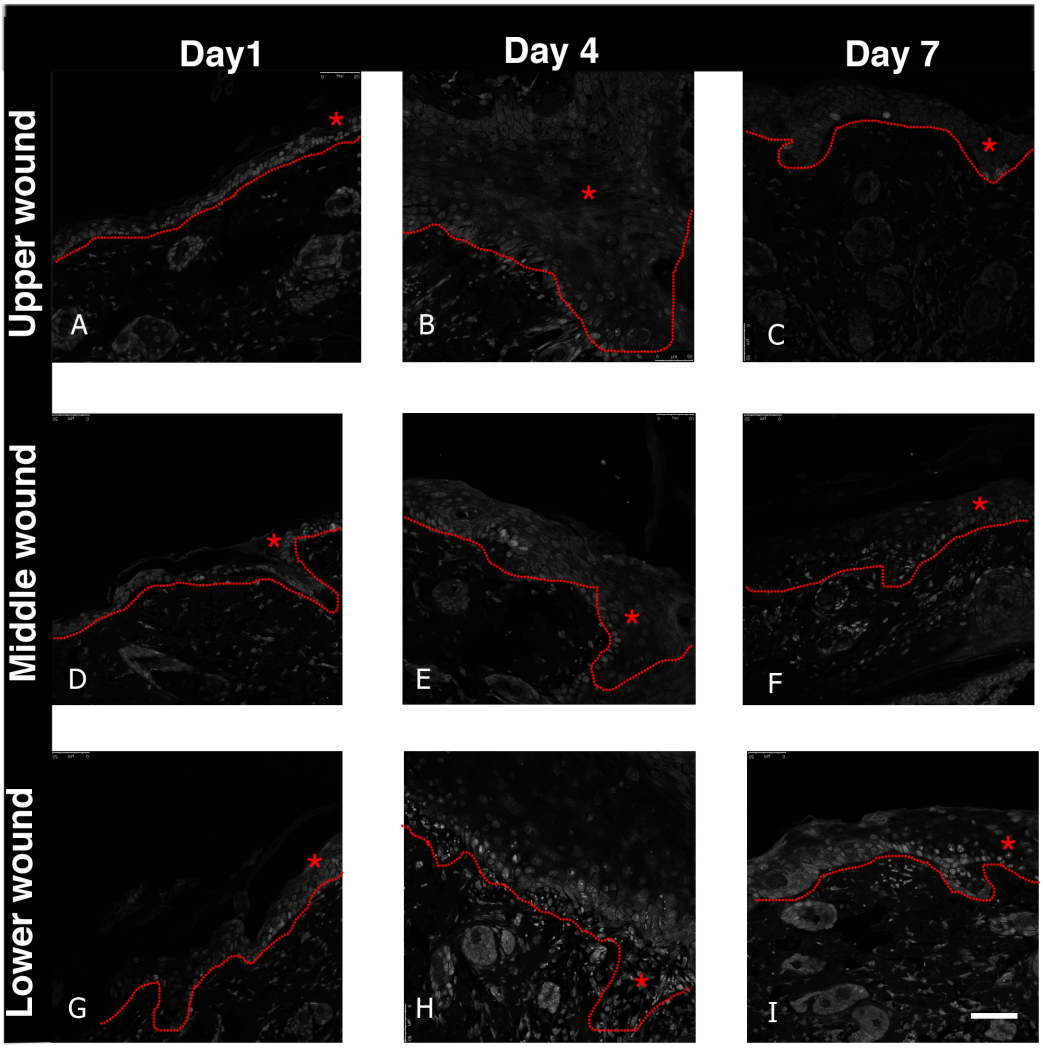


Figure 5.6. The epidermal and dermal Ki67-positive cells

Location of the wound may be functional in the proliferation 4 mm full-thickness wounds were created in 7-week-old wild type mice at different locations (Upper-posterior (A-C) middle posterior (D-E) and lower-posterior (G-I)). Sections of wounded skin were collected 1,4 and 7 days post wounding and stained with antibodies to Ki67 (White). The red dashed line mark the epidermal-dermal boundary; red asterisk marks wound site. Scale bar equal 200 microns.

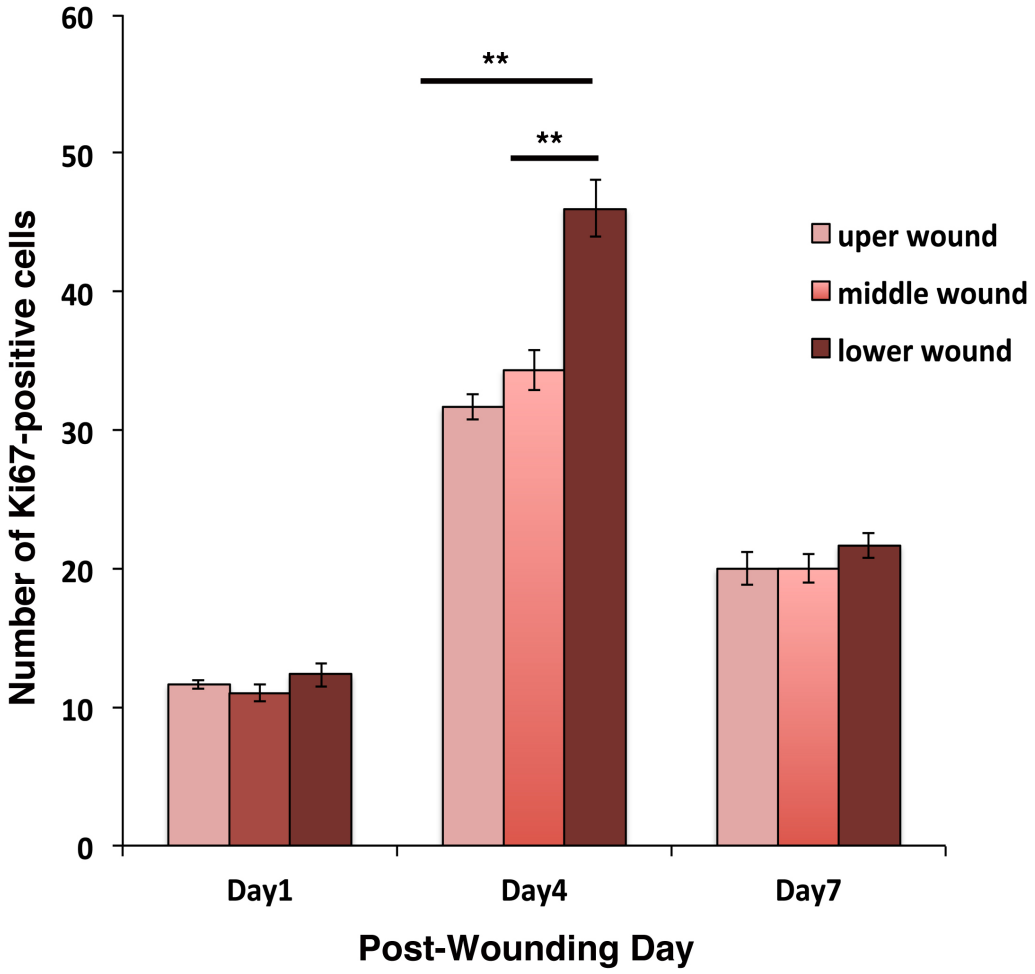


Figure 5.7. Quantification of the total Ki67 positive basal epidermal and dermal cells

Graph shows the number of Ki67 positive basal epidermal and dermal cells within 500 microns of the different regional wounds at 1, 4 and 7 days post wounding. Mean ± SEM ; T-test ,**P value<0.005.

5.3.3 No Changes in the Notch1 activity at different regional wounds

Findings in previous chapters suggest that activated Notch1 and Notch2 levels are highest in the inflammatory and proliferation phase of the wound response. The link between the wound healing and the effects of Notch activity during the healing process has been confirmed (Chigurupati et al., 2007). Previously, this study showed that throughout the skin wounding, Notch1 and Notch2 activity increased steadily in the upper stratum spinosum in wounded skin until day 4 post wounding, at which point activation subsided but remained detectable at day 7. To examine the possibility of site-dependent Notch1 activity on wounded mouse back skin by 4dpw, western blot analysis was employed. These data did not detect greater increase in Notch1 activity in wounded skin at different regions during wound healing (4dpw) (Figure5.8).

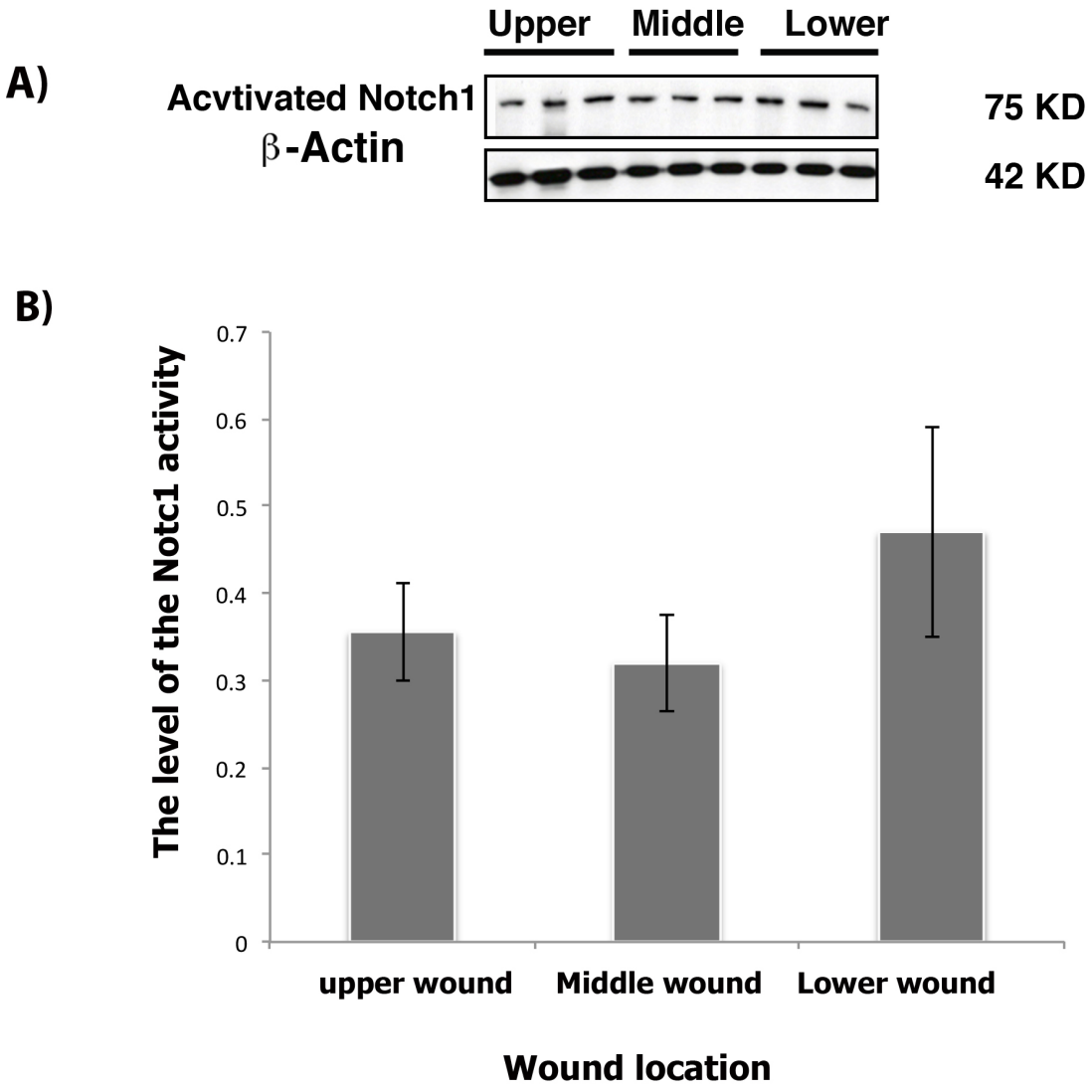


Figure 5.8. Relative levels of activated Notch1 at different locations

A) Representative immunoblot, showing relative levels of activated Notch1 (75 kDa) and B-actin (42 kDa) in lysate (20 mg/lane) in wounded skin samples from different locations of mouse back skin. B) show the quantitative analyses of immunoblot data. mean values ± SEM, n=3.

5.3.4 No detectable change in production of inflammatory cytokines in wounds at different regions

Immediately after injury, during the early inflammation phase, platelets and fibronectin accumulate to form a clot at the injured site. This clot acts as a source from which several chemotactic substances are released, including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), and immunoregulatory cytokines, providing a provisional matrix that allows cell migration (Amadeu et al., 2003, Gosain and DiPietro, 2004). An exaggerated inflammatory response results in increased production of pro-inflammatory cytokines in the initial wounding response. Wounds within an environment of sustained inflammation, matrix degradation and reduced growth factor activity, present with reduced tissue repair, cellular proliferation and angiogenesis (Chen et al., 1997, Harding et al., 2002, Medina et al., 2005). Levels of inflammatory cytokines, including tumour necrosis factor-α (TNF-α) and transforming growth factor- β (TGF-β), were compared between wounds at three different locations (Figure 5.9). Tumour necrosis factor-α mRNA at all wound sites was significantly upregulated ($p=0.0001$), compared to that in un-wounded skin at day 4 post wounding, although there was no significant difference between wounding locations. TGF-β levels in all wounded areas was significantly ($p<0.005$) increased compared with un-wounded skin; however, the expression level was higher at days 4 ($p<0.005$) and 7 ($p<0.05$) compared control at all locations, with no significant difference between the regions (Figure 5.10).

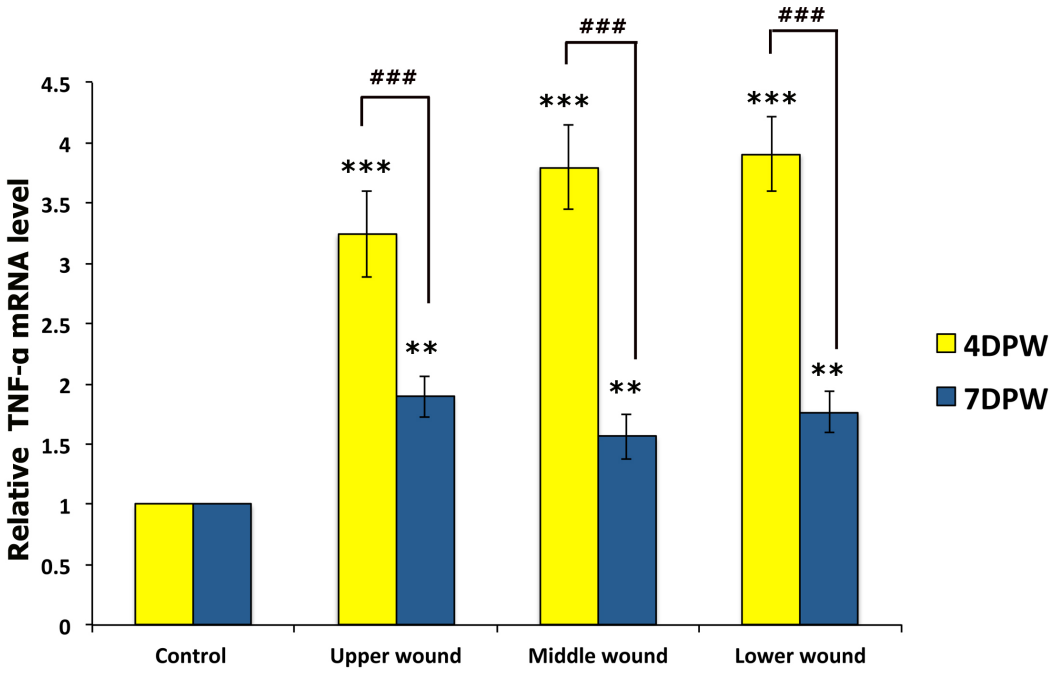


Figure 5.9. Relative TNF-α mRNA level

mRNA was isolated from unwounded controls or wounded skin from different sites of wild type mice back skin collected 4 and 7 dpw. Relative mRNA levels of TNF-α were quantified by q-RT-PCR. mRNA levels were normalised unwounded control. Mean ± SEM (standard error of the mean). ** p value <0.005,*** p value<0.0005, show a significant difference between upper and middle wounds, compared with the lower wounds. (Representing the comparisons between each location at 4dpw or 7dpw with un-wounded control skin). ###p value <0.0005, representing the significant difference between wounds at different location on 4dpw with their equals on 7dpw. Student's t-test, n=3 mice. Wounds were collected from 3 mice and 2 wounds per mice at each location at each time point. Data presented here is the average of 3 different biological replications in 3 runs of q-PCR with 3 technical replication at each run.

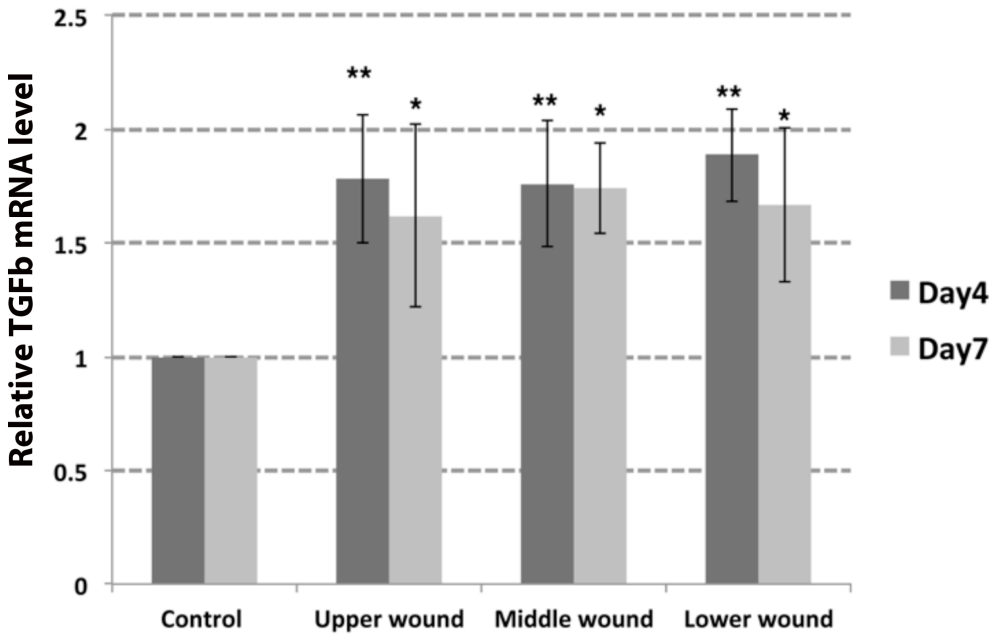


Figure 5.10. Relative TGFβ mRNA level

mRNA was isolated from unwounded controls or wounded skin from different sites of wild type mice back skin collected 4 and 7 dpw. Relative mRNA levels of TGFβ were quantified by q-RT-PCR. mRNA levels were normalized unwounded control. Mean ± SEM (standard error of the mean). *pvalue<0.05,** p value<0.005, indicate a significant difference between upper and middle wounds, compared with the lower wounds. (Representing the comparisons between each location at 4dpw or 7dpw with un-wounded control skin). Sstudent's t-test, n=3 mice. Wounds were collected from 3 mice and 2 wounds per mice at each location at each time point. Data presented here is the average of 3 different biological replications in 3 runs of Q-pcr with 3 technical replication at each run.

Epidermal T cells have been shown to play unique roles in tissue homeostasis and repair in mice through local secretion of distinct growth factors in the skin. Previous research showed that epidermal T cells contribute to the effective healing of acute wounds and are functionally defective in patients with non-healing wounds (Eming et al., 2007, Sonnenberg et al., 2011). Therefore it can be hypothesised that the alteration of wound healing at different anatomical locations is related to the number of T cells at the wound sites. To visualise of T cells, skin sections were analysed by using CD3, to detect all T cell subsets. Dendritic epidermal T cells (DETCs) were detected in the interfollicular epidermal basal layer with dendritic morphology, while a few CD3+ T cells were also detected in dermis (Figure 5.11), but this was not statistically significant compared to different regions (Figure 5.12).

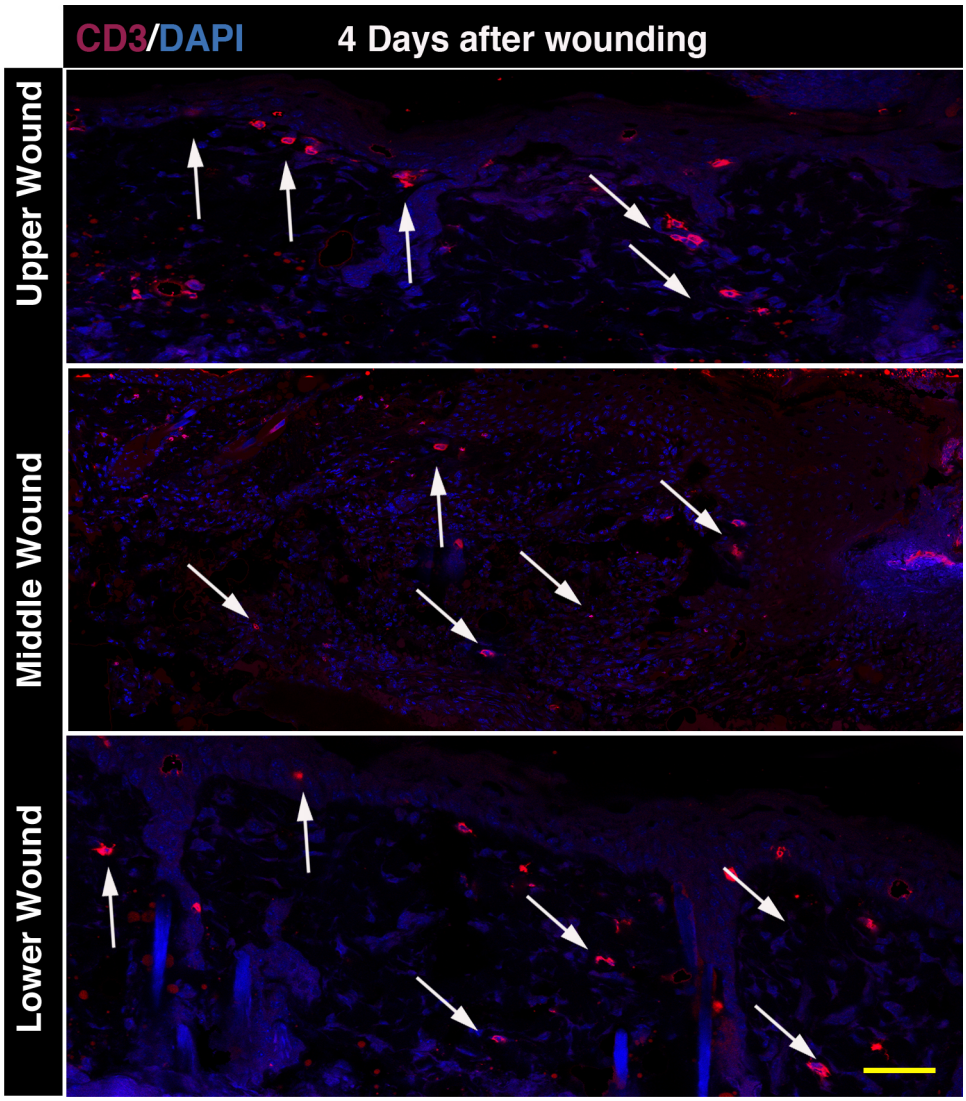


Figure 5.11. Immunostaining with CD3 and DAPI

Representative immunohistochemistry of a cross-section in the different regions of injured back skin day 4 post-wounding. Immunostaining with CD3 and DAPI. Scale bar = 100 μm

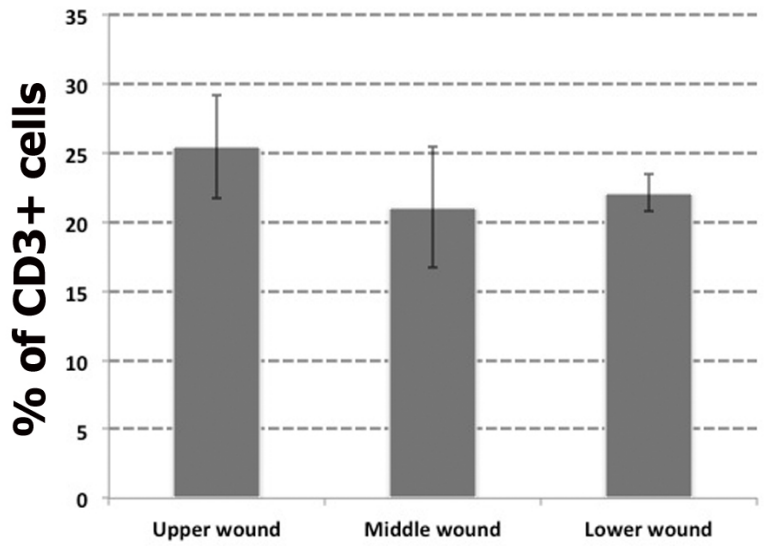


Figure 5.12. Quantification of CD3 positive T cells within 500 microns of the wound site

Graph to show the percentage of CD3 positive T cells within 500 microns of the wound site from 3 different locations at 4dpw (n=3 mice for each location). Mean ± SEM (standard error of the mean). All comparisons were non-significant.

5.3.5 SM22 expression and local MMP-1 differ at different location

It has been reported that the up-regulation of SM22 expression in the injured tissue may be related to a multistep modification process in which fibroblasts are converted to myofibroblasts (Faggin et al., 1999). SM22 alpha is a protein that is expressed specifically in adult smooth muscle. Approximately one week after wounding, the provisional matrix is replaced by neo-formed connective tissue, essentially composed of small vessels, extracellular matrix, and fibroblastic cells that become activated and differentiate into myofibroblasts. Myofibroblasts are recognised to play a central role in closing the wound tissue, through their capacity to produce a strong contractile force possibly generated within stress fibers (Faggin et al., 1999). Therefore it was hypothesised that the fibroblasts maturation into myofibroblasts in mouse back skin wounds, may depend upon the anatomical locations of wounds in mouse back skin. Immunohistochemistry showed that SM22α levels were weak and confined to the dermis at day 4 post wounding (Figure 5.13), but increased until day 7 in all regional wounds (Figure 5.13). At day 7 post-wounding, in lower and middle body wounds, SM22α immunoreactivity was detected in the dermal area of the wound edges and labelling was diffuse in the areas away from the wound bed. In contrast, in wounds located at the upper body, SM22α was not localised on the cell membrane in the dermal cells as much as lower and middle wounds (Figure 5.13).

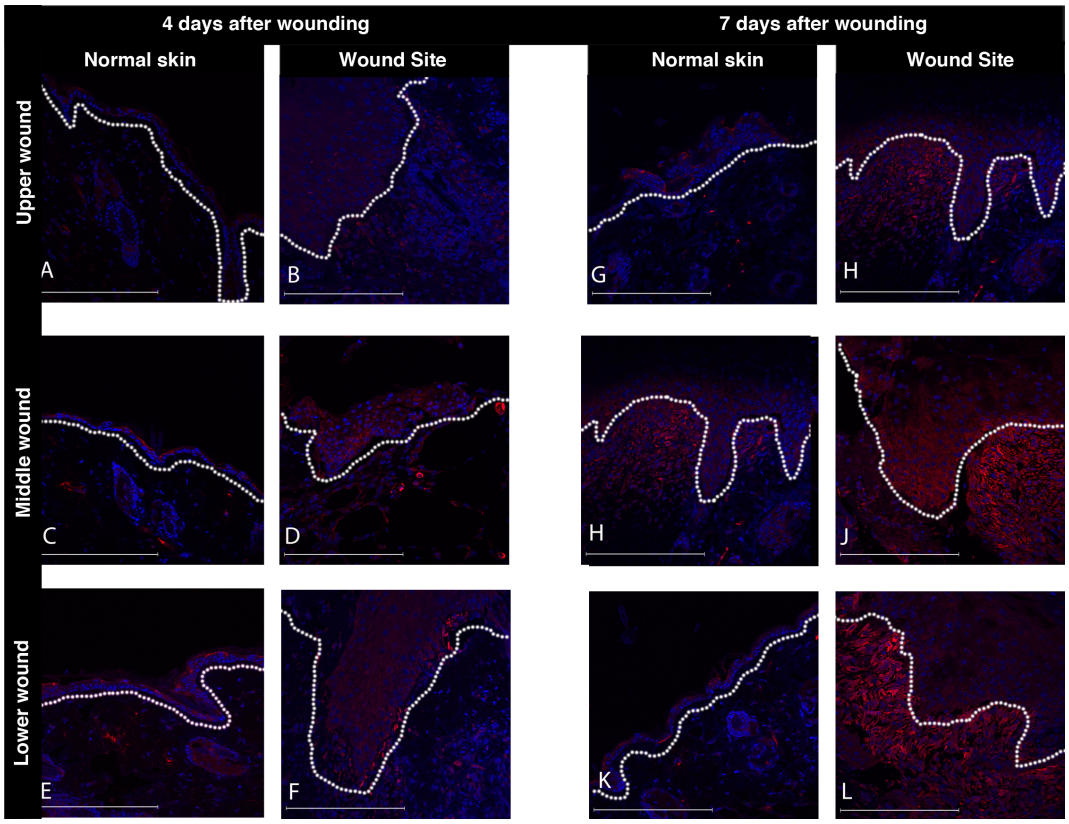


Figure 5.13. Immunostaining of mouse back skin with SM22α

Representative immunohistochemistry of a cross-section in the wound at different regions (upper, middle and lower) of mouse back skin at day 4 and 7 post-wounding. Immunostaining of mouse back skin with SM22α (red and DAPI (blue). Scale bars equal 100 microns.

Among the many gene products that are critical for the restoration of normal tissue architecture, several members of the matrix metalloproteinase (MMP) family function as positive regulators of repair processes. It has been reported that MMP-1 is essential *in vitro* for the migration of corneal epithelial cells over a type I collagen matrix (Daniels et al., 2003). The expression level of MMP-1 was examined at different sites of mouse injured back skin. The presence of MMP-1 was highly localised behind the leading edge of the basal epithelium and dermis at lower body wounds, compared to the upper and middle body located wounds where it was not present (Figure 5.14). These data may suggest that local MMP-1 expression may influence the regional wound healing responses.

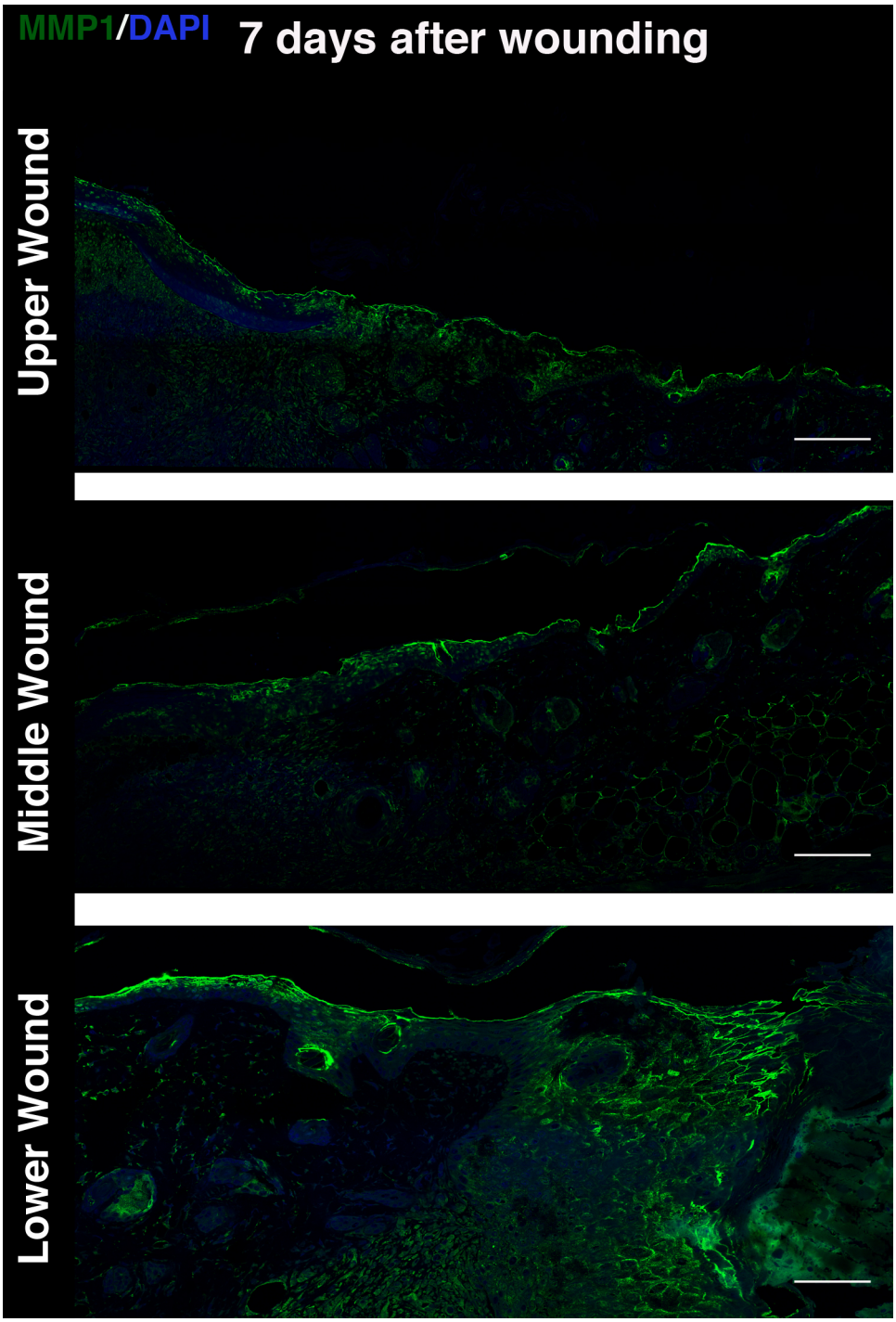


Figure 5.14 Immunostaining of mouse back skin with MMP1

Representative immunohistochemistry of a cross-section at the different injured regions of mouse back skin at day 7 post-wound. Immunostaining of mouse back skin with MMP1 (green) and DAPI (blue). Scale bar = 100 μm.

5.4 Discussion

A substantial quantity of work regarding wound healing has been published from various points of view (Broughton et al., 2006a). However, it seems that the occurrence of regional differences in wound closure has not been reported in mice. Clinically, it has been observed that burns heal differently depending on their anatomical location (Wang et al., 2009). In addition, previous studies have reported that hypertrophic scarring occurs frequently in areas of high tension and movement, such as anterior surfaces of the neck, shoulder and chest wall, and flexor surfaces of the extremities, but virtually never appears in certain body areas such as the scalp, eyelid, palm, and sole (Greenhalgh, 2005, Greenhalgh, 2012, Matsumura et al., 2001). In this chapter, it was noted that mouse dorsal lower body wounds healed better. These investigations clearly indicate that there is a regional difference in wound closure rate of the mouse skin. The rate of the healing and wound closure was higher in the lower posterior region than healing rates in the anterior and in the middle-posterior region. This would suggest the existence of a posterior-anterior gradient in epidermal response to injury.

One might suspect that the differences in healing might be partly due to differences in skin proliferation. Keratinocytes in the wounded margins begin to proliferate behind the actively migrating region during early days post injury (Singer and Clark, 1999). In this project, keratinocyte proliferation was significantly increased in posterior region wounds compared with in anterior and in the middle-posterior region wounds at the relatively early stages of wound healing (1–4 days post wounding), suggesting an involvement of regional proliferation in skin wound healing.

Interestingly, in middle-posterior and anterior region wounds, the level of keratinocyte proliferation and migration appear to approach that of posterior wounds after day 4 post-wounding. The reduction in wound size appeared to also follow this trend; wounds in middle-posterior and anterior region were larger in the early days after wounding, but their size approached that of posterior region after day 5. These observations suggest that the differences in keratinocyte migration and proliferation in the early stages of wound healing in this study may be influenced by wound location.

Re-epithelialisation and wound contraction are two essential factors during the healing process. Myofibroblasts that express SM22, are believed to be one the main factors affecting contraction formation (Desmouliere et al., 2005, Greenhalgh, 2012). It is unknown whether or not more expression of SM22 directly results in faster contraction in posterior wounds compared with anterior and middle wounds. What is clear is that these wounds close earlier by re-epithelialisation and cell migration compared with anterior and middle wounds. In contrast to the data described in this investigation, an earlier study using a full-thickness excision porcine model, still reported wound contraction to be dependent on wound location, however less contraction in lateral-caudal was observed in comparison to lateral-cranial locations (Hinrichsen et al., 1998). Hinrichsen et al., (1998) demonstrated that healing time was longer in lateral-caudal than lateral-cranial wounds, but no histological or other clinical wound healing assessments were provided.

It is well recognised that extended inflammatory responses delay wound healing. In this study, no wounds showed a typical over-inflammatory response in all considered locations of the skin. The level of CD3 positive T cells and the amount of the relative mRNA expression of TNF α in wounds from all locations did not show any significant difference. Therefore, it is unlikely that the differences in healing between the cranial and caudal wounds are attributable to impaired inflammatory factors.

The matrix metalloproteinases (MMPs) are proteins from metalloproteinase enzyme family that facilitate biological reactions. MMPs play a critical role in wound healing (Baker and Leaper, 2003). During the normal process of the wound healing MMPs are produced from activated inflammatory cells at the wound site, as well as epithelial cells, fibroblasts and vascular endothelial cells. At each stage of the normal wound-healing cascade, MMPs play essential and beneficial roles. During the inflammation phase MMPs are involved in removal of the damaged extracellular matrix (ECM) and bacteria, in the proliferation phase MMPs are responsible for degradation of the capillary basement membrane for angiogenesis and the migration of epidermal cells. During the remodelling of the wound healing process, MMPs contribute to contraction of scar ECM and remodelling of scar ECM (Baker and Leaper, 2003). Involvement of MMPs in angiogenesis and contraction has been considered as the key

factors that MMPs control during the wound healing process. MMPs degrade the basement membrane that surrounds capillaries, which allows vascular endothelial cells to migrate from capillaries near the wound and to establish new blood vessels in the wound bed (Muller et al., 2008). MMPs secreted by myofibroblasts are necessary for contraction of newly synthesised scar ECM. MMP-1 has been previously been introduced as a key member of MMPs family in the wound healing process. MMP-1 is essential for the migration of epithelial cells over a type I collagen matrix (Daniels et al., 2003). In this project, a close relationship between MMP-1 and the rate of the wound closure was established, as the MMP-1 expression after wounding was significantly marked in posterior wounds compared to other locations. These data is suggest that MMP-1 effects the wound closure rate, however whether this is directly or indirectly remains to be determined.

In conclusion, these data showed that wound location is involved in wound healing associated with keratinocyte proliferation and differentiation in response to skin injury. Further understanding of the anatomical location's role in wound healing may reveal new therapeutic strategies in this area. It is conceivable that alterations in proliferation and keratinocyte migration at different sites of the body represent true regional differences in epidermal wound closure in response to injury. Assuming the validity of this suggestion, the question arises why such a regional difference in proliferation and migration accrues after injury. One possible explanation is the existence of an anterior-posterior gradient of MMP inhibitors, wound contraction disruption, collagen deposition and proliferation inhibitors. The faster healing in posterior compared with anterior locations observed in this study is significant. However, there remains cause to examine the regional disruption of wound healing factors in the mouse skin, this problem remains the subject of future investigation.

6 General discussion

Previous studies have established that in adult skin Notch signalling sustains the epidermal barrier by promoting cell-autonomous keratinocyte terminal differentiation (Blanpain and Fuchs, 2006). Notch1 and Notch2 receptors are described as the main Notch signalling mediators in postnatal skin (Rangarajan et al., 2001).

Recent studies of Notch in adult tissue homeostasis have also shown that Notch orchestrates communication between the epithelial and immune compartments of the skin to prevent chronic inflammation and inflammatory skin disease (Dumortier et al., 2010, Goud and Deshpande, 2011). Although it seems that the role of Notch is well established in immune and inflammatory diseases, little is known about the role of this signalling pathway in normal physiological processes, such as cutaneous wound healing.

Firstly, work performed previously provides strong evidence that Notch is involved in wound healing, however the specific feature or the site of action was unspecified (Ambler and Watt, 2010, Chigurupati et al., 2007). The results presented in this study demonstrate distinct, temporal activation of Notch1 and Notch2 in the wound site with maximal Notch2 activity occurring at 24 hours after injury during the acute inflammatory phase of normal healing. This work showed that wounding caused in an increase in Notch1 activity with peak activity detected 4 days post wounding. By seven days post wounding, when the epithelium has closed, the remodelling phase has initiated and the risk of microbial contamination has reduced, the activity of both Notch1 and Notch2 return to unwounded skin levels.

It has been reported that epidermal Notch activity affects multiple processes that are related to wound healing (Nicolas et al., 2003). For example, epidermal Notch activity increases the production of TNFα, CCL20, IL22 and IL23 during the inflammatory and proliferation phase of the healing process. Maintenance of the epidermal stress response is now accepted as an established role for Notch activation in adult skin (Nicolas et al., 2003).

Contribution of Notch signalling in inflammatory processes, including the activation of macrophages (Monsalve et al., 2006) and dermal T cells infiltration (Ambler and

Watt, 2010) drew attention to determine the consequence of continued epidermal Notch activation during the healing process on wound closure. By using a transgenic mouse (K14NICDER) where Notch can be controllably activated in the epidermis, this work demonstrated a mechanism by which epidermal Notch activity was induced before wounding and during the healing process to determine the effects of over-activation of Notch on wound closure. Skin wounding activates the Notch pathway in the basal and suprabasal epidermis. Injury induced Notch1 and Notch2 activity, as well as upregulation of TNF-α and TGF-β. Forced epidermal over-activation of Notch, stimulated the production of IL22 and IL23 and Mip1, which subsequently influences epidermal proliferation and macrophage recruitment. Stimulation of SM22, and MMP1 are also the major consequence of over-activation of epidermal Notch signalling (Figure 6.1). Hence, although previous research showed that the epidermal Notch activation via a 4-hydroxy-tamoxifen-inducible transgene caused epidermal thickening and inflammation, via dermal accumulation of T lymphocytes (Ambler and Watt, 2010), continuous Notch activity before and after wounding was not helpful to wound closure rate.

Non-healing chronic wounds (Ulcers) or hyperthropic scars are the most established wound-healing disorders in the clinic. The most common clinical entities in association with chronic wounds are diabetes mellitus, venous insufficiency, pressure necrosis and vacuities (Eming et al., 2007). The prolongation of the inflammatory phase throughout the healing process has been described as one of the most significant causes of the non-healing wounds (Agren et al., 2000, Eming et al., 2007). Remaining in a chronic inflammatory state generates a cascade of tissue responses that amplify the hostile microenvironment within the wound. Immune suppression is the only reported effective treatment for most systemic and metabolic defects affecting the individual's inflammatory reaction and wound healing (Eming et al., 2007).

Presented data in this thesis showed that inflammatory phase elongation caused by the over-activation of Notch signalling, may contribute to impaired repair mechanisms of the K14NICDER mouse model. This result may have been accomplished by interference with the repair mechanisms increasing the inflammatory response (Figure 6.1).

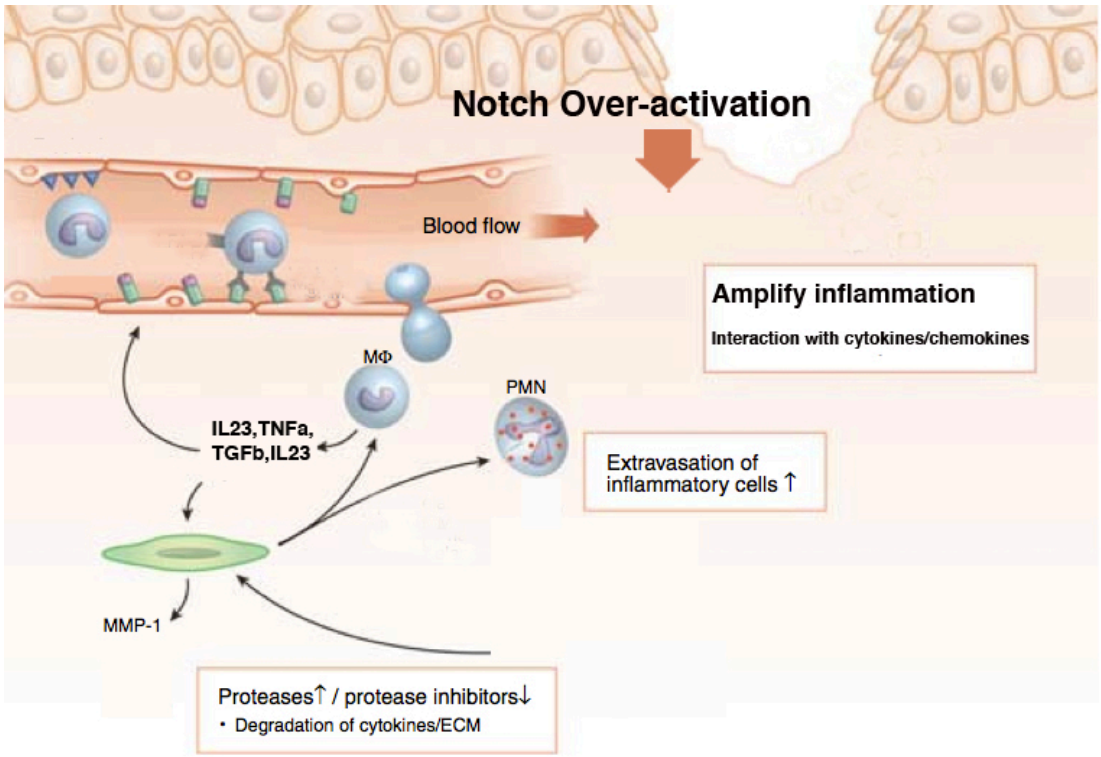


Figure 6.1. Model of molecular and cellular mechanisms in response to prolonged epidermal Notch activity.

Modified from (Eming et al., 2007).Wounds with epidermal over-activated Notch fail to progress through the normal pattern of wound repair, but instead remain in a state of chronic inflammation predominantly characterised by abundant inflammatory cells infiltration. Persistant Notch activation and inflammatory cells play a major role in the generation of proinflammatory cytokines (TNF-α, IL- 22 and IL23) and increased expression of MMPs (MMP- 1) that leads to degradation of structural proteins of the extracellular matrix crucial for repair (Eming et al., 2007) .

Indeed, there is convincing evidence that unbalanced protease activity is one of the major underlying mechanisms of non-healing wounds (Pa- lolathi et al., 1993; Harris et al., 1995; Saarialho-Kere, 1998; Barrick et al., 1999). The expression and the

activity of various MMP classes, including collagenases (MMP-1, MMP-8), have been shown to be highly upregulated in chronic venous stasis ulcers (Saarialho-Kere, 1998; Norgauer et al., 2002). The data presented in this thesis demonstrated the expression and the activity of collagenase (MMP-1) to be highly upregulated in response to over-activity of Notch signalling in K14NICDER wounded skin.

In a normal wound healing process, activated keratinocytes at the wound edge, fibroblasts, endothelial cells, as well as invading neutrophils and macrophages are considered as potent inducers of MMP expression. In chronic wounds proinflammatory cytokines have been shown to down regulate the expression of tissue inhibitor of metalloproteinases, thus creating an environment with a relative excess of MMP activity. The release of pro-inflammatory signals from the blood clot and the local damaged tissue results in the infiltration of inflammatory cells to the wound site in normal healing. Pro-inflammatory signals together with signals from invading inflammatory cells, control the vital re-epithelialisation and connective tissue contraction of the wound (Desmouliere et al., 2005, Hunt et al., 2000). Indeed it was recently reported that Notch signalling is involved in infiltration of inflammatory cells into the damaged tissue and plays a key role in the inflammatory cell responses during the wound healing (Arumugam et al., 2006, Chigurupati et al., 2007). The significance of Notch signalling in the recruitment and activation of leukocytes to the site of injury in mice was reported previously (Arumugam et al., 2006).This investigation has demonstrated that over-activation of Notch signalling resulted in activation of transcription factors that control the expression of proinflammatory cytokines (IL-22, -23, and TNF-α), chemokines and proteolytic enzymes, including MMP-1. Elevated levels of various serine proteinases, the expression and the activity of various MMP classes, including collagenases (MMP-1, MMP-8) and the activation of transcription factors that control the expression of proinflammatory cytokines (IL-1, -6, and TNF-α) and chemokines (Wenk et al., 2001) are all defined within the chronic wound microenvironment, and act to amplify the persistent inflammatory state of non-healing wounds (Grinnell et al., 1992; Grinnell and Zhu, 1996; Barrick et al., 1999).

Based on these observations, the similarity between the consequences of over-activation of Notch and exacerbation of chronic wound conditions has been indicated.

In addition, it is a well-accepted concept that successful therapy toward healing of the chronic wound must disturb the proinflammatory cycle. Blocking Notch pathway in tissue repair might be an important avenue to develop protective strategies, which shield the regenerative tissue from damage caused by the chronically inflamed microenvironment of the non-healing wound. The excessive and unbalanced inflammation characterising the chronic wounds and wounds with over-activated Notch suggests a promising target for future therapeutic interventions.

In summary, various clinical and experimental studies confirm the critical role of inflammation during repair. The balance between a complex network of several leukocyte cell subsets and numerous pro- and anti- inflammatory mediators is an essential factor to accomplish appropriate tissue homeostasis during healing.
Over-activation of Notch results in deregulation of critical parameter inflammatory mediators that impair the quality of healing in mouse skin wounding. The potential implications of Notch activity in skin wound healing inflammatory response are wide, from macrophage activation and recruitment, pro-inflammatory cytokine expression, epidermal proliferation and migration and ECM deposition. Nevertheless, precise understanding of Notch's contribution in inflammation is still poorly characterised. Moreover, before the findings outlined in this study can be applied to development of possible therapies, further elucidation of the players, the functions and the regulatory events involved in the specificity and control of the Notch pathway in skin wound healing and inflammatory response.

6.1 Further Research

In order to continue this investigation, there are two key points that require further key focus. Firstly, investigation into the effects of over activation of Notch signaling on further time points during healing process of K14NICDER wounded skin. Following the observation that the effects of prolonged Notch activity differ between K14NICDER mice before wounding as well as during the healing process to those treated only before wounding, similar investigations with K14NICDER mice, Notch activity from early inflammatory stage up to day seven post wounding would be of

significant interest.it would also be of interest to investigate the rate of the wound closure in mice treated with 4OHT for three weeks after wounding.

Secondly it is key to investigate the consequences of blocking of Notch signalling by using experimental mouse models. This would be particularly useful to answer the regulation of wound closure and Notch activity and inhibition. Diabetic foot ulcers represent one of the most feared and invalidating complications of diabetes with high financial pressure for the healthcare system. For the moment there is no specific therapy available and it has become a priority to develop novel rational therapeutic strategies based on new pathophysiological mechanisms. Further work would be interesting to test the hypothesis that Notch signalling plays a role in the pathogenesis of diabetic foot ulcer and to determine whether pharmacological inhibition of Notch signalling with γ-secretase inhibitors can ameliorate the wound healing process in a diabetic animal model.

In addition, data presented in chapter 5 of this thesis, has demonstrated that wound healing in mouse back skin is highly dependent on the anatomical site of the lesion. This investigation examined the reason of different rate of healing on wounds located at different anatomical regions of the mouse body. Data presented in this chapter showed that lower body wounds healed significantly better than those in the upper posterior, or middle to posterior of the mouse back skin wounding model. These presented data; might be explained by the existence of an anterior-posterior gradient of protease inhibitors, or a faster wound contraction, and collagen deposition in the wounds located in the lower body regions. However, the regional disruption of wound healing factors in the mouse skin remains the subject of future investigation. Previous studies presented that exercise activity has been shown to have a positive effect on wound healing. It has been reported that a relatively short-term exercise intervention is associated with enhanced rates of wound healing among healthy older adults. Thus, more activity and movement of the lower body region in mice may be an important component to promote wound healing (Keylock et al., 2008). Previous research confirmed that health factors appear to have an effect on both immune function and wound healing. Among the most commonly studied health criteria (e.g., smoking, diet, exercise), physical exercise is especially relevant because of its influence on multiple components of the wound-healing system, including associations with immune and endocrine function (Keylock et al., 2008). Thus, it would be interesting

to evaluate the influence of lower body activity, tail stretch and movement, and whether they influence wound closure in mice.

Hair follicles undergo continuous cycles of growth and regression throughout life, and thus contain huge regenerative potential. Over recent years it has become evident that hair follicles play a major role in skin wound healing. For example, it has been shown that hair follicle growth stage has a profound effect on healing. Recent studies have shown that progenitor cells residing in hair follicles (HFs) are able to contribute to this re-epithelialization of wounds in *vivo* (Ansell et al., 2011). A significant acceleration of healing during the anagen phase of HF cycling in *vivo*, associated with alterations in epithelial, endothelial, and inflammatory cell types has been reported (Ansell et al., 2011).

Hair follicles during most of anagen appear grey or black, while hair follicles in telogen have no pigment and the skin becomes pink. Patchy regions (domains) of black and pink are revealed, corresponding to areas of hair growth and quiescence. These domains are seen on both the dorsal and ventral sides of the body. They appear because pelage hair follicle populations in the mouse do not cycle independently, but rather show coordinated hair cycle stages within the same domain and discordant stages among adjacent domains. Interestingly, these domains develop into complex patterns from cycle to cycle as age progresses (Plikus and Chuong, 2008). Although, the hair growth cycle was considered during this project and mice that were chosen for wounding experiments were all in their telegen phase, many questions remain unanswered: What are the signals that allow hair follicle cells to contribute to healing? Is it possible to control the contribution of hair follicles to healing? Does differed anatomical location has different hair growth stages? Do the different hair growth stages affect the rate of the healing at different anatomical locations? As previously stated, to aid in understanding of differed anatomical wound closure and the distribution of hair follicles at different location, it would be advantageous to investigate longitudinal sections of a single skin strip to facilitate more precise molecular profiling of hair cycle stages at different locations. Finally, it will also help the development of new models to fully determine the relationship between the hair pattern formation in living mammals and regional wound closure. A comprehensive analysis using varied techniques including molecular and cellular analysis, surgery

and histological phenotyping of wounds, could be combined with the gene expression profiling of hair cycle and wound healing at different anatomical locations. The aim would be the identification and manipulation of signals involved in the hair cycle contribution to wound repair and location, lineage tracing of hair follicle cells during healing, and understanding of the hair follicle contribution to pathological wound repair at different anatomical locations.

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