Deficiency of Seven in Absentia Homologue (Siah) and its Effect on Wound Healing

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Anatomy and Physiology)

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

James Nthenge Musyoka

29/03/2011
Abstract

Wound healing in the skin is a complex and well orchestrated process involving the interaction of numerous cell types as healing progresses through phases involving inflammation, proliferation and remodelling. Impairment to the wound repair process can lead to delayed (chronic wounds) or excessive healing (fibroproliferative disorders). Because of the loss of function in scarring tissue, the difficulty in healing chronic wounds and high cost of wound care, there is considerable interest in the factors involved in the regulation of normal healing. Following injury, vascular damage often occurs resulting in low oxygen tension (hypoxia). Slight changes in systemic and cellular oxygen concentrations induce tightly regulated response pathways that attempt to restore oxygen supply to cells and modulate cell function in hypoxic conditions. Most of these responses occur through the induction of the transcription factor hypoxia inducible factor-1 (HIF-1) which regulates many processes needed to carry out tissue repair processes during wound ischaemia. For this reason HIF-1 is viewed as a positive regulator of wound healing and a potential regulator of tissue fibrosis. Seven in absentia homologue proteins (Siah) are a family of E3 ubiquitin ligases that tag HIF-hydroxylases for degradation during hypoxia and thus increase HIF-1α availability. In this context, Siah deficient cells are unable to adequately stabilise HIF-1α under hypoxic conditions. We hypothesized that Siah would have a regulatory role in early wound healing where hypoxic microenvironments are common and that Siah would be important for cell functionality in hypoxia. Thus, this thesis aims to address the role of Siah2 in dermal wound healing in vivo by examining the effect of Siah2 deficiency using Siah2 knockout (transgenic) mice and in vitro through the study of cultured dermal fibroblasts (murine Siah2-deficient fibroblasts and human fibroblasts). The results indicate that in the absence of Siah2, cutaneous wound repair is significantly impaired, albeit transiently. Specifically, macrophage invasion, re-epithelialisation, matrix deposition, fibroblast-myofibroblast transition, angiogenesis and wound contraction are delayed or reduced in Siah2 deficient wound repair. Furthermore, Siah2 knockout fibroblasts showed impaired functionality under hypoxic conditions (specifically collagen synthesis, αSMA induction) and an impaired response to exogenous TGFβ (delayed cell migration and reduced collagen synthesis). Moreover, transient inhibition of Siah in human dermal fibroblasts using a transfected Siah1/2 inhibitor (phyllpod, a Drosophila protein) reduced the
functionality (VEGF release, collagen induction, cell migration and TGF beta receptor type 1 induction) of otherwise healthy dermal cells. The results suggest for the first time, a role for Siah proteins as positive regulators of early wound healing through their regulation of HIF-1α fate and possibly through HIF-1 independent mechanisms. Aberrant wound healing is a concern of epidemic promotions and current strategies are far from ideal. In conclusion, this thesis demonstrates that Siah is a positive regulator of wound healing and cell functions related to healing and we propose that these findings may have important clinical implications for both impaired and over-elaborate forms of healing that result in scarring.
Acknowledgement

This thesis is as much mine as it is to the people in my life who have helped in one way or the other to make it possible. Firstly, I would like to thank my primary supervisor Assoc Prof. Ian Darby for his wisdom and encouragement during my candidature and for the many interesting conversations and social activities, they were fun.

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Last but not least, I dedicate this PhD to the most important person in my life, my lovely wife Roshan Lee Musyoka without whom I would have not made it this far. Thanks for the constant encouragement and belief in me. You deserve an honorary doctorate for your patience, understanding and administrative contributions to this work.
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<td>β-actin</td>
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<td>Connective tissue growth factor</td>
<td>CTGF</td>
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<tr>
<td>Proliferating Cell Nuclear Antigen</td>
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<td>Von Hippel–Lindau tumour suppressor protein</td>
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Chapter 1

Introduction to hypoxia and tissue repair
1.1. Introduction

Wound healing is a highly orchestrated sequence of complex overlapping events that are precisely timed to repair damage, prevent infection and restore function of tissue after injury or insult. These events are classically divided into inflammatory, proliferation and remodelling phases (Clark, 1996). In postnatal full-thickness wound repair regardless of the type of injury be it burns, pathological damage, toxin related, autoimmune or physical trauma the inevitable outcome is scarring and some subsequent loss of tissue function (Darby and Hewitson, 2007). In contrast, fetal wound healing before the third trimester proceeds with little or no scarring (Longaker et al., 1990). Healing is thought to become more adult like after the third trimester correlating with the development of the immune system (Longaker et al., 1990, Frantz et al., 1993). Scar free fetal wound healing is the subject of intensive research aimed at unlocking strategies that may achieve a similar outcome in adult wound healing (Siebert et al., 1990).

Wound healing involves a complex series of interactions between cells, chemical signals, extracellular matrix proteins and microenvironments collectively termed “dynamic reciprocity” (Singer and Clark, 1999). Invasion by inflammatory cells debrides the wound of damaged tissue and prevents infection, while releasing soluble factors that help to stimulate chemotaxis of fibroblasts and endothelial cells to form the granulation tissue (Singer and Clark, 1999). Simultaneously, rapid migration of keratinocytes also helps to cover and seal the wound from the external environment to further prevent infection. Alteration to the precise nature of these events leads to defective wound healing or abnormal scar formation (Clark, 1996).

Abnormal wound healing clinically presents mostly as fibroproliferative disorders (keloids and hypertrophic scars) and more commonly chronic wounds (diabetic ulcers, pressure sores and venous ulcers). These defects impose a great burden to patients in terms of morbidity and quality of life and also place a substantial financial burden on
global health care systems (Sen et al., 2009). Fibroproliferative lesions are often painful and prone to develop into contractures which can impose severe restrictions to mobility and quality of life (Roseborough et al., 2004, Bayat et al., 2003). Ulcers can be painful, may be socially isolating because of appearance or smell and are often prone to infection which increases the risk of amputations (Cavanagh and Bus, Barron et al., 2007).

Sadly, current wound care strategies are still far from ideal, owing mostly to our limited understanding of the complex mechanics involved in wound healing. An urgent approach to wound healing research must be adopted to further our understanding of wound healing and wound care strategies (Sen et al., 2009). In this chapter, the general mechanics of wound healing are reviewed with emphasis on the role of hypoxia and its signalling pathways on the wound healing milieu. Furthermore, the potential roles of hypoxia regulating transcription factor, HIF-1, and its associated proteins including prolyhydroxylases (PHDs) and seven in absentia homologue (Siah) in wound healing are discussed in further detail.

1.2. Normal wound healing

After tissue insult, platelet aggregation initiates the coagulation cascade that converts fibrinogen to fibrin fibres which then helps to stabilize the plug. This forms a temporary barrier and scaffold that acts as a provisional matrix which is critical in providing both a transient structure to the wound bed and anchorage for infiltrating cells (Laurens et al., 2006). Activated platelets release cytokines which accumulate in the provisional matrix and create a chemotactic gradient that can be tracked by infiltrating inflammatory and mesenchymal cells. These cytokines and growth factors likely act as a pool that can be used until autocrine and paracrine secretion of soluble factors take over once inflammatory cells have infiltrated the provisional matrix (Laurens et al., 2006). Transforming growth factor beta (TGFβ) is an example of a matrix bound latent growth
factor present during the early inflammatory phase and can be enzymatically or mechanically activated later (Wipff et al., 2007, Hynes, 2009).

Inflammatory cells (neutrophils and macrophages) infiltrate the provisional matrix in large numbers to debride the wound site, removing debris and bacteria. The wound sites are often avascular during the inflammatory phase owing to the disruption of blood vessels and then rapid infiltration of metabolically demanding cells occurs, thus ischaemia develops causing tissue hypoxia (Remensnyder and Majno, 1968). Inflammatory cells preferentially accumulate in these hypoxic areas which is critical in the restoration of aseptic tissue conditions and in the release of chemotactic factors that stimulate re-epithelialisation, and the formation of the granulation tissue (Leibovich and Ross, 1975, Knighton et al., 1983, Bosco et al., 2008).

Following the onset of inflammation, keratinocytes from the wound margin migrate rapidly over the provisional matrix to re-epithelialise and seal the wound site (Ortonne et al., 1981, Takashima and Grinnell, 1985). In response to released factors keratinocytes differentiate into a migratory phenotype by down-regulating their desmosome and hemi-desmosome integrin links, at the cell-cell and cell-basement membrane contact points as well as increasing expression of actin filaments that enable mobility (Odland and Ross, 1968, Krawczyk and Wilgram, 1973, Gabbiani et al., 1978). This may show as a purse-string arrangement of actin filaments which aid in coordinated movement and rapid closure of the epithelial defect adult (Danjo and Gipson, 1998) and fetal wound healing (Cowin et al., 2003).

Replacement of provisional matrix with granulation tissue is critical to restoring tissue integrity, architecture and function. As fibroblasts and endothelial cells infiltrate the provisional matrix, they lay down extracellular matrix and blood vessels respectively which eventually form the granulation tissue. The granulation tissue is later remodelled mechanically by contracting fibroblasts, loss in cellularity via apoptosis follows and there
is modification to extra-cellular matrix by matrix metalloproteinase (MMPs) (Gabbiani et al., 1971, Desmouliere et al., 1995, Darby et al., 2002). Typically in adult mammalian full-thickness wounds, matrix synthesis exceeds the remodelling attempt resulting in scar formation, failure to achieve the original architecture and reduced tensile strength of the resulting scar tissue (Clark, 1996). Essential functions of the skin in thermoregulation tend to be impaired by scar formation, which may have varying effects depending on the size of scar (Wilson et al., 1971). For a schematic representation of wound healing phases and cell recruitment refer to Figure 1-1 below.
Figure 1-1. Schematic overview of the overlapping phases of wound healing over time and the peak of different cells during these phases. Wound healing is classically divided into three phases: inflammation (acute or chronic), proliferation (fibrogenesis and angiogenesis) and remodelling. Migration of wound repair cells into the wound site is sequential and co-ordinated in a time depended manner. Different cell types peak and co-exist at different time points allowing for spatio-temporal regulation of different wound healing phases. Eventually wound contraction and loss in cellularity through apoptosis resolve the granulation tissue and the matrix fibrillar mass is degraded by metalloproteases. The resulting mature wound is relatively acellular and avascular (Adopted from (Greenhalgh, 1998))
1.3. Inflammation

Inflammation acts as a protective attempt to remove microbes and injurious debris. In addition, inflammatory cells in particular macrophages are widely appreciated as important regulators of cytokines and growth factors during wound healing (Leibovich and Ross, 1975). In adult wound repair, macrophages and their released factors typically persist in the wound bed and act as regulators of the following proliferation and remodeling phases as part of an overlapping chronic inflammatory phase. This is in contrast to scarless fetal wound healing which tends to be characterized by a short inflammatory phase and rapid wound resolution (Blakytny et al., 2004, Martin and Leibovich, 2005). So although the inflammatory process is thought to be important in normal wound healing its prolonged effects may be important for scar formation in adult animals (Blakytny et al., 2004).

1.3.1. The coagulation cascade and platelets in wound repair

The inflammatory phase is typically preceded by the coagulation cascade which is considered to be the initiator of the wound healing process (Singer and Clark, 1999). It is essential in haemostasis, activation of inflammatory cells and growth factor release/activation. The coagulation cascade leads to aggregation of platelets and the conversion of fibrinogen to fibrin. Together with fibronectin these events form a stable plug (clot) which helps to re-establish haemostasis by preventing further blood loss (Laurens et al., 2006). The clot also establishes a provisional matrix that can act as a scaffold for infiltrating cells to adhere to through specialised cell matrix receptors called integrins (Albelda and Buck, 1990, Clark et al., 1982b, Clark et al., 1982a). In coordination with other cell surface molecule receptors, integrins allow inflammatory and mesenchymal cells to migrate and proliferate towards the wound centre along a chemotactic gradient of released factors and chemical mediators. Activated platelets within the fibrin clot are essential in the release of multiple factors including interleukins 1 (IL-1), IL-6, platelet-derived growth factor (PDGF), transforming growth factors α...
(TGFα) and TGFβ (Werner and Grose, 2003). It is well accepted that some growth factors are released into the matrix in latent or bound form which provides both storage and an additional platform for their regulation. Activation of latent growth factors occurs in different ways but mostly through cleavage by proteolytic factors or mechanical force. Specific examples are the activation of latent TGFβ1 by platelet released furin-like proprotein convertases or by integrin associated matrix metalloproteinase (MMPs) (Blakytny et al., 2004, Wipff and Hinz, 2008). Mechanical activation of latent growth factors by cells is also possible, for example the activation of latent TGFβ from extracellular matrix through myofibroblast contraction (Wipff et al., 2007).

1.3.2. Neutrophil recruitment

Following the formation of the haemostatic plug, neutrophils (polymorphonuclear leukocytes) rapidly accumulate in the damaged tissue acting as the first line of defence against local infection. They initialise debridement of damaged tissue and matrix components by synthesizing and secreting hydrolases and reactive oxygen intermediates (Clark, 1996). Neutrophils typically reach their peak 24 to 48 hours post injury, after which their numbers reduce considerably through apoptosis and macrophage-induced phagocytosis (Engelhardt et al., 1998, Newman et al., 1982). Macrophage-induced phagocytosis of apoptotic neutrophils is an essential stimulant for the initial release of TGFβ1 which regulates the function of fibroblasts and endothelial cells and their chemotaxis into the wound site (Peters et al., 2005).

The bulk of neutrophils transmigrate to the wound site from the circulatory system along a gradient of released soluble factors which increases leukocyte-endothelial adherence (Engelhardt et al., 1998, Gillitzer and Goebeler, 2001, Bevilacqua, 1993). These cell-cell interactions occur mainly through endothelial selectins (P and E-selectin) and neutrophil integrin molecules expressed on the cell surface (Bevilacqua, 1993). The expression of these adhesion molecules is regulated by a mixture of non-specific and
neutrophil-specific pro-inflammatory factors produced by preceding platelets, pathogens and resident inflammatory cells.

During wound healing highly specialised C-X-C chemokines mediate regional specific extravasation of neutrophils and precise tissue homing (Engelhardt et al., 1998). These include growth related oncogene–α (GRO-α) and interleukin-8 (IL-8) which bind CXCR1 and CXCR2 neutrophil cell surface receptors respectively (Baggiolini et al., 1994). Following injury, IL8 and GRO-α expression is rapidly up-regulated and released by local macrophages and neutrophils in response to TNFα and IL-1 and expression peaks after 24 hours, correlating with the influx of neutrophils observed during this period (Engelhardt et al., 1998).

In addition to these secreted factors, the tissue ischaemia encountered in healing wounds is considered to be stimulatory for the recruitment of neutrophils (Palluy et al., 1992). Inflammatory cells in general (neutrophils and macrophages) preferentially accumulate in regions of ischaemia such as damaged and pathological tissue. Hypoxia stimulates the up-regulation of leukocyte-endothelial adhesion molecules, selectin and β2-integrin subunit resulting in increased tethering, rolling and diapedesis (Wood et al., 2000)(Rainger et al., 1995). In addition, hypoxia up-regulates IL8 presentation and release by endothelial cells which further enhances neutrophil specific recruitment to hypoxic tissue (Wang and Lin, 2010).

1.3.3. Relevance of neutrophils in wound healing

The relevance of neutrophils in wound healing has been intensely debated over the years. Using an anti-neutrophil serum approach, earlier studies on neutrophil-depleted wounds showed no significant difference in cutaneous wound healing kinetics when compared to control wounds, suggesting that neutrophils were dispensable during healing (Simpson and Ross, 1972). Since then depletion studies utilizing CXCR2 and CD-18 transgenic mice, which both fail to recruit neutrophils, have had phenotypes with
delayed or defective wound healing (Devalaraja et al., 2000, Peters et al., 2005). The lack of apoptotic neutrophils in CD-18 deficient wounds is thought to deprive macrophages of apoptotic neutrophils, their main stimulant to secrete TGFβ1, resulting in impaired myofibroblast transformation and reduced wound contraction (Peters et al., 2005). A similar explanation may also be plausible for the defect in CXCR2 mice which display a similar phenotype. Additionally, neutrophil function is impaired in diabetes as a result of hyperglycaemia and is restored by insulin treatment and euglycaemia, pointing to a possible role for altered neutrophil function in diabetic healing impairment (Weringer et al., 1982, Wierusz-Wysocka et al., 1988). This would further support the role of neutrophils in normal wound healing. The discrepancy in results from animal experiments is surprising given that some of the approaches used in these studies target neutrophils specifically. Highly selective and tissue specific deletion models are now possible using Cre-lox systems and may better serve to draw a conclusion on the relevance of neutrophils in cutaneous wound healing.

1.3.4. Macrophage recruitment

Macrophages infiltrate the wound site in parallel to neutrophils, initially in smaller numbers, and eventually (2-3 days post injury) become the predominant inflammatory cell in the wound (Leibovich and Ross, 1975). They remain in wounds until the remodelling phase as part of a chronic inflammatory phase of wound repair. Like neutrophils, macrophages are essential during host defence and tissue debridement but are also necessary regulators of the proliferation and remodelling phases of wound repair (Kurihara et al., 1997, Leibovich and Ross, 1975, Mirza et al., 2009). This largely relies on their ability to express and release cytokines into the wound bed that regulate fibroblast function and angiogenesis (Leibovich and Ross, 1975, Leibovich and Ross, 1976, Mirza et al., 2009). These cytokines include platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), fibroblast growth factors (FGF) and VEGF (IL-1) (Werner and Grose, 2003). This cocktail of soluble factors potently stimulate
proliferation, migration and differentiation of keratinocytes, fibroblasts and endothelial cells thus promoting wound closure, angiogenesis, ECM deposition and remodelling.

Macrophages infiltrate the wound bed mainly from the circulation and to a lesser extent from local or adjacent unwounded tissue (Clark, 1996). Successful macrophage extravasation requires specific cell-cell adhesion molecules including immunoglobulins such as VCAM-1, integrins such as α4β1 and selectins which include P and E isoforms (Beekhuizen and van Furth, 1993). Platelet and neutrophil released factors increase the expression of these molecules on the cell surface of endothelial cells and macrophages which enables increased tethering, rolling and adhesion of monocytes on endothelial cells along a chemotactic gradient of specific cytokines and growth factors (Beekhuizen and van Furth, 1993). Specific monocyte chemokines bind to the C-C motif chemokine family of receptors (Yoshimura et al., 1989a, Yoshimura et al., 1989b). Most notably, CCL2 (or monocyte chemo-attractant protein -1 (MCP-1)) which acts on the C-C receptor 2 (CCR2), is thought to be the most specific cytokine during monocyte homing into the wound site. CCL2 is expressed by up to 20% of total cells within and around the wounded area in response to TNF-α and IL1 and peaks about 24 hours post injury (Engelhardt et al., 1998). Other C-C binding chemokines may include RANTES (or CCL5) and macrophage inflammatory protein -1 (MIP-1) but appear to be dispensable during normal dermal wound repair (Low et al., 2001). The importance of C-C binding chemokine in wound healing is effectively demonstrated in CCL2-null mice which exhibit impaired macrophage invasion and subsequent delays in wound healing characterised by aberrant re-epithelialisation, collagen synthesis and revascularisation (Low et al., 2001).

1.3.5. Relevance of macrophages in wound healing

Despite their central role in regulating nearly all phases of wound repair, the relevance of macrophages in wound healing in recent years has been intensively debated. In particular, the question as to whether infiltrating macrophages impede or promote
wound healing has received much attention. Earlier studies had established the importance of macrophages and their released factors in early wound healing by demonstrating impaired healing and delayed fibroblast infiltration in macrophage depleted wounds following anti-macrophage serum and steroid treatment post injury (Leibovich and Ross, 1975). However, these findings were challenged by a demonstration of complete healing in the absence of macrophages and neutrophils, as described in the antibiotic rescued newly born PU null mouse which heal despite the lack of multiple macrophage and granulocyte progenitor cell lineages (Martin et al., 2003). Wound healing in the PU null mouse follows an embryonic pattern of repair with reduced scarring (Martin et al., 2003). However experiments in adult mice have not been performed using this transgenic line because PU null mice die 48 hours after birth in the absence of antibiotic treatment. Furthermore, wound healing in adult mice and humans is associated with prolonged inflammation, including the persistence of macrophages through almost all phases of wound healing. Numerous studies have continued to describe impaired wound healing in models with defective monocyte/macrophage recruitment or function. Impaired cutaneous wound healing is observed in granulocyte/macrophage colony-stimulating factor knockout mice which show reduced neutrophil and macrophage recruitment (Fang et al., 2007). Moreover, deletion of macrophage signalling molecule such as MyD88 that enable the release of macrophage derived angiogenic growth factors, also cause significant delays in wound healing (Macedo et al., 2007). Recently, selective and specific ablation of macrophages using Cre-lox transgenic mice with deleted CD11b showed elaborate defects in all phases of wound healing (Mirza et al., 2009). Additionally, macrophages from CD11b null mice show impaired synthesis of relevant growth factors such as, TGFβ1 and VEGF, highlighting the regulatory role of macrophages in the cytokine environment of the healing wound (Mirza et al., 2009).
1.3.6. Hypoxia and macrophages

The impact of local inflammatory cells in wound healing is also heavily influenced by micro-environments encountered by these cells such as hypoxia. Macrophages preferentially accumulate in hypoxic areas which is characteristic of most lesions including tumours, keloids and cutaneous wounds (Bosco et al., 2008). Macrophages accumulate in large numbers in such sites and then respond rapidly to the hypoxia present with altered gene expression, thought mostly to be mediated through up-regulation of the transcription factor, hypoxia-inducible factor (HIF) (Cramer et al., 2003). Adaptation of macrophages to hypoxia may involve increasing expression of trans-endothelial adhesion proteins (integrins) and cytokines such as TNF-α and IL1 which enhance the ability of macrophages to cross the vascular endothelium and migrate into hypoxic lesions (Kalra et al., 1996, Li et al., 1999). Hypoxia also alters the expression of some macrophage receptors allowing them to be more responsive to increased inflammatory cytokines (Ghezzi et al., 1991, Scannell et al., 1993). For example, the increased release of TNF-α and IL-1 by numerous local cells within the wound bed including resident fibroblasts, macrophages, endothelial cells and keratinocytes immediately after injury. It is plausible that macrophages increase receptors during hypoxia making them more sensitive to chemokines and enhances their migration to hypoxic sites. Interestingly, wounds are not shown to be hypoxic at day 1 as determined immunochemically by hypoxyprobe® staining, a widely used marker of tissue hypoxia (Haroon et al., 2000). Although, this may also be due to poor perfusion failing to deliver the marker to the wounded tissue. It is instead suggested that hypoxia increases gradually and is detectable at about 3 days post injury, correlating with the maximal peak in macrophage infiltration (Haroon et al., 2000). Hypoxia is further sustained until day 7 to 10 correlating with a rapid increase of proliferating mesenchymal cells that enter the wound (Lokmic et al., 2006, Haroon et al., 2000). The importance of hypoxic stimuli in macrophage infiltration during wound healing is demonstrated in heterozygous HIF-1α deficient mice which show considerable delays in myeloid cell
infiltration as early as day 2 post injury, a consequence of reduced hypoxia signalling (Zhang et al., 2010b).

As hypoxia in the early wounds increases, macrophage-derived factors are likely to be increased and provide additional stimuli for attracting macrophages and mesenchymal cells such as fibroblasts and endothelial cells. Described hypoxia-induced macrophage derived soluble factors include TGFα, TGFβ, VEGF, FGF, PDGF, TNF-α, IL-1 and IL-8 (Jackson et al., 2007, Kuwabara et al., 1995, Albina et al., 1995, Hempel et al., 1996, Scannell et al., 1993). This mixture of growth factors and cytokines is likely to have an additive effect on early wound healing events such as re-epithelialisation and granulation tissue formation.

In addition to enhancing macrophage infiltration and cytokine release, hypoxia through HIF-1 also enhances the phagocytic potential of myeloid cells, increasing their ability to debride damaged tissue and rid lesions of micro-organism such as bacteria (Cramer et al., 2003). In wound healing, phagocytosis of apoptotic neutrophils by macrophages is an important event in the release of soluble factors including TGFβ, which plays a major role in regulating formation and remodeling of granulation tissue (Peters et al., 2005). Overall, the effects of hypoxia on macrophages are therefore likely to be important in accelerating wound healing.

1.4. Proliferation: Re-epithelialisation, Angiogenesis and Fibrogenesis

Re-epithelialisation and formation of granulation tissue form the proliferative phase of wound repair. It is a complex phase of wound repair which relies on complex interactions between inflammatory cells, fibroblasts, keratinocytes and endothelial cells as well as the extracellular matrix components and released cytokines. Here, soluble factors released into the fibrin clot by inflammatory cells, in particular platelets and macrophages, stimulate the simultaneous and cooperative migration, proliferation and
differentiation of keratinocytes from the existing uninjured epidermis to close the wound as well as fibroblasts and endothelial cells from both resident and circulating progenitor lineages to form the granulation tissue (Singer and Clark, 1999). As they migrate into the wound site mesenchymal cells interact with each other in an organised but complex fashion through the release of specific paracrine and autocrine factors to allow successful transition from provisional to a mature granulation tissue.

1.4.1. Re-epithelialisation

Rapid restoration of the epithelial barrier helps to seal the wounded area from the external environment and prevents further infiltration by micro-organisms. Successful re-epithelialisation relies on rapid proliferation, differentiation and migration of keratinocytes from the edges of the wound to cover a fibronectin rich provisional matrix (Clark et al., 1982a). Shortly after injury, keratinocytes undergo significant subcellular modifications, from an otherwise predestined terminally differentiated phenotype to one that is highly migratory. These modifications include disassembly of hemidesmosomal integrin links between epidermis and basement membrane, retraction of intracellular tonofilaments and keratin filaments, dissolution of most desmosomes, and formation of peripheral cytoplasmic actin filaments which can extend and contract creating lamellipodia and filopodia (Santoro and Gaudino, 2005). Regulation of the keratinocyte migratory phenotype during wound healing requires cooperative responses by multiple factors including integrins, matrix components (fibronectin and collagen), cytokines and specific cell-cell interactions (Santoro and Gaudino, 2005). Re-epithelialisation may also be accelerated by wound microenvironments, such as tissue hypoxia, which are encountered as re-epithelialisation progresses over a mostly avascular provisional matrix (O'Toole et al., 1997, Ridgway et al., 2005). As the migrating edges meet, regeneration of the basement membrane occurs and keratinocytes lose their migratory phenotype, returning to a resting state (Santoro and Gaudino, 2005). After maturation of the granulation tissue, the wound is remodelled through contraction, reassembly of matrix and loss in cellularity through apoptosis (Desmouliere
et al., 1995). Keratinocytes terminally differentiate and undergo apoptosis, most likely in a TGFβ-mediated manner. (Greenhalgh, 1998, Amendt et al., 2002)

1.4.1.1. Matrix and re-epithelialisation

Interactions between keratinocytes and matrix components are critical during re-epithelialisation. In vitro, fibronectin and collagen especially, have profound stimulatory effects on keratinocyte migration even in the absence of growth factors, while growth factor induced migration remains dependent on the availability of matrix components (Li et al., 2004). However in vivo the lack of growth factor stimulation as observed following mutation to keratinocyte growth factor receptor (FGFRIIIb) results in delay and abnormalities in re-epithelialisation, an indication that growth factors are also important in accelerating wound closure (Werner et al., 1994).

The interaction between matrix components and keratinocytes occurs mostly through integrins which are heterodimeric cell surface receptors consisting of α and β subunits. There are 24 known α/β combinations that can be achieved to specifically recognise different matrix proteins through recognition of specific amino acid sequences such as RGD sequences (Clark, 1996). Activation of integrin receptors in migrating keratinocytes allows for interaction with a variety of matrix proteins interspersed in newly formed matrices and pre-existing matrices at the wound margin (Giancotti, 1997). Keratinocytes can increase their repertoire and vary their expression of integrins accordingly as matrix components change in the maturing wound (Larjava et al., 1993). For example, migration of keratinocytes on fibronectin rich provisional matrix requires a different profile of integrins to that of keratinocytes migrating on fibrillar collagens present at the wound margin and in maturing granulation tissue or on superficial wounds such as blisters where the dermis may not be breached (Collo and Pepper, 1999, Putnins et al., 1999, Dumin et al., 2001)
In the unwounded epidermis α6β4 integrins are an essential structural component of hemidesmosome anchoring keratinocytes to the basement membrane (Laminin-322), as demonstrated in patients with junctional epidermolysis bullosa and α6β4 gene deficient mice that are prone to epidermal blistering (Carter et al., 1990a, Vidal et al., 1995). α2β1 and α3β1 integrins are highly expressed in unwounded and resting epithelium where they are proposed to facilitate cell-cell adhesion (Carter et al., 1990a, Carter et al., 1990b). Furthermore, α2β1 acts as the receptor for type I and II collagen required for keratinocyte migration during wound healing (Dumin et al., 2001). High expression of α2β1 in quiescent keratinocytes (unwounded tissue) is proposed to be a pre-emptive measure to allow attachment and rapid migration of keratinocytes on underlying collagen if the basement membrane is breached during injury and inhibition of α2β1 using neutralising antibodies impairs re-epithelialisation by human keratinocytes (Parks, 2007, Dumin et al., 2001).

Following injury, the α6β4 integrin subunits are modified by phosphorylation to allow keratinocytes to detach and expression of fibronectin and collagen receptors α5β1 and α2β1 respectively is increased to allow migration and proliferation at the wound edge and onto newly formed matrix (Dans et al., 2001, Larjava et al., 1993). Other integrins expressed during re-epithelialisation include fibronectin receptor αVβ1 and vitronectin receptors αvβ5 and αvβ6 (Larjava et al., 1993). The interaction between integrins and matrix during migration also requires cleavage of the matrix components by matrix metalloproteases (MMPs). MMPs bind to their respective integrins and catalyse a three part cycle involving ligation, cleavage and separation which functions as a “motor” to maintain keratinocyte movement and directionality (Dans et al., 2001, Parks, 2007). The relevance of this mechanism is observed during α2β1-mediated keratinocyte migration, which can be halted when the ability of α2β1 to bind to its ligand is blocked, if MMP1 is inhibited or its expression reduced, or if cells are plated on collagenase-resistant type-1 collagen (Dumin et al., 2001). Regulation of integrins and MMP activity during migration is extensively influenced by availability of matrix substrates but also by a host of growth factors.
factors present in the wound (Gailit et al., 1994, Zambruno et al., 1995, Koria and Andreadis, 2007).

1.4.1.2. Re-epithelialisation and cytokines

A battery of growth factors produced by inflammatory cells such as platelets and macrophages play an important role in stimulating re-epithelialisation. Growth factors released into the provisional matrix are often bound to matrix substrates requiring activation by proteolysis. Growth factors that modulate keratinocyte migration include TGFα, FGF2, IL8, keratinocyte growth factor (KGF [FGF7]), IL-1α and TGFβ, to mention a few (Gailit et al., 1994, Li et al., 2006). In vitro, TGFα shows the highest potency in promoting keratinocyte migration and proliferation, showing a similar potency to that of complete serum which contains a mixture of factors (Li et al., 2006). However, re-epithelialisation during wound healing is more likely to involve a co-operative response from a cocktail of growth factors and is evident by the lack of wound phenotype in TGFα and KGF null mice which show normal re-epithelialisation following injury (Humble et al., 1998, Guo et al., 1996). Indeed, during wound healing TGFβ, TGFα and KGF act cooperatively to promote a balance between proliferation and migration, which is important for normal and organised re-epithelialisation to occur. All three growth factors, promote keratinocyte migration while TGFα and KGF promote hyper-proliferation (Gailit et al., 1994, Li et al., 2006). TGFβ has a net inhibitory effect on keratinocyte proliferation, an effect that is suggested to keep the re-epithelialisation attempt organised by preventing unregulated proliferation (Amendt et al., 2002). Consequently, increased proliferation and reduced apoptosis is evident in dominant-native TGFβ type II receptor (TGFRII) mutant basal keratinocyte transgenic animals and mouse lacking Smad3 (Amendt et al., 2002) (Ashcroft et al., 1999), TGFRII and Smad3 being required for TGFβ signal transduction.

The regulation of integrins by growth factors is important for the detachment, migration and proliferation of keratinocytes during re-epithelialisation. Phosphorylation of
serine/threonine and tyrosine residues of α6β4 integrin subunit (β4) by macrophage stimulation proteins and growth factors such as epidermal growth factor (EGF) induces hemidesmosome disassembly which is a critical process in keratinocyte detachment from the basement membrane (Santoro and Gaudino, 2005). Following detachment, KGF and TGFβ induce the expression of α5, α2 and β1 integrin subunits to increase migration on the fibronectin and collagen in the newly formed matrix (Gailit et al., 1994, Koria and Andreadis, 2007). Conversely, the release and activation of these factors from their matrix bound substrates is also regulated by integrins and MMPs expressed by migrating keratinocytes at the leading edge. In keratinocyte and fibroblast cultures, the activation of matrix-bound latent TGFβ is shown to be a αvβ6 integrin-dependent mechanism (Munger et al., 1999, Annes et al., 2002). The relevance of this is demonstrated by reduced TGFβ1 activation on epithelium at the leading edge following injury or infection and accelerated wound healing in αvβ6-deficient animal models (Annes et al., 2002, Munger et al., 1999, Xie et al., 2009).

1.4.1.3. Re-epithelialisation and hypoxia

Re-epithelialisation of the wound may also be influenced in part by acute hypoxia as migration of keratinocytes proceeds over an avascular provisional matrix. Hypoxia can be detected on re-epithelialising sheets during cutaneous wound healing in the early proliferative phase of wound healing using pimonidazole adduct staining (hypoxyprobe) (Haroon et al., 2000, Lokmic et al., 2006). Alterations in the expression of metabolic proteins, adhesion proteins, receptors and released factors are required during hypoxia to enhance or continue cell functions such as migration, differentiation and proliferation. Cellular adaptations to hypoxia are regulated mostly by HIF-1 mediated pathways (Semenza, 1999).

Indeed, basal keratinocytes migrating over the provisional matrix show increased expression of HIF-1α and the downstream target Glut-1 on the re-epithelialising sheet during cutaneous wound healing (Elson et al., 2000). Increased Glut-1 expression is
associated with increased glucose intake into the cell and is necessary for the provision of metabolic energy required for increased cell migration and proliferation during hypoxia. Furthermore hypoxia up-regulates expression of migratory integrins such as αvβ6 and closely associated MMPs (MMP-2 and MMP9) in keratinocyte cultures which correlates to their enhanced migration on fibronectin (Ridgway et al., 2005). Increased αvβ6 may also correlate to increased activation of latent TGFβ (Munger et al., 1999). The expression of paracrine acting factors such as VEGF is also increased in a HIF-1 dependant manner in keratinocytes and is likely to have a stimulatory effect on angiogenesis during tissue hypoxia (Weir et al., 2011). Deficiency of HIF-1, for example in aged keratinocytes, reduces TGFβ activity, MMP expression and impairs migration in hypoxia (Xia et al., 2001). Furthermore cutaneous wound healing in HIF-1 heterozygous null mice is characterised by impaired wound healing and delayed wound closure (Zhang et al., 2010b). Overall, hypoxic conditions appear to be important for rapid re-epithelialisation during wound healing which is essential for restoring aseptic conditions and an epithelial barrier. Additionally, hypoxia may also be important for the release or activation of keratinocyte derived paracrine factors (including VEGF) that are important for stimulating other wound healing events such as angiogenesis.

### 1.4.2. The formation of the granulation tissue

Following the inflammatory response, the restoration of tissue integrity and vasculature are fundamental functions of granulation tissue formation. Initially, tissue integrity is temporarily restored by the provisional matrix which also provides a scaffold for cell migration and a reservoir of chemotactic factors released by inflammatory cells. Fibroblasts infiltrate the wound and release a variety of extracellular matrix components that provide further anchorage for cell adhesion and restore tissue architecture and tensile strength (Clark, 1996). Furthermore, fibroblasts synthesize and release a variety essential paracrine and autocrine factors required for the successful formation and remodelling of the granulation tissue (Denton et al., 2009). As fibroblast numbers increase, a shift in regulatory processes controlled by inflammatory cells to processes
regulated mostly by fibroblast-derived factors occurs as the wound matures. Eventually endothelial cells and smooth muscle cells proliferate and differentiate in the wound bed and form new vessels that sprout into the granulation tissue to provide oxygen and nutrients for the healing tissue (Singer and Clark, 1999). Then once matured, the granulation is remodelled mechanically, enzymatically and cellularity becomes reduced (Gabbiani et al., 1971, Desmouliere et al., 1995, Darby et al., 2002, Darby et al., 1990). Mechanisms involved in the formation of the granulation tissue are complex due to the interplay of signals and interactions between macrophages, fibroblasts and endothelial cells in the granulation tissue as well as paracrine factors exchanged between epithelial cells overlaying the wound and mesenchymal cells. Moreover, the effects of different tissue microenvironments such as hypoxia which can alter cell behaviour and the outcome of wound healing are still not well understood.

1.4.3. Angiogenesis:

Angiogenesis is an essential component of wound healing, morphogenesis and pathological processes such as tumour growth and includes the cellular processes of adhesion, invasion, migration, proliferation and differentiation of vascular endothelial cells to form capillary tubes (Clark, 1996). Angiogenesis is critical for the provision of nutrients and oxygen to growing and regenerating tissue (Niinikoski, 1969, Remensnyder and Majno, 1968). During the tissue repair process, endothelial cells from surrounding undamaged blood vessels invade, migrate, proliferate and form tube-like structures that sprout and split to form new capillaries throughout the newly formed granulation tissue. Circulating cells derived from the bone marrow and distant vessel walls, for example circulating endothelial progenitor cells, provide an additional source of cells that promote angiogenesis. Indeed depletion of endothelial progenitor cells impairs wound healing, as demonstrated in experimental models of diabetic wound healing (Botusan et al., 2008). Angiogenesis is a complex process involving multiple growth factors, cell-matrix interactions, cell to cell interactions most of which are still incompletely understood. In addition to this, angiogenesis is greatly stimulated by tissue
hypoxia following disruption to the original vasculature and the rapid influx of metabolically demanding wound repair cells such as inflammatory cells (Remensnyder and Majno, 1968). Increased angiogenic responses in ischaemia are part of tightly regulated homeostasis feedback mechanism regulated predominately through HIF transcription factors (Semenza, 1998).

During the early phases of wound healing HIF-1 regulates the invasion of circulating angiogenic cells, which include a heterogeneous population of cells including endothelial progenitor cells, as well as myeloid, mesenchymal and hematopoietic stem cells which promote vascular growth and remodelling through the production of angiogenic cytokines (Zhang et al., 2010b). Furthermore, HIF-1 up-regulates the expression of angiogenic paracrine factors such as VEGF and TGFβ by different cells types including macrophages, keratinocytes and fibroblasts (Jeon et al., 2007, Weir et al., 2011, Distler et al., 2007). The increased expression of matrix molecules such as fibronectin and collagen following stabilisation of HIF-1 in dermal fibroblasts has also been demonstrated and is likely to enhance angiogenesis during wound healing (Distler et al., 2007, Steinbrech et al., 1999). Consequently, ablation or reduction of HIF-1 impairs angiogenesis while constitutive expression or up-regulation of HIF-1 improves or accelerates granulation tissue formation and improves wound healing (Zhang et al., 2010b, Botusan et al., 2008).

1.4.3.1. Angiogenic cytokines

Angiogenesis is regulated by a variety of autocrine and paracrine factors produced by cells in and adjacent to the granulation tissue. The most studied and most potent of the angiogenic factors is the heparin binding glycoprotein, vascular endothelial growth factor (VEGF), which exerts its biological effect through cell surface receptors VEGFR-1 and VEGFR-2, selectively expressed on endothelial cells (Connolly et al., 1989, de Vries et al., 1992). In response to tissue ischaemia and inflammatory cytokines, VEGF expression and release is increased by a variety of cells types including neutrophils,
macrophages, fibroblasts, keratinocytes and endothelial cells, with the effect to provide vasculature for newly formed granulation tissue (Jeon et al., 2007, Berse et al., 1999, Ghezzi et al., 1991, Weir et al., 2011, Steinbrech et al., 1999, Nogami et al., 2007). VEGF potently stimulates endothelial cell proliferation, migration and vessel sprouting by stimulating the up-regulation of various matrix-binding integrins including αvβ3, α1β1 and α2β1 involved in enhancing endothelial cell to cell and endothelial cell to matrix binding, a process required for proliferation and tube formation (Senger et al., 1997, Senger et al., 1996). Furthermore, expression of integrin-associated MMPs is increased by VEGF in endothelial cells and vascular smooth muscles cells allowing ligation and cleavage of matrix which is necessary for cells to migrate, proliferate and form tubes through the matrix (Iwasaka et al., 1996).

Loss or reduction in VEGF expression or binding to its receptors results in defective vessel formation which can have a significant impact on tissue formation including that seen in development, wound healing as well as tumourogenesis. Loss of VEGF in transgenic mice causes severe cardiovascular and vascular abnormalities resulting in embryonic lethality (Ferrara et al., 1996). Similarly, inactivation of VEGFR-1 or VEGFR-2 in transgenic murine models causes embryonic lethality and impedes the development of functional blood vessels (Ferrara et al., 1996). Furthermore, mini-pump mediated delivery of VEGF neutralising antibody into subcutaneous wound environments retards vascular development and inhibits granulation tissue formation (Howdieshell et al., 2001). Conversely, VEGF-targeted vascular therapy may be beneficial in controlling the outcome of some pathological events associated with VEGF over-expression such as fibrosis and cancers (Wang et al., 2008a, Gounant et al., 2009). Anti-VEGF monoclonal antibodies are currently in clinical trials for scar prevention post glaucoma related surgical interventions (Bochmann et al., 2011, How et al., 2010). Additionally, due to underlying microvascular dysfunction associated with diabetes and difficulties in forming granulation tissue, application of VEGF has been used to stimulate mobilisation
of circulating progenitor endothelial cells and promote angiogenesis in diabetic wound healing (Galiano et al., 2004, Kirchner et al., 2003).

Angiopoietin-1 (Ang 1) is a heparin binding glycoprotein from the same family as VEGF with potent angiogenic capacity and is important for the maintenance, growth and stabilization of new vessels (Werner and Grose, 2003). Ang-1 exerts its effect through tyrosine kinase Tie-2 receptors which are unique to endothelial cells and critical during development as demonstrated by lethality and severe retardation to vascular integrity of Tie-2 deficient mice (Sato et al., 1995). Ang-1 is regulated by Ang-2 which antagonizes its effects through competitive ligand binding of the Tie-2 receptor (Sato et al., 1995). Consequently, over-expression of Ang-2 in transgenic models results in lethality and severe vascular defects in a manner resembling phenotypes in Ang-1- or Tie-2-deficient transgenic models (Maisonpierre et al., 1997). In contrast, over-expression of Ang-1 in rodent models significantly increases vessel sprouting and similarly, delivery of matrix-bound Ang-1 into cutaneous diabetic wounds promotes improved healing through the enhancement of angiogenesis, lymphangiogenesis and blood flow (Suri et al., 1998, Cho et al., 2006). Ang-1 and Tie-2 expression are increased in fibroblasts-myofibroblasts and endothelial cells during the early phases of wound healing and expression regresses as the granulation tissue matures and scar formation occurs (Staton et al.).

FGFs are heparin binding mitogenic proteins suggested to play a role in angiogenesis through stimulation of endothelial cell proliferation and migration (Werner and Grose, 2003). They exert their effect through activation of tyrosine kinase receptors FGFR-1-4 which are expressed by a variety of cell types (Johnson and Williams, 1993). Expression of FGF1, FGF2, FGF5 and FGF7 is found in normal and unwounded skin, and expression of these factors is increased after skin injury suggesting a regulatory role during wound healing (Werner and Grose, 2003). Furthermore, FGF2 is found in human and porcine wound fluid particularly in the early stages after injury (Chen et al., 1992, Breuing et al., 1997). Angiogenic effects of FGFs have been reported in endothelial cell cultures where
exogenous FGFs promote cell migration (Montesano et al., 1986). Furthermore, FGFs increase the expression of plasminogen activators (which convert plasminogen to plasmin) and MMPs which has been shown to enhance endothelial cell migration on matrix (Montesano et al., 1986). FGFs are also mitogenic for other wound healing cells such as fibroblasts and keratinocytes in culture, which can in turn produce angiogenic factors in vivo. Additionally, the role of FGFs in regulating wound healing responses is corroborated by a number of studies where local application of FGF1, FGF2, FGF4, FGF7 (KGF) and FGF10 stimulated tissue repair (Werner and Grose, 2003). However, numerous FGFs appear to be dispensable during angiogenesis, evidenced by the lack of an angiogenic phenotype after injury in several FGF null mice, include FGF1, FGF2 and FGF7 which show no obvious vascular defects or complications when compared to wild type littermates (Miller et al., 2000). That said, delivery of FGF2 neutralising antibodies in cutaneous wounds causes a striking reduction in cellularity and vascularization compared with the granulation tissue formed in control IgG-treated wounds (Broadley et al., 1989). Cross-reactivity between FGF2 neutralising antibodies with other members of the FGF family was not excluded and this is likely to be the reason for the strong phenotype observed, given the partial phenotype in the FGF2 null model (Miller et al., 2000).

TGFβ1 is a broad range cytokine that acts through a heterodimeric serine threonine kinase receptor complex consisting of Type I (TGFRI) and II (TGFRII) subunits expressed on a multiple cell types (Massague, 1998). TGFβ1 is renowned for its ability to potently induce angiogenesis and fibrogenesis during wound healing and pathological fibrosis. Roberts et al was the first to show rapid induction of severe fibrogenic and angiogenic events following subcutaneous injection of TGFβ1 into normal skin (Roberts et al., 1986). Following this, subcutaneous neutralization of TGFβ was shown to decrease spontaneous vascular growth and collagen deposition in a model of wound healing (Fajardo et al., 1996). Moreover, wound healing of TGFβ1 null mice show marked delays in angiogenesis and granulation tissue (Crowe et al., 2000). In culture, TGFβ rapidly
stimulates endothelial cell migration and differentiation allowing tube formation and sprouting in collagen lattices, but inhibits proliferation and migration of endothelial cell monolayer cultures implying that TGFβs regulates cell-matrix interactions involved in angiogenesis (Iruela-Arispe and Sage, 1993). Additionally, the regulation of angiogenesis-related integrins by TGFβ has been suggested. For example, the increase in expression of α2β1 and α5β1 integrins induced by TGFβ provides activated endothelial cells with an enhanced capacity to migrate through their underlying basement membrane and invade interstitial or newly-formed matrices, and it has been hypothesized that this is an essential process during angiogenesis (Enenstein et al., 1992). Furthermore, up-regulation of matrix proteolytic activity (via MMPs) is required for “motorized” function of integrins as endothelial cells invade the extracellular matrix, and this may also be regulated by TGFβ to promote angiogenesis. It also well appreciated that TGFβ can stimulate neutrophils, macrophages and fibroblasts to up-regulate and release paracrine factors in particular VEGF, which can further potentiate stimulation of angiogenesis (Jeon et al., 2007).

1.4.3.2. Extracellular matrix and angiogenesis

Endothelial cell to matrix interactions are vital for successful angiogenesis (Basson et al., 1992, Madri and Williams, 1983). The extracellular-matrix acts as a vital scaffold, a reservoir for growth factors and cytokines as well as a mediator of intracellular signals. The relevance of cell to matrix interactions in angiogenesis can simply be demonstrated by the contrast in behaviour following growth of endothelial cells in three-dimensional type I matrix or basic monolayer cultures. When embedded in matrix endothelial cells attach, elongate, proliferate and differentiate to eventually form tubes with a lumen resembling capillaries, while monolayer cultures in contrast fail to differentiate (Madri and Williams, 1983, Montesano et al., 1983). Consequently, the formation of matrix and the interaction of different matrix components with endothelial cells, is thought to be critical for successful angiogenesis during wound healing and development and may have important implications in angiogenesis in fibrotic diseases and tumourigenesis.
Interactions between matrix proteins and endothelial cells occur predominantly through a variety of integrin receptors which can be up-regulated or down-regulated by cytokines and other factors as cells encounter overlapping matrix components in the healing wound. For example, VEGF selectively increases interactions between dermal endothelial cells and provisional matrix by selectively inducing the expression of cell surface integrins α1β1, α2β1 and αvβ3 while α5β1 expression can be modulated by TGFβ1 (Collo and Pepper, 1999, Senger et al., 1997, Senger et al., 1996). Since ligand specificity differs between different integrins, endothelial cell migration, proliferation, differentiation and survival differs in both temporal and spatial manner as wound healing progresses (Perruzzi et al., 2003). Endothelial cells will initially bind to fibronectin in the provisional matrix and later attach to fibroblast-deposited type I collagen as the granulation tissue develops. This may be achieved through transiently expressing fibronectin specific integrins including αvβ3 and α5β1 which allow endothelial cells to adhere to fibronectin and later overlap with expression of α1β1 and α2β1 integrins to allow attachment and function on type I and III collagen (Collo and Pepper, 1999, Perruzzi et al., 2003). Failure or inhibition of these overlapping integrin expression inhibits angiogenesis during wound healing and prevents cell survival despite rescue attempts by angiogenic cytokines such VEGF (Perruzzi et al., 2003, Senger et al., 1997).

Integrins are vital transducers of bidirectional cell signals after ligand (matrix) binding. Outside-in signalling informs cells about the matrix environment while inside-out signals are important for integrin activation and function (Clark, 1996). Type I collagen–integrin (α2β1) dependent activation of intracellular p44/P42 mitogen activated protein kinases (Mapk) pathways is an example of an outside-in signal which induces gene expression associated with endothelial cell survival. Inhibition of p44/42 or α2β1 interrupts integrin mediated signalling and subsequently reduces endothelial cell viability and response to exogenous VEGF (Perruzzi et al., 2003). The importance of ligand-integrin
associated inside-out and outside-in signalling is also reported in integrin β3 mutant transgenic mice which fail to form complete capillaries in response to VEGF stimulation (outside-in signalling) (Mahabeleshwar et al., 2006). Furthermore, phosphorylation of VEGF receptor-2 is significantly reduced in endothelial cells expressing mutant β3 integrin leading to impaired integrin activation (inside-out signalling) in these cells (Mahabeleshwar et al., 2006). Targeting integrin based interactions is viewed as an important therapeutic strategy for the future in the treatment of pathologies where angiogenesis is a contributing factor including abnormal wound healing, fibrotic disorders and cancers.

1.4.3.3. Angiogenesis - cell to cell interactions:

Irrespective of the physiological process (wound healing or development) or pathological condition (fibrosis or cancer), angiogenesis represents a complex multi-step process in which capillary endothelial cells sever their normal cell to cell attachments, migrate and proliferate in perivascular extracellular matrix in which they eventually reform cell to cell associations and create new vessels (Albelda and Buck, 1990). As discussed earlier, cellular adhesion between cells and matrix predominately occur with through integrin receptors which are vital for angiogenesis (1.4.3.2). Cell-specific cell to cell adhesion molecules localized to endothelial cell intercellular junctions are equally important for angiogenesis. These intercellular junctions are made up of transmembrane proteins anchored to actin cytoskeleton which provide structural and functional support during vessel formation. Furthermore, it is increasingly becoming evident that endothelial cell to cell interactions play an important role in inducing and integrating intracellular signals involved in cell growth and survival as well as matrix and cytoskeleton remodelling. Numerous cell junction proteins have been identified and extensively reviewed including vascular endothelial (VE) and neural (N) cadherins, junction adhesion molecule (JAM), occludins, PECAM-1 and claudins (Liebner et al., 2006). Loss or inhibition of these cell to cell adhesion molecules impairs functionality of blood vessels and blocks endothelial cell tube formation. For example, anti-VE cadherin
augments vessel permeability and decreases integrity by disrupting VE-cadherin homotypic adhesion and clustering (Corada et al., 1999). Furthermore, loss of VE-cadherin in transgenic mice causes embryonic lethality characterised by defective organization of endothelial cells in large vessels and impairment of angiogenesis (Vittet et al., 1997, Gory-Faure et al., 1999). Similarly, neutralisation with monoclonal anti-PECAM-1 antibodies has been shown to inhibit cytokine-induced angiogenesis in subcutaneous implants (DeLisser et al., 1997). In addition, PECAM-1 null mice, although viable, show significant defects in subcutaneous implant and tumour-related angiogenesis (Cao et al., 2009). Interestingly, anti-PECAM-1 treatment does not alter the rate of wound closure or angiogenesis in excisional cutaneous wounds, however, a similar result is yet to be confirmed using PECAM-1 null mice (Cao et al., 2009).

Besides providing structural functions, endothelial cell to cell intracellular signalling through adhesion molecules has been proposed to be important for sustaining vessel integrity and regulating angiogenesis. For example, VE-cadherin clustering and complexing with VEGF-receptor 2 is required for VEGF induced activation of phosphatidly inositol 3-kinase (PI3), AKT and Bcl2 pathways which promote cell survival and inhibit endothelial apoptosis during angiogenesis (Carmeliet et al., 1999). Deficiency or truncation of VE-cadherin induces endothelial apoptosis and abolishes VEGF induced survival signals (AKT kinase and Bcl2) via reduced complex formation with VEGF receptor-2, beta-catenin and phosphoinositide 3 (PI3)-kinase, causing impaired vascular remodelling and maturation (Carmeliet et al., 1999).

1.4.3.4. Hypoxia and angiogenesis

Blood vessels function as conduits for the delivery of oxygen and nutrients in all physiological and pathological states. Following full-thickness cutaneous injury, vessel function is compromised leading to acute tissue hypoxia and the hypoxic state is sustained further due to rapid influx of inflammatory and mesenchymal cells with a high metabolic demand for oxygen (Remensnyder and Majno, 1968). Local relative hypoxia
has been observed in wounds by direct measurement of local oxygen pressure and its necessity in maintaining good angiogenesis during wound healing has been well-defined (Knighton et al., 1981). Hypoxia is well known to induce the up-regulation of major angiogenic factors (VEGF, Ang-1, TGFβ1 and FGF-2), the recruitment of inflammatory and progenitor endothelial cells as well as inducing the migration of fibroblasts and keratinocytes which coordinate the angiogenic effort (Steinbrech et al., 1999, Cramer et al., 2003, Berse et al., 1999, Weir et al., 2011, Albina et al., 1995, Jeon et al., 2007, Harmey et al., 1998, Kuwabara et al., 1995, Zhang et al., 2010b). Cellular adaptive responses to hypoxia are mediated by HIF-1, which induces transcriptional responses that include expression of angiogenic factors by hypoxic cells and expression of cognate receptors for these ligands by vascular cells and their progenitors (Semenza, 2010). Expression of virtually all critical angiogenic growth factors required for local activation of vascular cells during angiogenesis is induced by HIF-1, including VEGF, stromal derived factor 1 (SDF-1), Ang 2, PDGF and TGFβ (Forsythe et al., 1996, Loh et al., 2009, Distler et al., 2007, Semenza, 2010). Although TGFβ expression and responsiveness is increased during hypoxia and impaired in HIF-1 deficient cells, it is not known whether HIF-1 is a direct or indirect activator of TGFβ gene expression (Distler et al., 2007). The released factors also serve as homing signals for mobilization and recruitment of circulating angiogenic cells (CACs) (progenitor endothelial cells and bone marrow derived myeloid cells) from distant sites such as bone marrow and vessels from other adjacent tissue (Botusan et al., 2008).

Impairment of HIF-1-dependent responses to hypoxia is a major factor contributing to the impaired vascular responses seen in wound ischaemia. For example, defective HIF-1 signalling has been identified in ageing- and diabetes-related impaired angiogenesis during wound healing, and correction by constitutive expression or stabilization of HIF-1 significantly improves the angiogenic defect and accelerates wound healing (Botusan et al., 2008, Liu et al., 2008). Conversely, prolonged hypoxia and over stimulation of HIF-1 associated responses are viewed as an important mechanism of elaborate angiogenesis
in pathological conditions. High levels of HIF-1 and its downstream targets (such as VEGF) are reported in a variety of pathological conditions including keloid scars, system sclerosis and liver cirrhosis (Zhang et al., 2006, Distler et al., 2004, Bozova and Elpek, 2007). Consequently, inhibition or ablation of HIF-1 signalling is shown to reduce angiogenesis and collagen deposition in some fibrotic models (Distler et al., 2007, Bozova and Elpek, 2007, Moon et al., 2009). Furthermore, impaired angiogenesis, reduced mobilisation of endothelial progenitor cells and delayed wound healing has recently been demonstrated in HIF-1 heterozygous null mice (Partial HIF-1 deletion)(Zhang et al., 2010b). These studies confirm at a molecular level that acute hypoxia responses mediated through HIF-1 are required for good angiogenic responses during wound healing, but may also contribute to abnormal angiogenesis that drive pathological forms of tissue repair.

1.4.4. Fibrogenesis: Fibroblasts and myofibroblasts in wound repair

Fibroblast functions are critical in formation of granulation tissue which restores integrity to damaged tissue, and its eventual remodelling to form mature scar tissue (Gabbiani et al., 1971). Furthermore, it is generally accepted that defective reconstruction of connective tissue by fibroblasts/myofibroblasts in pathological states leads to tissue deformation that characterises many pathological fibroses (Hinz and Gabbiani, 2003a). In addition, due to loss of tissue functionality associated with scar formation and fibrosis, concerted efforts are being made to understand mechanisms that regulate fibroblast functions and might be targets for anti-fibrotic treatments (Wilson et al., 1971, Darby and Hewitson, 2007). The involvement of several autocrine and paracrine factors, matrix interactions, mechanical stimuli (tension) and cell to cell interactions in fibroblast function have been defined over the years, with particular focus on their involvement of fibroblast to myofibroblast differentiation which is appreciated as a major event in scar formation and fibrosis.
Fibroblasts are embryologically of mesenchymal origin and display a spectrum of phenotypes including non-contractile fibroblasts, semi-contractile proto-myofibroblasts and contractile myofibroblasts (Tomasek et al., 2002). The contractile phenotypes are mostly evident following activation by cytokines, extracellular matrix (splice variant fibronectin) and mechanical stimuli present in different physiological and pathological states including wound healing, fibrosis and tumourigenesis (Hinz et al., 2007, Desmouliere et al., 1993, Darby et al., 1990, Schmid et al., 1998, Serini et al., 1998, Tomasek et al., 1992). In addition to the contractile features of activated fibroblasts, prototypical myofibroblasts are further distinguished by their expression of contractile actin stress fibres, these are composed of alpha smooth muscle actin (αSMA), linked in a linear fashion through transmembrane fibronexus junctions to protruding filamentous fibronectin fibres (Singer et al., 1985, Desmouliere et al., 1993). Myofibroblasts are further distinguished from smooth muscle cells predominantly by their lack of expression of smooth muscle cell markers including desmin and smooth muscle cell heavy chain myosin. Specific fibroblast markers are problematic but may include fibroblast specific protein-1 (FSP-1) first suggested by the Neilson group (Strutz et al., 1995).

Following tissue insult, fibroblasts infiltrate the provisional matrix from local mesenchymal connective tissue and bone marrow derived circulating progenitor lineages in response to released inflammatory factors (Leibovich and Ross, 1975, Abe et al., 2001). Furthermore, epithelial mesenchymal transition (EMT), a well-defined origin of fibroblasts from differentiated epithelial cells in non cutaneous tissue repair, has been proposed as a source of fibroblasts in cutaneous wound repair. Briefly, EMT requires the organised dedifferentiation of epithelial cells through loss of polarity, adherence to underlying basement membrane and tight junctions (Clark, 1996). Recent reports described EMT in human skin wound healing by induction of bone morphogenic protein-2 (BMP-2) through addition of exogenous TNFα to grafted explants (Yan et al., 2010). However, whether this occurs in vivo and how much EMT contributes to the
fibroblast/myofibroblast population in wound healing and fibrosis in human skin remains contentious.

To repopulate the damaged tissue, quiescent fibroblasts from local and circulatory origins become activated in response to matrix substrates (fibronectin) and growth factors deposited in the wound by inflammatory and resident cells, acquiring a migratory phenotype with cytoplasmic actin but only partial contractile ability (photo-myofibroblast) (Hinz and Gabbiani, 2003b). Fibroblast adhesion to and migration on matrix also requires expression of appropriate integrin receptors for the variety of substrates encountered in the evolving matrix including fibronectin and type I collagen (Hakkinen et al., 1994, Saalbach et al., 1997). As fibroblasts migrate to the wound centre they replace provisional matrix with granulation tissue matrix (mostly type I and III collagen) whilst simultaneously releasing proteolytic factors and their inhibitors which ensures an organised formation of granulation tissue through deposit and remodelling (Clark, 1996). Furthermore, autocrine and paracrine factors released by activated fibroblasts take over from overlapping inflammatory-derived factors becoming the central regulators of subsequent proliferation and tissue remodelling events (Denton et al., 2009). With increasing mechanical tension generated by tissue remodelling, mechano-perception via cell-matrix junctions (fibronexis and focal adhesions) become activated causing protomyofibroblasts to further differentiate into myofibroblasts characterized by expression of α-SMA (Tomasek et al., 1992, Singer et al., 1985, Hinz et al., 2003). In addition to tension, the joint actions of TGFβ1 and fibronectin splice variant ED-A are also required for this phenotypic change (Serini et al., 1998, Desmouliere et al., 1993).

Importantly, the presence of myofibroblasts in most physiological and pathological states of wound repair is accompanied by excessive production of collagenous extracellular matrix (Hinz et al., 2007). An exception to this is fetal wound healing which proceeds while maintaining a tight balance between matrix production and remodelling.
by fibroblasts leading to complete matrix resolution and “scarless healing” (Rolfe et al., 2005). The over stimulation and persistence of myofibroblast in response to cytokines and growth factors is accepted as a key mechanism by which the balance between matrix synthesis and degradation is lost leading to the development of scar tissue and ongoing, often irreversible fibrotic disease (Schmid et al., 1998).

Cutaneous wound healing involves transient expression of αSMA and contraction by tissue myofibroblasts which later disappear through massive apoptosis resulting in a loss of cellularity, formation of mature scar tissue and some loss to tissue functionality (Desmouliere et al., 1995, Darby et al., 2002, Darby et al., 1990). However, in extensive burn wound repair, myofibroblast activity persists and leads to hypertrophic scarring and tissue deformation, characterized by excessive matrix bundles and fibrotic tissue contractures (Ehrlich et al., 1994). Detrimental loss of tissue function is characteristic of fibrosis affecting vital organs where myofibroblast-generated contractures and over production of collagenous matrix is evident; examples include idiopathic lung fibrosis, various forms of liver fibrosis including cirrhosis and renal (interstitial) fibrosis (Phan, 2002, Lan, 2003, Desmouliere et al., 2003).

1.4.4.1. Growth factors and fibroblast-myofibroblast function

Fibroblast activity during wound repair is initially regulated by matrix composition and a range of growth factors and cytokines mostly released into provisional matrix by preceding inflammatory as well as resident mesenchymal cells (Leibovich and Ross, 1975, Leibovich and Ross, 1976, Mirza et al., 2009). Active growth factors initiate the chemotaxis of fibroblasts from local mesenchyme and progenitor cell sites, while latent factors sequestered in the new matrix can be activated later to regulate fibroblast function as they populate the wound tissue (Clark, 1996, Blakytny et al., 2004, Wipff et al., 2007, Murphy-Ullrich and Poczatek, 2000, Munger et al., 1999). Furthermore, as fibroblasts become activated they begin to release soluble factors in an autocrine and paracrine fashion allowing regulation of their own functions as well as those of other
wound repair cells such as vascular endothelial cells and keratinocytes (Clark, 1996, Denton et al., 2009). Eventually, fibroblast-derived factors take over from inflammatory cytokines in the regulation of granulation tissue formation and its subsequent remodelling. Over the years a variety of growth factors and their cognate receptors, with specific and overlapping effects on fibroblasts function and wound repair have been identified. The most well-defined include growth factors belonging to the family of PDGFs, FGFs, and TGFβs. Furthermore, growth factors from the connective tissue growth factor/cysteine-rich 61/nephroblastoma over-expressed (CNN) family as well pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) have gained increasing importance in the regulation of fibroblast function.

PDGFs comprise of a family of homodimeric or heterodimeric growth factors including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD which exert their effect through tyrosine kinase receptors comprised of homo or heterodimers of α- and β-chains (Werner and Grose, 2003). The α-chain displays high affinity for all PDGF isoforms while the β has a high affinity for PDGF BB (Heldin and Westermark, 1999). PDGF is one of the first growth factors deposited by degranulating platelets and is proposed to be a key factor in granulation tissue formation (Senior et al., 1983). PDGF receptors are expressed in many wound cell types and it is suggested that it mostly operates in a paracrine fashion (Singer and Clark, 1999). Dermal fibroblasts are one of the major target cells of PDGF in the initiation and propagation of wound healing in the skin (Senior et al., 1983). Migration of dermal fibroblast is significantly enhanced by addition of exogenous PDGF-BB and to a lesser extent by the PDGF AB isoform in vitro (Siegbahn et al., 1990). Furthermore, application of PDGF-BB in cutaneous wound healing induces the influx of inflammatory cells and fibroblasts, stimulates fibroblast proliferation and granulation tissue formation (Pierce et al., 1989)(Pierce et al., 1991).

Deletion of PDGFR-β gene retards key dermal fibroblast functions including migration, proliferation and survival (Gao et al., 2005). Lethality in PDGF-BB and PDGFR-β
homozygous mice has restricted a full determination of the role of endogenous PDGF on
fibroblast function in a wound healing scenario (Sariano p 1994 and Leveen P, 1994). However, mice generated from the blastocyst chimeras that contain both wild type and
PDGFR-β null cells show a significant reduction in the contribution of PDGFR-β null
dermal fibroblasts in the formation of the granulation tissue following subcutaneous
injury (Crosby et al., 1999).

Importantly, augmented PDGF levels have been linked with a variety of wound healing
disorders. PDGF ligand and receptor expression is reduced in age- and diabetes-related
forms of impaired healing which further supports its role in fibroblast function and
granulation tissue formation in normal healing (Beer et al., 1997, Ashcroft et al., 1997).
Consequently, treatment of impaired wound healing defects with PDGF has had
beneficial effects in clinical trials and its use is now approved for the treatment of
human diabetic ulcers (Nagai and Embil, 2002). In contrast, augmented PDGF
production might also be involved in the pathogenesis of various fibroproliferative
disorders. Examples of this include hypertrophic and keloid scars, which both show
elevated levels of PDGF expression, which may be a contributing factor in the over-
stimulation of fibroblast proliferation and production of collagenous matrix in these
disorders (Haisa et al., 1994, Niessen et al., 2001).

As mentioned earlier, FGFs are heparin binding mitogenic proteins suggested to play an
important role in angiogenesis (see section 1.4.2.1). In addition to their angiogenic and
re-epithelialising stimulatory effects, the importance of FGFs in the chemotaxis and
regulation of fibroblast function including matrix production and remodelling during
wound repair is demonstrated through a variety of models. For example, constitutive
release of anti-FGF2 neutralising antibodies from subcutaneously implanted polyvinyl
alcohol sponges showed a striking reduction in cellularity, vascularization of granulation
tissue and collagen levels in normal wound healing, suggesting that endogenous FGF2
plays an important role in wound healing (Broadley et al., 1989). Furthermore,
exogenous application of FGF2 accelerates granulation tissue formation in impaired wound disorders such as ischaemic and diabetic wounds, by increasing fibroblast proliferation and collagen accumulation independently as well as synergistically with TGFβ (Uhl et al., 1993, Tsuboi and Rifkin, 1990, Broadley et al., 1988).

In addition to promoting granulation formation by fibroblasts FGF2 appears to be a negative regulator of the myofibroblast phenotype. Single application of FGF2 on cutaneous wounds antagonises fibroblast to myofibroblast transition, accelerates granulation tissue remodelling, restores tensile strength and improves scar outcome (Spyrou and Naylor, 2002). Furthermore, artificial dermal transplants lined with FGF2 showed reduced α-SMA-positive cells and increased myofibroblast apoptosis (Akasaka et al., 2007). Moreover, administration of FGF2 induces down-regulation of α-SMA and accelerates myofibroblast apoptosis in open skin wounds (Ishiguro et al., 2009).

Surprisingly, while most FGFs seem to be increased following injury and their applications appear to modulate different fibroblast functions associated with wound healing such collagen synthesis and remodelling, they appear to be mostly dispensable, as evidenced by a lack of wound phenotype in a number of FGF null wound healing models, including FGF1 and FGF7(aka KGF) which are thought to be important in wound healing (Miller et al., 2000, Guo et al., 1996). Moreover, FGF2 null mice show a partial but significant delay in wound healing characterised by reduced production of collagenous matrix and delayed wound contraction (Miller et al., 2000). Functional redundancies and structural similarities between FGF family members have been implicated in the lack of phenotype following single gene deletions. Consequently, it is not surprising that mutation of FGFR2IIIb, a receptor for several FGF isoforms causes a severe impairment to wounding healing (Werner et al., 1994).

Interleukin 6 (IL-6) is an important pro-inflammatory cytokine and its expression is strongly increased during the inflammatory phase of dermal wound healing (Grellner et
IL-6 is detected in wound fluid and its expression is detected in a variety of cell types within cutaneous wounds including neutrophils, macrophages and fibroblasts (Peteiro-Cartelle and Alvarez-Jorge, 1999, Gillitzer and Goebeler, 2001). Although the role of IL-6 during wound healing is mostly to induce leukocyte infiltration, there is a growing body of evidence that suggests that it might also directly and indirectly regulate some fibroblast functions. For example, in culture recombinant IL-6 induces procollagen gene expression and directly induces collagen production in adult human dermal fibroblasts (Duncan and Berman, 1991). Furthermore, IL-6 knockout mouse wounds show reduced TGFβ-1 gene expression which correlates to delayed deposition of collagenous matrix implying an indirect effect of IL-6 on fibroblast function through the induction of TGFβ1 (Gallucci et al., 2000). In addition, IL-6 can directly induce and inhibit α-SMA expression in dermal fibroblast cultures in a dose-dependant manner (Gallucci et al., 2006). Surprisingly however, IL-6 knockout mice show reduced wound contraction despite early augmentation of α-SMA expression in comparison to wild type littermates (Gallucci et al., 2000). The mechanism underlying dysregulated expression of α-SMA in this phenotype is still not clear but suggests that IL-6 might be important in preventing early differentiation of fibroblasts during wound healing. It is plausible that reduced TGFβ1 and reduced mechanical tension due to reduced cellularity and reduced granulation tissue formation are a contributing factor in delayed wound contraction of IL-6 knockout mice, despite their elevated expression of α-SMA. Overall these studies demonstrate that IL-6 can regulate important fibroblast functions including collagen production and α-SMA expression which are highly relevant in both wound healing and fibrotic diseases. Further characterisation of IL-6 in functional assays such as matrix contraction studies might also be useful in defining its effects on myofibroblast phenotypic modulation.

CTGF is a matricellular protein that contains 38 conserved cysteine residues that is characteristic of members belonging to the CNN family (Werner and Grose, 2003). CNN members are involved in embryonic development and gene deletion or global
inactivation of CTGF leads to defective endochondral bone formation, respiratory failure and perinatal lethality (Kawaki et al., 2008). In addition to its developmental roles, CTGF has emerged as an important regulator of cell proliferation, migration, differentiation, angiogenesis, matrix production and cell adhesion during physiological conditions such as wound repair as well as pathological states such as fibrosis and tumourigenesis.

In the unwounded dermis, CTGF expression is undetectable and only induced following insult (Igarashi et al., 1993). In normal cutaneous wound healing, CTGF is markedly increased by TGFβ-activated fibroblasts in the granulation tissue, where it is suggested to regulate proliferation and extracellular matrix production (Igarashi et al., 1993). Furthermore, CTGF is an important downstream inducer of TGFβ-mediated effects. In mice, subcutaneous injection of TGFβ1 results in increased CTGF expression in dermal fibroblasts and induces fibrosis at the site of injection (Frazier et al., 1996). Comparable effects can be observed after subcutaneous injection of CTGF (Frazier et al., 1996).

Importantly, over-expression of CTGF is observed in a variety of chronic fibrotic disorders affecting multiple organs including skin, liver and kidney. In skin, persistent over-expression of CTGF is observed in biopsies of keloid scars and hypertrophic lesions (Igarashi et al., 1996). While in culture, dermal fibroblasts derived from keloids and hypertrophic lesions show increased basal expression of CTGF as well as augmented CTGF expression in response to TGFβ (Shi-wen et al., 2000, Colwell et al., 2005).

The role of CTGF in cutaneous wound healing and fibrosis has also been tested using inhibition and depletion studies. In vitro CTGF induces matrix contraction of TGFβ1 primed fibroblasts in the presence of mechanical tension through up-regulation of α-SMA and attachment through increased MMP expression while inhibition of CTGF reduces these responses and subsequently reduces contraction (Daniels et al., 2003, Garrett et al., 2004). Furthermore in vivo, antisense inhibition of CTGF mRNA limits hypertrophy, reduces scarring and reduces myofibroblast persistence in the later phase
of rabbit cutaneous wound healing (Sisco et al., 2008). Surprisingly, it does this without hampering or delaying early events such as re-epithelialisation and initial granulation tissue formation (Sisco et al., 2008). Taken together, this data suggests CTGF is likely to be an important inducer of mid to late phase fibroblast functions in wound healing including myofibroblast differentiation, enhanced matrix production and wound contraction.

TGF-β1 is released in large amounts by platelets and macrophages during the acute inflammatory phase of healing and is undoubtedly the most important regulator of fibroblast function during wound healing. In particular, its role in inducing production of collagenous matrix and growth factors, fibroblast to myofibroblast transition and granulation tissue remodelling have been convincingly demonstrated over the years through addition and depletion studies.

TGFβ1 is pivotal to chemotaxis, collagen production and subsequent formation of granulation tissue by fibroblasts. This was first demonstrated by application of TGFβ1 to normal skin and its induction of rapid fibrosis with massive invasion by inflammatory cells, endothelial cells and fibroblasts with excessive collagenous matrix production in vivo and in vitro (Roberts et al., 1986). Furthermore, healing of rodent cutaneous incisional wounds is accelerated by TGFβ1 in a dose dependant manner and is evident through increased infiltration of macrophages and fibroblasts, increased tensile strength and increased collagen deposition (Mustoe et al., 1987). Exogenous TGFβ has also been used to augment healing in impaired wound disorders such as diabetic wounds by restoring collagen content of granulation tissue and improving tensile strength of the tissue (Broadley et al., 1988).

It is generally accepted that fibroblast to myofibroblast transformation is a key event in the physiological reconstruction of collagenous matrix after injury and it is also implicated in the generation of deformations that characterise fibrosis (Gabbiani, 2003).
In normal cutaneous wound healing, transient expression of αSMA correlates to fibroblast-myofibroblast transition and wound contraction (Darby et al., 1990). However, in fibrotic disorders αSMA expressing myofibroblasts persistent for long periods and are associated with increased fibrosis and detrimental tissue contractures (Schmid et al., 1998). TGFβ1 is central to the myofibroblast phenotype through its ability to induce de novo expression of αSMA in granulation tissue fibroblasts and quiescent fibroblasts both in vivo and in vitro (Desmouliere et al., 1993, Serini and Gabbiana, 1996, Montesano and Orci, 1988). These findings implicate TGFβ1 in wound contraction and retractile phenomena observed during fibrotic diseases.

The importance of endogenous TGFβ1 in fibroblast function during wound healing has been demonstrated in several inhibition and deletion studies. For example, neutralisation of endogenous TGFβ1 with antibodies in rodent cutaneous incisional wounds has been shown to reduce granulation tissue formation and overall scarring (Shah et al., 1995). Paradoxically in this incisional model, healing is not delayed and tensile strength remained unaffected implying that the net effect of endogenous TGFβ1 was on scar formation and not the rate of healing. In contrast, marked delays in granulation tissue formation and wound contraction are observed in immunodeficient TGF-β1 null mice excisional wound models (Crowe et al., 2000). Additionally, selective deletion of TGFRII in dermal fibroblasts results severe abnormalities during wounding with diminished fibrillar collagen and granulation matrix deposition, impaired myofibroblasts transition and reduced contraction (Denton et al., 2009). While in culture TGFβRII null mouse fibroblasts fail to migrate, contract lattices or express de-novo α-SMA following addition of exogenous TGFβ (Denton et al., 2009). Inhibiting TGFβ and its signalling cascade is viewed as an important strategy for treatment of fibrotic disorders. For example, limiting of TGFβ receptor binding though retroviral delivery of a dominant negative TGFRII has been shown to reduce scar hypertrophy in scarring rodent models (Reid et al., 2007). Furthermore, temporal neutralisation of TGFβ1 and 2 with their
cognate monoclonal antibodies has been shown to reduce hypertrophic scarring in a rabbit ear cutaneous model (Lu et al., 2005).

It is also well accepted that TGFβ augments the direct and indirect expression of fibroblast-derived paracrine and autocrine factors as well as their cognate receptors on different cell types to regulate a variety of wound healing events including angiogenesis and re-epithelialisation. For example, activation or over-expression of SMAD3, a downstream effector of TGFβ signalling, in dermal fibroblasts has been shown to up-regulate the release of VEGF and augment expression of FGF-2 receptors in vitro and to accelerate wound healing in dermal ulcer models by increasing angiogenesis and re-epithelialisation (Sumiyoshi et al., 2004). Furthermore, TGFβ1 up-regulates VEGF release by dermal fibroblasts in temporal micro-environments such as hypoxia, which in vivo is likely to have an additive effect on angiogenesis during wound repair and also in fibrotic diseases where hypoxia is common (Berse et al., 1999). In addition to VEGF, TGFβ1 induces the expression and release of autocrine CTGF in fibroblasts which augments their proliferation and their extracellular matrix synthesis, a process that has been linked to several fibrotic disorders as well as having a role in normal wound healing (Frazier et al., 1996). Importantly, TGFβ1 induces further expression of TGFβ1 and its cognate receptors in dermal fibroblasts resulting in an autocrine loop, a mechanism that extends and amplifies its action during the repair process (Van Obberghen-Schilling et al., 1988). In normal wound healing TGFβ auto induction may be useful in enhancing angiogenesis and granulation tissue formation while later, in scar resolution, there is loss of TGFβ over-expressing fibroblasts through apoptosis (Desmouliere et al., 1995, Schmid et al., 1998). In contrast, augmented TGFβ receptor expression and persistent autocrine induction of TGFβ1 is viewed as an important mechanism by which myofibroblast survival and activity is prolonged in fibrotic lesions, including hypertrophic scars, where excessive production of collagenous matrix and poor resolution of granulation tissue is observed (Schmid et al., 1998). Additionally, impaired forms of wound healing, such as chronic wounds, show aberrant TGFβ signalling, decreased TGFβ
receptor expression in fibroblasts and impaired granulation tissue formation, implying that reduced auto-induction by TGFβ is a possible mechanism in the pathogenesis of impaired healing (Kim et al., 2003, Wu et al., 1999).

1.4.4.2. Fibroblasts, integrins and the extracellular matrix

The function of fibroblasts during wound healing and fibrosis is critically influenced by their ability to interact with the extracellular matrix, a process mediated through cell-surface expressed matrix receptors (integrins)(Clark, 1996). Integrins are transducers of signals from outside into the cells and vice versa that regulate cell adhesion, migration, proliferation and differentiation as well as extracellular matrix remodelling (Thannickal et al., 2003, Clark, 1996), as discussed above in sections on re-epithelialisation and angiogenesis. Integrins can modulate the signalling cascade elicited by several growth factors through activation of intracellular pathways and influence cell function (Thannickal et al., 2003). Furthermore, integrins play a critical role in the activation of latent growth factors sequestered in the extracellular matrix as well as the activation of some growth factor receptors (Asano et al., 2005b, Wipff et al., 2007, Margadant and Sonnenberg, 2010). The role of integrins during myofibroblast differentiation and activation of latent TGFβ during wound healing and fibrosis has been especially studied over the years.

Granulation tissue fibroblasts differentially express integrins depending on the matrix substrate present as the wound heals (Saalbach et al., 1997). Following injury, the events of the coagulation cascade result in a provisional matrix rich in fibronectin which provides the initial anchorage for infiltrating wound repair cells (Clark et al., 1982a, Laurens et al., 2006). Adhesion and migration of fibroblasts on fibronectin during the early phase of wound healing is mediated through increased expression of fibronectin receptor α5β1 (Saalbach et al., 1997). As healing proceeds, collagen becomes the predominant substrate and granulation tissue fibroblasts show reduced functionality of α5β1 integrins and weakened interactions with fibronectin (Hakkinen et al., 1994).
Granulation tissue fibroblasts increase their expression of collagen binding receptors α1β1 and α2β1 integrins to establish collagen contacts (Saalbach A, 1997).

As tension in the granulation tissue develops, with increasing fibroblasts matrix deposition and remodelling, fibroblasts differentiate into myofibroblasts through expression of *de novo* α-SMA and the granulation tissue contracts (Darby et al., 1990). Integrins play a critical role in mechano-perception by acting as link between extracellular matrix and focal contacts (Hinz et al., 2003). Mechanical forces act on focal contacts and together with TGFβ and ED-A fibronectin splice variant, lead to expression and incorporation of α-SMA (Desmouliere et al., 1993, Serini et al., 1998, Serini and Gabbiana, 1996). Loss or inhibition of integrins impairs tension induced activation of the myofibroblast. For example, blockade of α2 and β1 subunits with respective antibodies abrogates TGFβ1 induced α-SMA expression in fibroblasts seeded under tension (Arora et al., 1999). Furthermore, mice with fibroblast-specific deletion of integrin β1 subunit show delayed cutaneous wound closure, less granulation tissue formation and reduced fibroblast to myofibroblast differentiation (Liu et al., 2010).

In addition to cell to matrix interactions, integrins play an important role in the activation of latent growth factors. In wound healing and fibrosis activation of latent TGFβ by fibroblast and myofibroblast integrins occurs through both a protease dependant and protease independent manner (Wipff and Hinz, 2008, Margadant and Sonnenberg, 2010). Integrin-dependant protease activation of TGFβ involves association of αV containing integrins with TGFβ latency complex and recruitment of MMPs (MMP2 and MMP9) to release TGFβ through proteolytic cleavage (Wipff and Hinz, 2008). In fibrotic disorders such as scleroderma, high amounts of active TGFβ are apparent and fibroblasts show increased integrin expression and enhanced cell surface proteolytic activity through MMPs (Asano et al., 2005a). Inhibition of integrins including αVβ3 reduces MMP expression and levels of active TGFβ (Asano et al., 2005b) while antibody antagonists of αVβ3 specifically inhibited granulation tissue formation in a transient
manner in porcine cutaneous wound healing (Clark et al., 1996). Integrin related protease independent activation of TGFβ can occur mechanically via integrin-mediated cell contraction. In vitro, the force generated by contracting myofibroblasts can exert conformation changes on the latent TGFβ complex bound by integrins (αVβ5, α8β1 and αVβ3) and cause mechanical release of active TGFβ (Wipff P, 2007). Conversely, inhibition of these integrins or loss of tension reduces TGFβ activation (Wipff P, 2007).

The relevance of integrin-dependent activation of TGFβ has been demonstrated in αV and β1 subunit null mice. Mice lacking αVβ6 and αVβ8 mirror abnormalities characteristic of TGFβ 1, 3 null mice including cleft palates, severe autoimmunity syndrome and loss of Langerhans cells (Aluwihare et al., 2009). Targeted deletion of β1 subunit in fibroblasts using Cre-lox systems caused significant reduction in activation of latent TGFβ1 by fibroblasts causing delayed closure and reduced granulation tissue formation in cutaneous wound healing in mice (Liu et al., 2010).

1.4.4.3. Hypoxia modulates fibroblast function

Tissue hypoxia is considered to be a major signal that initiates and regulates angiogenic responses in physiological and pathological conditions including normal and abnormal wound healing. Cellular adaptive responses to hypoxia are mediated mostly by HIF-1, which induces transcriptional responses that include expression of a variety of proteins that enable survival and function of hypoxic cells (Semenza, 2010). Response to hypoxia and activation of HIF-1 pathways by wound repair cells is critical for normal wound healing, so much so that failure to do so, contributes to impaired wound healing (Botusan et al., 2008, Zhang et al., 2010b). Additionally, sustained HIF-1 accumulation in fibroblastic cells represents an important mechanism in the pathogenesis of several fibrotic disorders. Over the years the role of HIF-1 and hypoxia on fibroblast function has been partially defined, particularly in the context of fibroblast cell migration, collagenous matrix production, fibroblast to myofibroblast differentiation and the release of paracrine and autocrine factors.
Acute hypoxia in the early phase of healing has been determined through direct PO$_2$ measurement and immunochemical assay (pimonidazole) in normal wound healing and correlates to an increase in expression of HIF-1 and downstream targets such as the glucose transporter -1 (Elson et al., 2000, Lokmic et al., 2006, Remensnyder and Majno, 1968). In culture acute hypoxia (0 to 72 hrs) enhances several fibroblast functions including migration, proliferation and production of collagenous matrix through up-regulation of TGFβ responses (Falanga and Kirsner, 1993, Falanga et al., 2002, Siddiqui et al., 1996, Steinbrech et al., 1999). Accumulating evidence point to a strong correlation between HIF-1 stabilisation and TGFβ signalling during enhanced fibroblast function in hypoxic tissue. Indeed increased HIF-1 signalling enhances fibroblast expression of matrix proteins (collagens and fibronectin), growth factors (TGFβ, CTGF, and VEGF) and TGFβ1 activating factors (thrombospondin-1) (Distler et al., 2007, Higgins et al., 2004). The relevance of HIF-1 in the regulation of these factors during hypoxia has been convincingly tested using HIF-1 depleted or inhibited fibroblasts (Distler et al., 2007, Higgins et al., 2004). The evidence suggests that HIF-1 is important in the regulation of dermal fibroblast function during angiogenesis and granulation tissue formation, events that occur under relatively hypoxic conditions. Indeed, recent studies of cutaneous burn wound healing in HIF1α heterozygous null mice showed a significant reduction in wound vascularization, perfusion and granulation tissue contraction (Zhang et al., 2010b).

In contrast to acute hypoxia, chronic ischaemia (not anoxia) has a net inhibitory effect on some pivotal fibroblast functions in the skin. In a cutaneous wound in an ischaemic limb model, persistent hypoxia was shown to reduce production of collagenous matrix and retard granulation tissue formation as well as impair αSMA expression and reduce contraction (Alizadeh et al., 2007). In culture, persistent hypoxia reduces collagen synthesis, αSMA expression, contractile ability and increases cell survival, which are pivotal elements required for effective wound healing and remodeling (Modarressi et al., 2010, Siddiqui et al., 1996). Re-oxygenation is shown to reverse the effects of
chronic hypoxia and restore αSMA expression and matrix production \textit{in vitro} (Modarressi et al., 2010, Siddiqui et al., 1996). This adds support to the use of hyperbaric oxygen in the treatment of some ischaemic wounds. Impaired TGFβ signalling is proposed as one of the mechanisms behind these hypoxia-induced defects (Modarressi et al., 2010). Indeed, fibroblasts from chronic ischaemic wounds show reduced TGFβ signalling, decreased TGFRII expression and unresponsiveness to stimuli from TGFβ1 (Kim et al., 2003). Ischaemia has also been suggested to reduce peptide growth factor receptor binding (Falanga et al., 1994). That said, it is still unclear how TGFβ signalling is impaired by persistent hypoxia, in the absence of complications associated with age and diabetes, and where hypoxic conditions cause HIF-1α stabilisation. It is conceivable that severe hypoxic conditions, such as chronic hypoxia and anoxia, induce HIF-1α stabilisation but repress HIF-1 transcription activity (Schmid et al., 2004). It is therefore tempting to speculate that the reduced fibroblast activity during chronic hypoxia is also in part due to HIF-1 transcriptional repression.

Impaired healing in aged and diabetes-associated wounds has been linked with defective responses to hypoxia. Briefly, dermal fibroblasts from aged patients show delayed migration in hypoxia and impaired TGFβ signalling in comparison to fibroblasts from young patients (Mogford et al., 2002). Furthermore, TGFβ1 shows a failure to stimulate healing in aged mice but stimulates healing in young mice with chronic ischaemic wounds (Wu et al., 1999). Growing research now indicates that impaired healing in aged and diabetic ischaemic wounds is partly caused by defective HIF-1 signalling and a reduced ability to respond to local tissue hypoxia (Botusan et al., 2008). Indeed, hyperglycaemia has been shown to impair HIF-1 stability and cellular function in primary dermal fibroblasts and endothelial cells (Catrina et al., 2004). Additionally, HIF-1α gene transcription is impaired in dermal fibroblasts of aged mice resulting in delayed angiogenic responses in ischaemic wounds (Loh et al., 2009). Defective granulation tissue formation and delayed dermal regeneration in diabetic mice wound models were also improved by preventing hydroxylation and thus increasing stabilisation of HIF-1α.
Fibrotic disorders characteristically develop chronic tissue hypoxia due to microvascular occlusion and high oxygen consumption (Kischer et al., 1982, Ichioka et al., 2008). In skin, this is observed in scarring disorders including keloids, hypertrophic lesions and scleroderma (Kischer CW, 1982)(Silverstein JJ, 1988). The persistence of myofibroblasts, augmented expression of growth factors (TGFβ1 and VEGF), their cognate receptors (TGFβRI / II and VEGFR) and excessive production of collagen matrix and tissue inhibitor of metalloproteinase (TIMP-1) are key features of skin fibrotic disorders. Increasing evidence now demonstrates that hypoxia through HIF-1 induction may be a contributing factor in the pathogenesis of certain fibrotic disorders. For example, accumulation of HIF-1 protein is observed in biopsies and cells from keloid scars and scleroderma suggesting a possible link between hypoxia and unregulated behaviour of fibroblastic cells in fibrotic disorders (Distler, 2007) (Zhang, 2003). In keloids persistent hypoxia up-regulates plasminogen activator inhibitor-1 (PAI-1) in a HIF-1-dependent manner *in vitro* and *in vivo*, correlating to excessive production of matrix by dermal fibroblasts (Zhang, 2003). Using fibroblasts from scleroderma patients and mice treated with long-term hypoxia, HIF-1 is shown to up-regulate expression of collagenous matrix and TGFβ1 while abrogation of HIF-1 or TGFβ1 inhibits the hypoxia-induced expression of extracellular matrix (Distler, 2007). The role for HIF-1 in the pathogenesis of fibrotic disease has been further supported by the discovery HIF-1α gene polymorphisms in systemic sclerosis cases of a French European Caucasian population (Wipff J, 2009). Additionally, chronic hypoxia and augmented HIF-1 accumulation in fibroblasts of non-cutaneous organs is considered to be an important mechanism in the pathogenesis of fibrosis including liver cirrhosis and kidney fibrosis. Furthermore, selective and tissue specific deletion of HIF-1α in liver using a Cre-lox system has been shown to reduce liver fibrosis through reduced myofibroblast transformation, reduced expression of PDGF and
PAI-1 and reduced production of collagenous matrix following bile duct ligation (Moon et al., 2009).

Overall, local relative hypoxic gradients and HIF-1 in normal healing are likely to be important for maintaining good angiogenic responses and granulation tissue formation by potentiating several fibroblast functions such as migration, proliferation, survival, growth factor release and matrix synthesis in the early phases of healing. Failure to respond to hypoxic stimuli, due to HIF deficiency, (as seen in age- and diabetes-related wounds) plausibly propagates chronic hypoxia, which appears to be detrimental to some dermal fibroblast functions such TGFβ signalling, matrix production and myofibroblast transformation. In contrast, chronic hypoxia and consistent HIF accumulation in fibrotic disease appears to potentiate the actions of fibroblasts causing increased myofibroblast differentiation and excessive matrix production. The susceptibility to fibrotic disease may also be higher in populations with an increased frequency of HIF-1α polymorphisms. Based on all the above, it would seem that targeting HIF-1 has several important clinical implications to tissue repair conditions where HIF-1 is impaired, deficient or over-expressed by fibroblastic cells.

1.5. Remodelling and scar formation

Once the granulation tissue has spread evenly across the wound site, the remodelling phase of wound repair begins. Remodelling is characterised by contraction of the granulation tissue, degradation of collagen and a significant loss in cellularity in the wound bed, the net result of which is a scar (Darby et al., 2002, Gabbiani et al., 1971, Desmouliere et al., 1995). Myofibroblasts expressing contractile actin (αSMA) align parallel to the direction of mechanical tension by adhering to collagen through focal adhesion proteins and integrins (Darby et al., 1990, Tomasek et al., 2002, Tomasek et al., 1992, Hinz et al., 2003). Myofibroblasts also make fibronexus connections to each other (Singer et al., 1985). TGFβ1 in combination with fibronectin splice variant ED-A FN and mechanical stress, stimulate contraction of αSMA bundles which significantly
reduces the granulation area (Desmouliere et al., 1993, Serini et al., 1998). Following contraction αSMA positive myofibroblasts and vascular cell numbers in the granulation tissue are reduced by the induction of apoptosis (Desmouliere et al., 1995). An up-regulation of iNOS (inducible nitric oxide synthase) which increases the production of free radicals in the granulation tissue may be one trigger for apoptosis (Darby et al., 2002). Additionally, increased collagen degradation due to increased expression of MMPs coupled with reduced expression of TGFβ1 and metalloproteinase inhibitors (TIMP-1) is thought to further promote remodelling (Darby et al., 2002) and may induce apoptosis through loss of growth factor signalling and changes in cell adhesion. However, despite all the efforts in remodelling, adult mammalian full thickness wounds inevitably produce a scar.

1.6. Abnormal wound repair

Injurious events that create tissue loss give rise to the healing process that eventually leads to restoration of tissue integrity, albeit with some loss in function due to scar formation. Tissue repair is a highly orchestrated series of complex events which may be subject to several abnormalities. These abnormalities can occur at different phases of wound repair and lead to different types of abnormal wounds. Clinically, abnormal wound healing manifests mostly as fibroproliferative disorders (keloid and hypertrophic scars) and chronic wounds (ulcers) (Clark, 1996). Fibroproliferative disorders are the result of an excessive or continual fibrogenesis response that outstrips the remodelling attempt (Craig, 1975). Fibroproliferative lesions are often painful, itchy and prone to develop into contractures (hypertrophic scar) which can impose severe restrictions to mobility and quality of life as well as social and psychological burdens. (Roseborough et al., 2004, Bayat et al., 2003). Chronic wounds are characterized by persistent inflammation, lacking a proliferative phase and increased proteolytic activity that prevents sufficient deposition of matrix components (Wilkinson et al., 1993, Palolahti et al., 1993). Chronic wounds can be painful, may smell and are often prone to infection which increases the risk of amputations (Cavanagh and Bus, Barron et al., 2007).
Abnormal wounds are difficult to treat and often impose a significant financial load on global healthcare system mostly due to the massive amount of hours required for wound care (Sen et al., 2009). In developed countries alone, it has been estimated that 1 to 2% of the population will experience a chronic wound during their lifetime, and with an aging population and increasing incidence of diabetes and obesity world wide, this figures are likely to increase sharply (Sen et al., 2009). In the US alone, the estimated costs cost of chronic wounding is in excess of 25 billion dollars (US) per annum. While in Australia chronic wounds are conservatively estimated to cost the healthcare system 500 to 600 million dollars per annum (Leach, 2004). In the US alone, the cost of anti-scarring drugs was conservatively estimated to be a 12 billion dollar (US) market (Sen et al., 2009). The cost of wound healing in general is only sure to rise with the increase global population and the rising cost of healthcare. Please refer to Figure 1-2 for typical gross images of abnormal wounds.
Figure 1-2. Representative gross images and histological images of typical abnormal wounds. (a.) Typical hypertrophic and (c.) keloid scars and (b.)(d.) their histology showing excessive amounts of disorganized collagen. (e.) Typical foot ulcer and (f.) histology showing poorly formed granulation tissue. Images were adopted from (a.)(b.)(Kokoska, 2010, Roseborough et al., 2004), (c.)(d.)(Berman, 2008, Sperling, 2010) and (e.)(f.)(Hichand, 2010, Moretti et al., 2009)
1.6.1. Hypertrophic and Keloids scars

Hypertrophic and Keloid scars are defined as raised skin scars characterised by a constitutively active proliferation phase of wound healing with a structural makeup that is highly vascular, with excessive deposition, mostly of type I collagen (Craig, 1975, Rudolph, 1987, Bayat et al., 2003, Aarabi et al., 2007). There are clear histological and immunochemical differences between the two fibroproliferative lesions. Hypertrophic scars contain distinct collagen bundles and high cellular density of myofibroblasts, an important feature in provoking contractures around areas prone to mechanical stress such as joints (Ehrlich et al., 1994). Keloids deposit large amounts of thick disorganised collagen fibres and seldom contain α-SMA expressing fibroblasts or provoke contracture (Ehrlich et al., 1994). Keloids are further distinguished by their ability to breach the margin of the original lesion and aggressive growth often likened to (benign) tumours (Rudolph, 1987). Keloids develop from small and large injuries and are particularly resistant to medical management, and are too aggressive for surgery owing to a high likelihood of recurrence (Rudolph, 1987). Hypertrophic scars may develop after deep and/or extensive cutaneous insults such as burns and surgical incisions and can be treated through grafting and surgical corrections.

In normal cutaneous healing, the complete formation of granulation tissue marks the beginning of the remodelling phase achieved through rapid granulation tissue contraction, degradation of matrix and loss in cellularity through apoptosis (Gabbiani et al., 1971, Darby et al., 2002, Desmouliere et al., 1995). In contrast, fibroproliferative disorders show failure to resolve the connective tissue formation phase of normal healing. Fibroproliferative disorders are often multifactorial with a variety of underlying and secondary factors contributing to the wound healing defect. The underlying factors may include genetic predisposition and age, while secondary effects may include persistent auto-induction of growth factors, defective apoptotic pathways and increased tissue hypoxia (Schmid et al., 1998, Ladin et al., 1998, Kischer et al., 1982, Clark et al., 2009).
The temporal expression of growth factors is important in orderly and organised regulation of event during wound healing and deregulated expression of growth factors can alter these orderly events resulting in abnormal wound healing. Biopsies of fibroproliferative lesions often show elevated levels of growth factors and their cognate receptors. (Schmid et al., 1998, Peltonen et al., 1991). For example, elevated levels of both TGFβ and its receptors (TGFβRI/TGFβRII) have been well defined and suggested to lead to increased auto-induction of TGFβ and excessive production of collagenous matrix (Peltonen et al., 1991, Schmid et al., 1998). Furthermore, increased auto-induction of TGFβ in hypertrophic scars has been correlated to the persistence of the myofibroblast phenotype and the possible development of fibrotic contractures (Schmid et al., 1998). In addition to TGFβ, other elevated growth factors have been identified and include FGF2, VEGF, PDGF and CTGF, suggesting a possible synergetic effect of multiple growth factors in the development of excessive scarring disorders. (Jiang et al., 2004, Haisa et al., 1994, Colwell et al., 2005). In cell culture, fibroblasts harvested from fibroproliferative lesions show increased sensitivity to growth factors and deposit increased amounts of collagen in comparison to normal dermal cells, which further supports an enhanced growth factor response paradigm in the development of these disorders (Haisa et al., 1994, Bettinger et al., 1996, Fujiwara et al., 2005, Colwell et al., 2005).

As mentioned above, in normal wound healing fibroblasts/myofibroblasts and endothelial cells undergo massive apoptosis during the remodelling phases leading to a loss in cellularity (Desmouliere et al., 1995). However, in keloid scars and keloid fibroblasts in culture, focal dysregulation of p53 expression and up-regulation of Bcl-2 is proposed to produce a combination of increased cell proliferation and decreased cell death via apoptosis (Ladin et al., 1998).
Keloids appear to be prevalent amongst populations of darker skinned people. This is now supported by evidence demonstrating familial autosomal dominant inheritance amongst African populations (Clark et al., 2009). Links to vitamin D deficiency amongst coloured people have been postulated as possible reason for predisposition to keloids (Cooke et al., 2005). Vitamin D is a robust endogenous anti-inflammatory agent, and due to high pigmentation, darker skinned individuals absorb less sunlight and metabolise less Vitamin D (Cooke et al., 2005). However, apart from evidence provided by one in vitro study, demonstrating some beneficial effects of introducing Vitamin-D derivatives into keloid cells (Zhang et al., 2010a), there is little evidence to support causal effects of Vitamin-D in the pathogenesis of keloid scarring.

Perivascular contraction by myofibroblasts and microvascular occlusion in hypertrophic scars and keloids lead to tissue hypoxia which has been suggested to be important in the pathogenesis of excessive scarring disorders (Kischer et al., 1982). Consistent elevation of HIF-1α protein levels are observed in keloid and scleroderma tissues compared with normal skin and has been linked with increases in fibrotic related factors such TGFβ1, thrombospondin-1, PAI-1 and VEGF (Zhang et al., 2003b, Distler et al., 2007). It is also tempting to postulate that HIF-1α gene polymorphisms, which have been correlated to increased susceptibility in scleroderma (Wipff et al., 2009), may potentially be involved in increasing susceptibility to other fibroproliferative disorders such as keloid and hypertrophic scars.

At present, fibroproliferative disorders have no cure and scarring is often permanent. Several treatments may be administered to manage and minimize the scarring outcome including anti-inflammatory drugs, glucocorticoid steroids, occlusive dressings containing silicone, calcium chelators and apoptosis inducers have been reviewed as the common non-invasive scar management approaches (Roseborough et al., 2004). However, their specificity to wound repair is lacking and systemic side effects may be a concern. Plastic surgery and skin grafts remains the most widely used approach in
management of large scars. But recurrence of lesions remains may be a concern with surgery. For example, keloid scars have a 70% recurrence rate within the first year of postoperative correction (van de Kar et al., 2007). Overall treatment of fibroproliferative scars via non-invasive methods remains unspecific and surgical approaches offer no guarantees. Targeting wound-specific factors would seem a better approach and may offer more targeted and specific treatment with minimal systemic adverse effects.

Research into wound specific factors including growth factors and chemokines has been the focus of the last 2 to 3 decades. These factors can alter the behaviour of wound repair cells such as macrophages, endothelial cells and fibroblasts/myofibroblast. Therefore targeting growth factor offers a specific approach to managing wound healing disorders. TGFβ1 is the most important growth factor in the wound milieu, through its potent stimulation of inflammation, angiogenesis, collagen synthesis and myofibroblast differentiation (Desmouliere et al., 1993, Roberts et al., 1986). Minimising the effects of TGFβ1 is viewed to be an important approach in the regulation of scarring and fibrosis as is demonstrated through several studies (Roberts et al., 1986, Distler et al., 2007). Neutralization of TGFβ1 and 2 with antibodies has been effective in reducing scarring in rabbit hypertrophic ears models and mice incision wounds (Shah et al., 1995, Lu et al., 2005). Furthermore, anti-sense TGFβ1 has been shown to reduce deranged TGFβ-Smad signal transductions, collagen synthesis, inhibit growth and reduce collagen receptors expression in keloid fibroblasts (Bran et al., 2010a, Bran et al., 2010b, Bran et al., 2010c). Delivery of anti-sense TGFβ1 oligonucleotides into third degree burns and postoperative wounds has been beneficial in reducing scar formation and hypertrophy (Luo et al., 2002, Cordeiro et al., 2003). TGFβ3 is thought to antagonise the fibrotic effects of TGFβ1-2 and its application on rodent and human cutaneous wounds is demonstrated to improve scarring (Shah et al., 1995). Human synthetic TGFβ3 was in the final phase of clinical trials for use in regeneration of normal skin and scar reduction after injury of surgery (Occleston et al., 2009, Occleston et al., 2008). Disappointingly perhaps, TGFβ3 has recently been reported to have failed phase III clinical trials (Press release, February,
2011) and will be unlikely to be pursued further as a therapy in the short term (Obstoj-Cardwell, 2011). Reduction of TGFβ ligand and receptor binding though retroviral delivery of a dominant negative TGFRII has been shown to reduce hypertrophy in scarring rodent models and is a potential therapeutic approach of the future (Reid et al., 2007).

In addition to targeting TGFβ, inhibition of its down stream inducers may be effective in regulating scarring. For example, inhibition of CTGF mRNA with anti-sense sequences is effective in limiting hypertrophy, reduces scarring and decreases myofibroblast persistence in the late phase of rabbit hypertrophic wound models, without hampering or delaying wound healing (Sisco et al., 2008).

Fibrotic disorders are usually diagnosed once tissue scarring is already in progress, and it is plausible that treatment will require the targeting of specific fibroblastic cell populations. For example, targeting of myofibroblast contractile apparatus through inhibition of αSMA polymerisation. Failure to assemble αSMA into stress fibres leads to a reduction in contractile ability and reduced collagen synthesis (Hinz et al., 2002). This might be of significance to patients with hypertrophic scars that have a high density of myofibroblasts and are prone to painful contractures (Ehrlich et al., 1994, Schmid et al., 1998).

1.6.2. Chronic ischaemic wounds

The vast majority of cutaneous chronic wounds clinically present as venous, pressure and diabetic ulcers (Mustoe, 2004). Chronic wounds show persistent inflammation devoid of a proliferative phase, poor deposition of matrix components and failure to heal in a defined time line (Wilkinson et al., 1993, Palolahti et al., 1993, Moor et al., 2009). The pathogenesis of chronic wounds is multifactorial and mostly associated to underlying conditions related to age and diabetes or both, for example, reduced gene transcription with increasing age causes reduced protein synthesis and non enzymatic
glycosylation of proteins plus microvascular dysfunction in diabetes can retard growth factor function and alter vascularization respectively (Wheaton et al., 1996, Algenstaedt et al., 2003, Sen, 2009). Fibrin cuffing of microvessels, oedema and increased venous pressure occurs in venous ulcers, pressure may be an issue in diabetic ulcers, and a common factor in most chronic wounds is varying degrees of local tissue ischaemia (Browse and Burnand, 1982, Mustoe, 2004, Mustoe et al., 2006).

Chronic ischaemia has got several important consequences to wound healing cells including reduced cell responsiveness to growth factors (Kim et al., 2003, Sen, 2009). Ischaemia has been suggested to reduce peptide growth factor receptor binding (Falanga et al., 1994). Treatment with oxygen (hyperbaric or topical oxygen) would therefore seem relevant and is currently one of many approaches used to improve healing in chronic ischaemic wounds. However, oxygen treatment alone is not sufficient when treating ischaemic wounds which are often chronic due to multifactorial causes (Zhao et al., 1994). Combination therapy, for example with hyperbaric oxygen and growth factors (PDGF and TGFβ1) has synergistic effects and improves healing outcomes of ischaemic wounds (Zhao et al., 1994). In addition, actin remodelling proteins such as Flightless 1 have now emerged as important regulators of wound repair and their manipulation has been shown to modulate wound healing in transgenic mice wound repair models, where over-expression of Flightless 1 impaired wound healing and deficiency improved and accelerated wound healing (Cowin et al., 2007). Furthermore, topical application of a Flightless inhibiting antibody has been shown to improve wound repair and therefore may be of therapeutic importance to conditions where wound healing is impaired (Cowin et al., 2007).

It is now well appreciated that underlying mechanisms such as age and diabetes have detrimental effects on HIF-1 expression or stabilisation therefore reducing the cellular responses during local tissue ischaemia (Liu et al., 2008, Catrina et al., 2004, Loh et al., 2009). Therapeutic approaches that stabilise HIF-1α would therefore be beneficial in
driving the expression of downstream HIF-1 target genes which enhance angiogenic responses and promote wound healing. Indeed, the use of drugs that inhibit HIF-1 hydroxylation have been beneficial in stabilising HIF-1α and improving wound healing in diabetic mice (Botusan et al., 2008). Correction of HIF-1α deficiency with electroporation-facilitated gene therapy increases wound healing, angiogenesis, and circulating angiogenic cells in diabetic aged mice (Liu et al., 2008).

Please refer to figure (See Figure 1-3) for schematic representation of therapeutic approach
Figure 1-3. Schematic of therapeutic approaches to improve healing in ischaemic wounds and reduce hypoxia induced fibrosis. Hypoxia, through HIF-1 stabilisation upregulated the expression of several factors (PAI-1, VEGF, TGFβ, CTGF) that promote wound healing. Over-expression of HIF-1 may lead to deranged expression of these factors and increase the risk of fibrosis. On the other hand, deficient HIF-1 stabilisation may impair adaptive ability in hypoxia and increase the risk of impaired healing. Targeting HIF-1 may have valuable therapeutic implications in both impaired and fibrotic wound healing. Adopted from (Botusan et al., 2008, Distler et al., 2007, Higgins et al., 2004, Hong et al., 2006, Zhang et al., 2006, Zhang et al., 2003b)
1.7. TGFβ1-Smad signalling in wound healing

TGFβ1 and its other mammalian isoforms TGFβ2 and 3, belong to a superfamily of growth factors that include bone morphogenic protein (BMP), nodals, activins, the anti Mullerian hormone and several other related proteins found in different classes of eukaryotes (Roberts, 1998, Massague, 1998). TGFβ1 is a multifunctional cytokine with broad ranging activities that are both contextually inhibitory and stimulatory to cell proliferation, extracellular matrix synthesis or degradation and mesenchymal-epithelial interactions during development, wound repair and carcinogenesis (Roberts, 1998, Massague, 1998, Verrecchia and Mauviel, 2002).

During wound healing TGFβ1 is released by a variety of cells including platelets, macrophages, keratinocytes and fibroblasts within and surrounding the wound bed. It is secreted in a latent form (L-TGFβ) that binds to extracellular matrix requiring proteolytic cleavage through various proteases including plasmin, thrombin and matrix metalloproteinase (Roberts, 1998). Mechanical activation of latent L-TGFβ can be achieved through integrin-dependant mechanisms during cell contraction (Wipff and Hinz, 2008, Wipff et al., 2007).

Bioactive TGFβ1 exerts its effect by binding to a subunit of a heterodimeric serine threonine kinase receptor complex consisting of a Type I (TGFRI) and II (TGFRII) (Massague, 1998, Piek et al., 1999). TGFβ1 binds to TGFRII which activates kinase activity leading to the recruitment and activation of TGFRI and the subsequent phosphorylation of receptor activated Smad-2 and 3 proteins (Massague, 1998, Piek et al., 1999, Massague and Chen, 2000, Massague and Wotton, 2000). Phosphorylated Smads (P-Smad)-2/3 complex with Smad-4 (translocator) and consequently become translocated to the nucleus to function as transcription factors and enable the expression of TGFβ-regulated genes (Massague and Chen, 2000, Massague and Wotton, 2000). Smad7 is a regulatory Smad and can inhibit Smad2/3 phosphorylation through
competition for the TGFRI binding domain, being the predominant mechanism through which TGFβ signalling is switched off (Nakao et al., 1997). A schematic of TGFβ-Smad signalling is represented below (Figure 1-4).

**Figure 1-4. A schematic of TGFβ-Smad signalling pathway.** Bioactive TGFβ binds to TGFRII receptor and activates kinase activity. Ligand bound TGFRII complexes with and phosphorylates TGFRI (transducer receptor). TGFRI kinases get activated which recruits and phosphorylates R-Smad (Smad-2/3). Phosphorylated R-smad complex with co-Smad (Smad-4) and translocates to the nucleus to function as a transcriptional regulator of TGFβ regulated genes. Smad-2/3 phosphorylation is negatively regulated by Smad-7 (Adopted from (Verrecchia and Mauviel, 2002))
The importance of TGFβ-Smads signal transduction, in physiological conditions such as development and wound repair, is demonstrated in various transgenic rodent models where proteins in the TGFβ-Smad signal chain have been deleted or mutated. For example, cutaneous models with deleted or abrogated TGFβ1, TGFβRII and Smad are all characterised by altered wound healing although some findings have been somewhat surprising. TGFβ null mice with suppressed auto-immune responses are characterised with defective wound healing (Crowe et al., 2000). Similar results are observed following specific over-expression of dominant negative TGFRII in dermal fibroblast populations in cutaneous mice models (Denton et al., 2009). Additionally, in such models Smad expression and phosphorylation is reduced or absent (Denton et al., 2009). However, wound healing studies from Smad null mice have been somewhat surprising and indeed contrary to TGFβ and TGFRII null mice studies.

Smad-2 is critical in development and its absence as demonstrated in Smad-2 null mice causes failure to achieve development of mesodermal cells and consequently prenatal lethality (Waldrip et al., 1998) which supports the role of the TGFβs and Smad-2 in development. The role of Smad-2 has not been properly tested \textit{in vivo} due to lethality of Smad-2 homozygous null mice and heterozygous Smad-2 null mice show no obvious phenotypes (Ashcroft et al., 1999). In contrast, Smad-3 homozygous null mice are embryonically sound and survive into adulthood without any obvious phenotype until challenged with injury (Ashcroft et al., 1999, Wang et al., 2007).

Paradoxically, Smad-3 null mice show accelerated wound healing characterized by rapid re-epithelialisation, an impaired local inflammatory response, reduced granulation tissue formation and normal responses to exogenous TGFβ, implying that Smad-3 negatively regulates wound healing (Ashcroft et al., 1999, Wang et al., 2007). Furthermore, fibroblasts from Smad-3 null mice produce normal amounts of collagen in response to TGFβ1, implying that Smad-3 is dispensable during granulation tissue formation during cutaneous healing (Ashcroft et al., 1999). Taken together, TGFβ-Smad-
2 signal transduction may be important in promoting wound healing while TGFβ-Smad-3 negatively regulates it. However, the significance of this is still not well understood.

### 1.8. Co-operation between hypoxia and TGF-β pathways

Local relative hypoxia occurs in all forms of wound healing. Normal wounds are often transiently hypoxic post injury and hypoxia regresses once a sufficient level of vascularization is achieved in the granulation tissue (Lokmic et al., 2006). Hypoxia in normal healing wounds is detected between 3 to 10 days post injury and also correlates with the increase in HIF-1α during the same period (Lokmic et al., 2006, Haroon et al., 2000, Elson et al., 2000). Inflammation, re-epithelialisation, angiogenesis and fibrogenesis are wound healing events that progress during acute tissue hypoxia. TGFβ plays a central and critical role in all phases of wound healing and its expression is high during tissue hypoxia (Lokmic et al., 2006). Thus the question of co-operation or synergy between hypoxia and TGFβ becomes relevant in our understanding of early wound healing. Please refer to schematic below (Figure 1-5).
Figure 1-5. Schematic overview of the overlapping wound healing phases, acute hypoxia and spatio-temporal expression of TGFβ1, HIF1α and Glut-1. Disruption of vessels and influx of inflammatory cells results in tissue hypoxia (low PO₂). HIF-1α and TGFβ levels are high during the early hypoxic phase of wound healing. Glut-1 is a downstream target of HIF-1 that increases cell metabolism and can be used as a marker of HIF-1 transcriptional activity. Co-expression of HIF-1 and TGFβ may be indicative of synergy between HIF-1 and TGFβ pathways during early wound healing. Adopted from (Elson et al., 2000, Haroon et al., 2000, Lokmic et al., 2006)

Dermal fibroblasts treated with anti-sense HIF-1α or TGFβ1 oligonucleotides both show inhibition of TGFβ1 expression in hypoxia which is further enhanced using a combination of both antisense sequences, indicating that hypoxia can induce TGFβ1 through both HIF-1-dependant and HIF-1-independent pathways (Distler et al., 2007).
However, it is not clear if TGFβ1 induction occurs directly through HIF mediated gene transcription and moreover, a HIF-1 promoter region (hypoxia response element) has yet to be defined on the TGFβ1 gene, as far as we know. Interestingly, TGFβ2 contains a hypoxia response element with an active HIF-1 promoter region that can be induced to increase autocrine TGFβ2 in hypoxia independently of Smad activation, an important process in the stimulation of vascular endothelial cells and angiogenesis (Zhang et al., 2003a). HIF-1 can also up-regulate the expression of thrombospondin-1, a TGFβ1 activating protein, which is a plausible indirect mechanism of TGFβ up-regulation and may then increase expression of collagenous matrix in dermal fibroblasts during hypoxia (Distler et al., 2007, Murphy-Ullrich and Poczatek, 2000). Furthermore, TGFβ1 has also been described to stabilise HIF-1α proteins through inhibition of HIF-1-prolyl-4-hydroxylases (PHDs) and consequently results in an increase in HIF-1 targets such as VEGF (McMahon et al., 2006). Such cross-talk between HIF and TGFβ pathways may provide a mechanism by which HIF-1 target genes may be expressed in the absence of oxidative stress (McMahon et al., 2006). Importantly, this also implies that HIF-1 may be important in some wound healing events, including tissue remodelling and wound contraction, which proceed in normoxic conditions where TGFβ1 expression is still relatively high. Furthermore, TGFβ expression in fibrotic disorders is often persistent (Distler et al., 2007, Schmid et al., 1998). Thus, increased HIF-1 expression through a TGFβ induced mechanism might be possible and is likely then to be a contributing factor in the pathogenesis of fibrotic disease.

Accumulating research suggests the possibility of cross-talk between HIF-1 and TGFβ pathways through direct physical interaction of HIF-1 and Smad transcription factors. It is well established that enhanced VEGF expression by hypoxic endothelial cells, macrophages and fibroblasts occurs in response to increased levels of TGFβ (Sanchez-Elsner et al., 2001, Jeon et al., 2007, Berse et al., 1999). Evidence suggests that this may partly involve a physical interaction between smad2/3-smad4 complex and HIF-1α at
the promoter region of genes containing both a hypoxia response element (HRE) and a Smad binding element (SBE), for example the VEGF gene (Sanchez-Elsner et al., 2001, Jeon et al., 2007). The physical interaction requires the recruitment of co-activator proteins such as 300 Kilo-Dalton co-activator protein (p300), which enables the formation of a HIF-1-Smad complex (Jeon et al., 2007) (Figure 1-6). It is plausible that similar synergistic mechanisms occur on other genes where the promoter regions contain both a HRE and a SBE. For example, HIF-1 up-regulates CTGF directly in hypoxia through a HRE on the promoter region of the CTGF gene (Higgins et al., 2004), a gene which also contains an active SBE that can be switched on in the presence of TGFβ1 (Higgins et al., 2004). Consequently, a synergy between Smad and HIF-1 during hypoxia would seem likely and may have important pathogenic implications in fibrotic diseases in general as well as tumourigenesis, where increased HIF-1 and CTGF expression is common (Higgins et al., 2004, Hong et al., 2006). A similar model of HIF-Smad interaction is suggested in the synergistic expression of erythropoietin-1 and TGFβ co-receptor, endoglin both important factors during angiogenesis (Sanchez-Elsner et al., 2004, Sanchez-Elsner et al., 2002)
Figure 1-6. Synergy of TGFβ and hypoxia involves a physical interaction between HIF-1 and Smad complex at the promoter region of HIF and TGFβ inducible genes. Complexing of HIF and Smad through a co-activator protein, p300, leads to synergistic expression of genes in hypoxia including VEGF and possibly other genes which contain both a SBE and a HRE sequence on the promoter region. Adopted from (Jeon et al., 2007, Verrecchia and Mauviel, 2002)
Interaction of TGFβ1 and HIF-1 pathways might also occur indirectly through activation of mitogen activated protein kinases (MAPK). For example, activation of HIF-1α requires phosphorylation through p42/p42 and p38 MAPK which can both be induced mechanically during oxidative stress and chemically through TGFβ1-induced pathways (Hur et al., 2001, Richard et al., 1999, Sato et al., 2002) (Figure 1-7). Consequently, reduced TGFβ1 induced p42/p42 MAPK phosphorylation is suggested to reduce HIF-1α stabilisation and TGFβ induced responses (migration) of aged cells exposed to hypoxia (Mogford et al., 2002). Inhibition of p38 MAPK has also been shown to reduce TGFβ1 induced CTGF expression in hypoxia, a process that partly requires induction through HIF-1 (Rong et al., 2005, Lee et al., 2009, Higgins et al., 2004). Activation of HIF-1α through MAPK has been demonstrated in fibroproliferative disorders. For example, activation of HIF-1 by several MAPK including p42/p42, PI3-K and AKT, has been shown to induce expression of PAI-1 in keloid fibroblasts (Zhang et al., 2004). Induction of PAI-1 inhibits matrix metalloproteinase activity and is an important marker of fibrotic disease. Activation of HIF-1 through MAPK in conditions where TGFβ and hypoxia are common may thus have important pathogenic implications.
Figure 1-7. A schematic of HIF-1α and TGFβ1 cross-talk through mitogen activated protein kinase (MAPK). TGFβ1 can induce responses through activation of MAPK and Smad dependent pathways. Response to hypoxia requires activation of HIF-1α through oxidative stress mediated activation of MAPK. TGFβ may also enhance HIF-1α activity through activation of MAPK. HIF-1α and smad complexes can also physical interact at the promoter region of some genes (e.g. VEGF and CTGF) to synergistically enhance their expression during hypoxia. Adopted from (Jeon et al., 2007, Verrecchia and Mauviel, 2002, Hur et al., 2001, Richard et al., 1999, Sato et al., 2002)
1.9. Regulation of hypoxia by HIF-1

Metazoan organisms ubiquitously express HIF-1, a helix loop-helix Per-Arnt-Sim family transcription factor. It is composed of a dimer of an alpha (HIF-1α) and a beta (ARNT or HIF-1β) subunit which bind together to acquire transcriptional properties predominantly under hypoxic conditions (Wang et al., 1995). To date, HIF-1 is still thought to be the master regulator of oxygen homeostasis and is described to regulates the transcriptional activity of hundreds of genes that promote angiogenesis, cell proliferation, increase metabolism and survival in hypoxic conditions (Semenza, 1998, Semenza, 1999). Of interest to the wound repair process are pro-inflammatory, angiogenic and pro-fibrotic genes that are up-regulated or enhanced by HIF-1 transcription factor activity including PAI-1, VEGF, GLUT1, erythropoietin, CTGF, TGFβ2 and PDGF, just to mention a few (Zhang et al., 2003a, Semenza, 1999, Higgins et al., 2004, Zhang et al., 2003b).

Furthermore, TGFβ1 is increased during hypoxia through both a HIF-1 dependent and independent manner, however it is not clear if HIF-1 can regulate TGFβ1 transcription directly (Distler et al., 2007).

The HIF-1α and β subunits are continuously expressed in cells at all times highlighting the importance of HIF-1 as an evolutionary adaptation. Its regulation is almost solely based on the rapid degradation or stabilisation of the HIF-1α subunit post translation (Kamura et al., 2000). In normal tissue oxygen conditions, HIF-1α is rapidly and continuously degraded post translation (Kamura et al., 2000). This is mainly due to the O₂-dependent hydroxylation of HIF-1α amino acid (aa) proline residues, Pro^{402} and Pro^{564}, in a reaction catalysed by HIF-Prolyl 4- hydroxylases (PHD) 1, 2 and 3 in presence of cofactors, O₂ and 2-Oxoglutarate (Bruick and McKnight, 2001). Hydroxylation of the amino acid residues results in conformational changes that allow HIF-1α to become a target of the Von Hippel-Lindau Tumor Suppressor Protein (pVHL), an important E3 ubiquitin ligase that binds to HIF-1α and complexes with elongin B, C and cullin 2.
resulting in HIF-1α ubiquitination and subsequent proteosomal degradation (Kamura et al., 2000). (Figure 1-8)

In addition to hydroxylation through PHDs, HIF-1α is also regulated through an asparaginyl-hydroxylase, FIH (factor inhibiting HIF-1) in an O₂ dependent manner. HIF-1α has two trans-activation domains, TAD-N (residues 531-575) and TAD-C (residues 786-826), which bind to co-activators including CREP binding protein (CBP) and p300 (Mahon et al., 2001). In the presence of O₂, FIH hydroxylates asparagine residue (ASN803) within the TAD-C, preventing the interaction of HIF-1α with the transcriptional co-activators, p300 and CBP, thereby decreasing the transcriptional ability of HIF-1 (Mahon et al., 2001). This is suggested to be a secondary oxygen regulatory mechanism for HIF-1α proteins that have evaded the PHD-mediated regulation.

In hypoxia HIF-1α is stabilised and its ubiquitination is limited through reduced oxygen cofactor and depletion of both PHDs and FIH through degradation by proteosomes (Nakayama et al., 2004, Fukuba et al., 2007). Seven in absentia homologues (Siah) 1 and 2, have E3 ubiquitin ligase activity and tag target proteins for degradation by proteosomes (Fukuba et al., 2007, Nakayama et al., 2004). In hypoxia, Siah activity is increased and correlates with the increased abundance and maintenance of HIF-1α and reduced stability of FIH and PHDs (Nakayama et al., 2004)(Fukuba et al., 2007). The relevance of Siah in the regulation of HIF-1 activity is demonstrated in Siah2 null cells which show marked reduction in HIF-1α stabilisation in hypoxia and reduced downstream transcription of hypoxia inducible genes (VEGF and GLUT-1)(Nakayama et al., 2004)

Following stabilisation, HIF-1 is activated by phosphorylation. Activation of HIF-1α in hypoxia occurs through stress activated MAPK such as p42/p42 and P38 MAPK (Hur et al., 2001, Richard et al., 1999) (Figure 1-8). These MAPKs can also be activated following cytokine-receptor binding (Sato et al., 2002). Thus it is plausible that MAPKs provide a potential avenue for the regulation of HIF-1 and its regulated genes by cytokines.
Following phosphorylation, HIF-1α dimerises with HIF-1β and is translocated into the nucleus where it binds to the hypoxia regulatory element (HRE) sequence on the promoter region of HIF inducible genes. Transcriptional co-activators p300 and CBP are also required to bind to trans-activation domains on the HIF-1α protein, enabling HIF-1 to be transcriptionally active (Figure 1-8). Under extreme (anoxia) or prolonged forms of hypoxia, p300 and HIF-1α interaction may be inhibited leading to HIF-1 transcription repression (Schmid et al., 2004). This occurs through induction of tumour repressor protein p53 which competes with HIF-1α for p300 co-activator (Schmid et al., 2004).

HIF1 is an important regulator of hypoxia induced events in several physiological and pathological conditions including normal and pathological wound healing. In normal cutaneous wounds HIF1 is important for good angiogenic responses, mobilisation of circulating angiogenic cells and wound contraction and consequently partial reduction of HIF1α is sufficient to impair wound healing (Zhang et al., 2010b). In fibrotic disease, over-expression of HIF-1 correlates to an increase in expression of pro-fibrotic factors associated with excessive production of collagenous matrix, while HIF-1 deficiency in aged and diabetic related ischaemic wounds correlates to reduced adaptive hypoxia responses and impaired healing (Kimura et al., 2008, Distler et al., 2007, Zhang et al., 2006, Zhang et al., 2003b, Zhang et al., 2004, Botusan et al., 2008, Liu et al., 2008). Consequently, positive (Siah proteins) and negative regulators (PHDs, pVHL and FIH) of HIF provide important therapeutic targets for drugs that could potentially be used to manipulate HIF-1 abundance in pathological conditions where over-expression or deficiency in HIF-1 correlates to pathogenesis. For example, PHD inhibitors can stabilise HIF-1α and subsequently drive expression of downstream HIF-1 target genes (Botusan et al., 2008). PHD inhibitors have been useful in improving healing of diabetic ischaemic wounds in mice and are now currently undergoing clinical trials for treatment of several human ischaemia based conditions including ischaemic wounds, anemia, ischaemic heart disease and pulmonary hypertension (Botusan et al., 2008, Smith and Talbot, 2010, Katschinski, 2009).
Figure 1-8. Schematic on HIF-1α degradation, stabilisation and activation. Normal physiological oxygen levels favour catalytic activity of FIH-1 and PHDs resulting in hydroxylation of HIF-1α and subsequent ligation with pVHL, which tags HIF-1α-OH for degradation by proteosomes. Low oxygen levels limit the catalytic activity of HIF-hydroxylase (PHDs & FIH) due to reduced substrate oxygen and an increase in the inhibitory effects of Siah proteins. Consequently hypoxia favours HIF-1α stabilisation, activation through MAPK, dimerisation of HIF-1α and HIF-1β and transcription of HIF-1 inducible genes. Adopted from (Kamura et al., 2000, Bruick and McKnight, 2001, Mahon et al., 2001, Nakayama et al., 2004, Fukuba et al., 2007, Hur et al., 2001, Richard et al., 1999).
1.10. Seven in absentia homologue

1.10.1. Introduction

The Siah proteins were first isolated and characterised from drosophila in the early 1990s and found to exist as three highly conserved genes in mice, Siah 1a, 1b and 2 (Della et al., 1993). The genes were initially shown to be widely expressed during development and also in adult tissue suggesting general functions, but shown to be elevated in olfactory epithelium, retina, forebrain, gonad and areas of proliferating cartilage during late development suggesting a critical role in the development of these specific areas (Della et al., 1993). The Siah family of proteins possess a RING finger structure with potent E3 ubiquitin ligase activity that controls the proteosomal degradation of a wide range of proteins including TNF-receptor associated factor 2 (TRAF2), synphilin-1, N-cor, m-cyb and β-catenin amongst others (Liu et al., 2001, Matsuzawa and Reed, 2001, Hu et al., 1997, Hu and Fearon, 1999, Li et al., 2002).

1.10.2. Generation of Siah transgenic mice and role of Siah in HIF-1α availability

Determination of the role of Siah family proteins in cell physiology has been possible through generation of Siah 1a and 2 knockout transgenic mice (Dickins et al., 2002, Frew et al., 2003). Siah1a homozygous (Siah1+/−) mutation leads to severe growth retardation, frequent lethality and impaired spermatogenesis (Dickins et al., 2002). In contrast, Siah2 homozygous (Siah2+/−) mutant mice are mostly phenotypically normal and grow to adulthood without any obvious complications (Frew et al., 2003). Heterozygous double mutants (Siah1+/−, Siah2+/−) have also been generated and are mostly phenotypically normal, suggesting redundancy between the two genes (Frew et al., 2003). Siah 1 and 2 homozygous double knock down mutation causes neonatal lethality which is indicative of the overlapping roles of Siah1 and 2 in growth and development (Frew et al., 2003). Additionally, the loss of Siah2 alone does not significantly impair described Siah2
associated activity such as degradation of TRAF2 and further supports the existence of functional redundancies amongst the Siah family of proteins (Frew et al., 2003).

Siah1/2 null cells also show relatively normal behaviour in culture in terms of cell cycle, proliferation and survival (Frew et al., 2002). However, when challenged with hypoxic conditions, Siah1 and 2 null cells showed reduced availability of HIF-1α and reduced expression of HIF-1 target genes (Nakayama et al., 2004, Fukuba et al., 2007). Complete failure to stabilise HIF-1 is observed in the double Siah1 and 2 knockout cells, suggesting that Siah is critical for HIF-1α availability in hypoxia (Nakayama et al., 2004). This phenomenon is explained by increased half-life of HIF-1 hydroxylases (PHDs and FIH) in the absence of Siah (Nakayama et al., 2004). Siah has a high binding affinity for HIF-hydroxylases (PHDs and FIH) and is critical for their ubiquitination–dependent degradation the consequence of which is the stabilisation of HIF-1α during hypoxia (Nakayama et al., 2004). More recent studies also point to the role of p38 MAPK in the activation and subcellular localisation of Siah under hypoxic conditions (Khurana et al., 2006). Phosphorylation of Siah2 by p38 MAPK, for example, significantly enhances its ability to degrade PHD3 in hypoxia (Khurana et al., 2006).

The relevance of Siah in HIF-1 stabilisation has been further tested using Siah specific inhibitors including the drosophila protein Phyllopod and vitamin K3 (menadione), both of which cause a marked reduction in HIF-1α abundance and the expression of HIF1 target genes (VEGF and GLUT1) (Moller et al., 2008, Shah et al., 2009).

1.10.3. The potential role of Siah proteins in wound healing

Local acute hypoxia and increased stabilisation of HIF-1 is important for good angiogenic responses and acceleration or maintenance of normal wound healing (Zhang et al., 2010b). Hypoxia through HIF-1 controls the expression of several important genes in multiple wound repair cells including macrophages, endothelial, keratinocytes and
fibroblasts. These genes may encode soluble factors (PAI-1, CTGF, VEGF, TGFβ), receptors (VEFGR) and metabolic proteins (Glut-1) that stimulate cell migration, differentiation, survival, matrix production and release of more soluble factors to collectively promote the healing process. Moreover, several examples of cross-talk between HIF-1 and TGFβ1 pathways have been reported and are likely to be important in wound healing. Consequently, altered HIF-1 expression may contribute to abnormal wound healing. Over-expression of HIF-1 and persistent hypoxia may contribute to pathogenesis of fibrotic disorders (keloids and scleroderma), while deficiency in HIF-1 may cause failure to respond to hypoxia and impair wound healing (aged and diabetic ischaemic wounds). Given that Siah is an upstream positive regulator of HIF-1 through its ability to degrade HIF-hydroxylases in hypoxia, it is plausible that Siah plays an important role in the regulation of early tissue repair events that proceed when the wound is markedly hypoxic. Furthermore, use of HIF specific PHD inhibitors in the treatment of impaired ischaemic wounds, implies that constitutive expression of Siah may also be beneficial in treatment of disorders where HIF-1 is deficient. Defective Siah expression may also represent a potential mechanism in the impairment of wound healing.

In addition to HIF-1 regulation, Siah is also an important positive regulator of RAS signalling. Inhibition of Siah has been shown to reduce Ras-mediated transformation and tumorigenesis in lung and pancreatic cancers models (Ahmed et al., 2008, Schmidt et al., 2007). This may be important during wound healing, given that Ras signalling is shown to be a downstream enhancer of TGFβ-Smad responses in normal cells, cancer cells and fibrotic disease (Yue and Mulder, 2000, Mulder, 2000, Leask and Abraham, 2004). Thus taken together Siah may regulate wound healing responses through both HIF-1 dependent and Ras mediated pathways. For this reason, the availability of Siah transgenic knockout mice offers a rare opportunity to test the role of Siah in wound healing. Thus, this thesis aims to address the role of Siah2 in dermal wound healing in vivo by examining the effect of Siah2 deficiency using Siah2 knockout (transgenic) mice
and *in vitro* through the study of cultured dermal fibroblasts (murine Siah2-deficient fibroblasts and human fibroblasts).
Chapter 2

**General Methods and Materials**
2.1. **Materials**

All reagents and chemicals for cell culture were obtained from Gibco Invitrogen (Aus), gas mixtures were from BOC (Aus), and all other chemicals were obtained from Merck and Sigma Chemical company (Aus), except those listed below:

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<th>Materials</th>
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<td>Church &amp; Dwight, Aus</td>
</tr>
<tr>
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<td>BioRad Laboratories, Aus</td>
</tr>
<tr>
<td>Opsite™ occlusive dressing</td>
<td>3M, Aus</td>
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<tr>
<td>pcDNA phyllopod expressing and control vectors</td>
<td>Kind gift from Dr Andreas Møller of Peter MaCallum Cancer Centre, East Melbourne</td>
</tr>
<tr>
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<tr>
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**Table 2-2. Antibodies and Sources**

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<tr>
<td>Mouse monoclonal anti-HIF1α</td>
<td>1:100(f), 1:500(w)</td>
<td>Abcam Inc, USA</td>
</tr>
<tr>
<td>Mouse monoclonal anti-SMAD2</td>
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<tr>
<td>Mouse monoclonal anti-αSMA</td>
<td>1:250 (f), 1:200(i)</td>
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<tr>
<td>Mouse monoclonal anti-PCNA</td>
<td>1:200 (f &amp;i)</td>
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<tr>
<td>Polyclonal rabbit anti-phosphorylated SMAD2</td>
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<td>Rabbit polyclonal anti-C3D1</td>
<td>1:50(i)</td>
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<tr>
<td>Rabbit polyclonal anti- TGFβ type II receptor</td>
<td>1:100(f)</td>
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<tr>
<td>Rabbit polyclonal anti-COL1A1</td>
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<td>Rabbit polyclonal anti-Glucose transporter 1 (Glut1)</td>
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<td>Rabbit polyclonal anti-HA</td>
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<tr>
<td>Rat monoclonal anti-F4/80</td>
<td>1:50(i)</td>
<td>Abcam Inc, USA</td>
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Key: f= flowcytometry, i= immunohistochemistry, w= western blotting, 

**Table 2-3. Instruments and sources**

<table>
<thead>
<tr>
<th>Instruments</th>
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<tr>
<td>BD FAC Calibur flow cytometer</td>
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<tr>
<td>Beckman Centrifuge</td>
<td>Beckman Coulter, USA</td>
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<td>Cabinet Laminar Flow Horizontal HSW180</td>
<td>Crown Scientific, Aus</td>
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Chapter 2

<table>
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<th>Manufacturer</th>
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<td>Humidified chamber (5% CO(_2), 95% air)</td>
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</tr>
<tr>
<td>Hypoxia chamber</td>
<td>RMIT, Aus</td>
</tr>
<tr>
<td>Leica (DMD108) micro-imager</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Leica Tissue processor</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Microtome (RM2125)</td>
<td>Leica Microsystems, Germany</td>
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<tr>
<td>Mini-PROTEAN CELL 3</td>
<td>Biorad Laboratories, USA</td>
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<tr>
<td>Molecular Imager ChemiDoc XRS System</td>
<td>Biorad Laboratories, USA</td>
</tr>
<tr>
<td>Olympus microscope BX50</td>
<td>Olympus, USA</td>
</tr>
<tr>
<td>Olympus polarizer</td>
<td>Olympus, USA</td>
</tr>
<tr>
<td>Semi-Dry Trans-Blot Electrophoretic Transfer Cell</td>
<td>Biorad laboratories, USA</td>
</tr>
</tbody>
</table>

2.2. **Dermal wound healing model**

2.2.1. **Animal procurement and care**

Male C57BL/6 background mice of either Siah2 homozygous knock out (-/-) or wild type (+/+ ) genotype and were a kind gift from Dr Andreas Möller of the Peter MacCallum Cancer Centre (East Melbourne, Victoria). The animals were bred and reared at the Peter MacCallum animal housing facility and transported to the RMIT Animal Facility prior to surgery. The mice were kept at standard and constant room temperature and constant day-night light cycle. Standard mice diet feed and water were available *ad libitum*. All transported animals were allowed to acclimatise for at least 48 hours before experimental procedures. All experiment procedures were in line with the required institutional animal experimentation ethics committee guidelines and the project approved (AEC# 0828).

2.2.2. **Animal preparation and surgical procedure:**

All mice were checked before the start of any procedures and utilized if they were alert, active, responsive and able to be handled. The mice were anesthetised with a Ketamine
(80mg/Kg) and Xylazine (5mg/Kg) cocktail formulated on the basis of an average mouse weight of 20g. The mice were shaved on the dorsum and then depilatory cream was applied to produce smooth hairless skin for wounding and to assist subsequent dressing adhesion. Depilatory cream (Nair) was used as described in the manufacturer’s instructions. The skin area was then disinfected with povidine-iodine solution and swabbed with alcohol (70% ethanol) before wounding with a full-thickness wound by punch biopsy (5mm radius) (Figure 2-1). Wounds were rinsed with sterile saline for irrigation, local anaesthetic (bupivacaine 0.25%) applied into the wound space and then the wound was dressed with surgical occlusive dressing (Opsite™, 3M). All mice were allowed to recover under a heating lamp. 24 hours post-wounding, all dressings were removed to limit variation caused by dressing detaching at different times. Wounds were then allowed to heal by secondary intention.
Figure 2-1. Schematic summary of sequence of events during dermal wound infliction from both an aerial and cross sectional perspective. 5mm radius (r) biopsy punches were used to create full thickness wounds on dorsum as illustrated in the cross section.
Figure 2-2. Illustration showing sequence and technique of wound extraction and trimming post healing. Cross section of wounds illustrates the desired orientation of the granulation tissue after trimming.
2.2.3. Animal sacrifice and sample extraction

After wounding, animals were killed at different time points (6, 9 or 12 days post wounding) by CO\textsubscript{2} inhalation and then cervical dislocation. Wounds were dissected and trimmed to include the granulation tissue of the wound and a 1-2mm margin of normal skin (Figure 2-2). Wounds were predominately harvested at day 6 in order to target described events related to hypoxia in early wound healing (Elson et al., 2000, Lokmic et al., 2006). Wounds were fixed in buffered formalin (10% v/v in PBS) for 24 hours prior to paraffin embedding. Mouse tails were disinfected with ethanol (70% v/v in dH\textsubscript{2}O) and collected for cell extraction (see description below).

2.3. Histology

2.3.1. Sample preparation and paraffin embedding

Tissue was transferred to sucrose-PBS solution (7% v/v) for 24 hours to remove fixative then transferred to 70% alcohol. The fixed tissue was then dehydrated by immersion in a series of ascending grades of alcohol (70%-100% ethanol, followed by 100% Xylene) using an automated tissue processor (Leica, Germany) and then embedded into paraffin blocks.

2.3.2. Histological sectioning

All wound sections were cut at a thickness of 4μm using a rotary microtome (Leica, Germany) and transferred onto Superfrost plus glass slides (Menzel, Germany). Slides were baked at 50° C overnight and used for general histological staining, immunohistochemistry and TUNEL (sections 2.4 to 2.6).

2.4. Histological staining

2.4.1. De-waxing of histological sections

All paraffin sections were de-waxed by immersion in two changes of xylene for 5 minutes and then hydrated by successive immersion in descending concentrations of alcohol
(100%, 90% 70%) and then transferred to water or phosphate buffered saline (0.01M, pH 7.4).

2.4.2. Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining was performed to visualise general histological structures of wound sections as previously described with minor modifications (Kiernan, 2001). Briefly paraffin embedded sections were de-waxed and rehydrated by successive immersion in alcohol (section 2.4.1). Sections were immersed in Harris’ haematoxylin for 2 minutes to stain the nuclei blue following by a 5 minute wash in running tap water. Sections were immersed in Scott’s tap water for 1 minute to ‘blue’ nuclear staining followed by immersion in running water for 2 minutes. Excessive staining was removed by short immersion in acid alcohol (1% HCL, 70% ethanol).

For better contrast, cytoplasm and matrix tissue were stained by immersion in 1% aqueous Eosin (w/v) for 1 minute followed by a 2 minute wash in running water. The sections were dehydrated by successive immersion in ascending grades of alcohol 70%, 90%, 100% followed by two successive immersions in xylene for 2 minutes each. Sections were mounted using xylene based mountant (DePeX, Merck).

H&E stained sections were used to measure the area of granulation tissue and calculate % re-epithelialisation using micro-imager (Leica, Germany). H&E stained sections were also used to count the number of vessel profiles within the granulation tissues.

2.4.3. Picrosirius red staining

Picrosirius red staining was performed to visualize collagen fibres using previously described methods (Constantine and Mowry, 1968). Briefly, sections were de-waxed and hydrated (see section 2.4.1) by immersion in xylene and descending grades of alcohol. After washing in water, sections were immersed in picrosirius red (0.1% w/v Sirius red stain dissolved in saturated picric acid) for 1 hour. This was followed by two quick washes
in acidified water (5% acetic acid v/v) to remove excessive staining and better differentiate the stain. Sections were quickly dehydrated by successive immersion in ascending grades of alcohol followed by immersion in xylene. Slides were mounted with Depex and visualised under polarised light using Olympus BX50 microscope.

To assess the level of collagen deposition, quantitation of polarized picrosirius stained areas of the wounds was performed. Staining intensity varies in polarised light depending on the thickness and maturity of the collagen fibres (Constantine and Mowry, 1968). Green to yellow birefringence was consistent with newly formed granulation tissue with fine collagen fibres, and intense yellow to red birefringence was characteristic of mature granulation tissue with thicker collagen fibres. Images of stained sections were taken and analysed using image analysis software (ImagePro Plus®). Collagen deposition was expressed as a percentage of staining within the margins of the wounded area.

2.5. Immunohistochemistry

In order to visualize different antigens expressed in the wound sections immunohistochemistry was utilized. Primary antibodies raised against αSMA, PCNA, CD31, F4/80 and Glut1 were used to visualize the myofibroblast phenotypic transformation, proliferating cells, neovascularisation, macrophage infiltration and glucose transmembrane transporter protein respectively. Similar staining protocols have been described previously in other wound healing studies (Darby et al., 1990, Vollmar et al., 2002, Elson et al., 2000, Hewitson and Darby, 2009).

2.5.1. Antigen retrieval of paraffin embedded sections

Briefly, paraffin embedded section were de-waxed and hydrated (see section 2.4.1) by immersion in xylene and descending grades of alcohol. Sections were immersed in PBS for 2 minutes and then into antigen retrieval solution (citrate buffer, 0.1M pH 6.0). The
solution was heated to 90°C for 10mins and allowed to cool to room temp. Sections were washed twice in wash buffer (0.01M PBS).

2.5.2. Blocking endogenous peroxidase activity

To prevent any non-specific reactions between endogenous peroxidase and 3, 3’-diaminobenzidine (DAB), endogenous peroxidase activity in tissue was quenched by a 10 minute incubation in hydrogen peroxide buffer (3% H2O2 : 0.01M PBS), followed by a thorough wash with wash buffer.

2.5.3. Blocking non-specific antigen binding

To prevent non-specific binding to proteins in sections a blocking step using bovine serum albumin (1% BSA in PBS) or normal goat serum (2% NGS in PBS) was applied. Blocking solution was applied for 30 to 60 minutes at room temperature and later tipped off the sections.

2.5.4. Primary antibody application

Antibodies were diluted in REAL® antibody diluent (Dako, Aus) based on optimised antibody concentrations that were determined using sections of mouse wound. Briefly, final antibody concentrations used were determined after trialling each primary antibody at various dilutions and incubation times. Optimal concentration was determined by the best contrast between positive specific staining versus non-specific background staining. Please refer to experimental chapters (see Chapter 3) for incubation conditions of individual antibodies. After the prescribed incubation times sections were washed thoroughly for a total of 10 minutes in three changes of wash buffer.
2.5.5. Secondary antibody application

Horse radish peroxidase (HRP) conjugated secondary antibodies against immunoglobulin G (IgG) of primary antibody hosts were used to probe for the applied primary antibodies. In all immunohistochemistry experiments unless otherwise stated, a prediluted dual link anti-mouse/anti-rabbit polymer conjugated to HRP was used as secondary antibody (DAKO EnVision™ System, Peroxidase Kit, Dako, Aus). This was applied to sections for 30 minutes at room temperature and thoroughly washed off at the end of incubation. The specificity of primary antibody and the level of background staining generated by the secondary antibody was tested on samples by omission of the primary antibody.

For antibodies raised in species other than rabbit or mouse, for example rat monoclonal anti-F4/80, sections were incubated after the primary antibody with a biotinylated secondary antibody. Vectastain ABC Elite Kit (Vector Laboratories, USA) was then used for the detection steps. Briefly, the primary antibody was detected using a biotinylated anti-rat secondary antibody for 30 minutes followed by a thorough wash. ABC Elite avidin-biotin-HRP was then applied for 30 minutes at room temperature and thoroughly washed off.

2.5.6. Horseradish Peroxidase (HRP) and 3, 3’-diaminobenzidine (DAB) colorimetric reaction.

The chromagen 3, 3’-diaminobenzidine (DAB) was used to visualise the localisation of probed antigens through HRP catalysed reaction. The colorimetric reaction turned DAB from clear to dark brown colour that could be visualised under a microscope. Sections were washed thoroughly in water and later counterstained with haematoxylin, then dehydrated in ascending grades of alcohol and mounted (See section 2.4.2). Images of stains were taken using DMD108 micro-imager (Leica Microsystems, Germany) and
analysed using image analysis software (ImagePro Plus®). Stains were expressed as a percentage of staining within the margins of the wounded areas.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used to visualise apoptotic cells at different stages of wound repair using previously described methods (Desmouliere et al., 1995) with minor alterations. Briefly, paraffin embedded sections were de-waxed and hydrated in alcohol (see section 2.4.1). Sections were permealized for 15 minutes in proteinase K solution (20μg/ml proteinase K in PBS) and washed thoroughly in wash buffer (0.01M PBS). Apoptotic nuclei were probed using the reaction of terminal deoxynucleotidyl transferase and biotinylated dUTP (Promega, USA) at 37°C for 60 minutes. The TUNEL reaction was stopped and sections thoroughly washed. Nuclei with incorporated biotin-dUTP were detected by incubation with HRP-conjugated streptavidin (1:500, dilution) for 30 minutes and then DAB colorimetric reaction to visualise staining (see 2.5.6). Sections were counterstained, dehydrated through alcohol and mounted. Apoptotic nuclei were visualised at higher magnification (x20) and images taken using the micro-imager.

2.7. Dermal fibroblasts and wound healing in vitro models

Dermal fibroblasts are critical for collagen deposition and wound remodelling. In vitro they have previously been used to provide valuable insights into the mechanisms that affect wound repair in a controlled and more easily defined setting (Falanga and Kirsner, 1993, Falanga et al., 2002, Steinbrech et al., 1999). In the thesis both primary mouse dermal fibroblasts (MDFs) and primary human dermal fibroblasts (HDFs) were used to investigate the involvement of Siah2 regulated pathways in established hypoxia and cytokine induced processes such as cell proliferation, migration, matrix synthesis, contraction and cytokine release.
2.7.1. Sample collection and preparation

For preparation of primary fibroblasts from Siah2 knockout and wild-type mice, tails were collected from sacrificed animals (see section 2.2.3), disinfected in alcohol (70% ethanol) and immersed in ice-cold sterile PBS containing antibiotics (5% penicillin: 5% streptomycin) for about 60 minutes. Tails were then washed thoroughly (4-5 washes) in ice cold buffer. The joined epidermis and dermis of the tail were removed by dissection under sterile conditions, washed and maintained in ice cold sterile PBS. In contrast, HDFs (Invitrogen Aus) were established cell lines acquired frozen in freeze mix media (10% DMSO, 20% Fetal bovine serum (FBS), specialised media)(Invitrogen, Aus) and transferred to standard culture media when required (see section 2.7.3).

2.7.2. Separation of dermis from epidermis and fibroblast isolation

Fibroblasts were isolated by enzymatic separation of dermis and epidermis followed by digestion of collagen matrix to isolate single cells following previously described methods with minor modifications (Wu et al., 2005). Briefly, the dermis and epidermis were separated by overnight digestion in dispase solution (10mg/ml dispase: 0.01M PBS) at 4°C. The dermis was then immersed in dispase- collagenase solution (4mg/ml dispase and 3mg/ml collagenase) for 45-60 minutes. Collagenase activity was quenched by dilution (1:15) in buffered fetal bovine serum (2% FBS, 0.01M EDTA. 0.1M PBS). The digested cells and buffer were then filtered and centrifuged to isolate the cell pellet. Supernatant was replaced with cell culture medium (DMEM, 5% FBS, 1% penicillin/streptomycin) (Figure 2-3).

2.7.3. Growth and maintenance of fibroblast cultures

MDFs/HDFs were grown in a standard cell culture humidified incubator (37°C, 5% CO₂, 21% O₂, humidified) and medium (DMEM, 5% FBS, 1% penicillin/streptomycin) changed every second day in line with standard cell culture protocols (Freshney, 2005).
MDFs/HDFs were grown until 70 - 80 % confluence and passaged based on experimental needs. Cells used between passages P1 to P4 for MDF and P1 to P5 for HDFs
Figure 2-3. Methodological overview of fibroblast extraction by enzymatic digestion. The process involves dissociation of tail dermal layer from cartilage and skeletal muscle, digestion of dermis to epidermis links (basement membrane) and further digestion of dermis in collagenase to release cells from the matrix. Detached cells can be filtered, washed and transferred to cell culture conditions.
2.7.4. Counting and seeding cells

As is standard for in vitro based assays using cells, cell counts are imperative in the generation of reliable data. Briefly, cultured cells were detached using trypsin (0.05% trypsin, 0.01M EDTA) and resuspended in equal volumes of trypan blue. Cell counts were performed on 1/400 mm haemocytometer and viability assessed by trypan blue exclusion. Average viable cell number (clear) in 4 large squares (1mm$^2$) was used to estimate cell number within a given suspension based on the following formulae:

\[
\text{Cell no.} = \frac{x}{\text{Sq} \times 10^4 \times D \times V}
\]

Where:
- \(x\) = number of cells
- \(\text{Sq}\) = number of large squares counted
- \(D\) = dilution factor
- \(V\) = Volume (ml)

To achieve equal cell density when comparing two groups of cells (i.e. knockout vs wild type) a dilution of the higher cell density group was performed. The dilution volume calculated using the following equations

\[
V_2 = \frac{C_1 \times V_1}{C_2}
\]

\[
V_d = V_1 - V_2
\]

Where:
- \(C_1\) = lower cell no.
- \(V_1\) = Volume with lower cell no
- \(C_2\) = higher cell no
- \(V_2\) = Volume of suspension that contains equal cell number as \(C_1\)
- \(V_d\) = Volume of medium required for dilution
2.7.5. **Pre-treatment of cells before experiments**

To reduce differences due to variation in cell proliferation and cell cycle, cells were serum starved before any experimental process following previously described methods (Suri et al., 1998, Sumiyoshi et al., 2003, Oberringer et al., 2008). Briefly, cells were washed once with sterile PBS and incubated with low serum medium (DMEM 1-2% FCS) for 4 to 6 hours then washed and treated according to the experimental condition to be tested (e.g hypoxia and TGFβ incubations). Please refer to method sections in experimental chapters (4 and 5) for specific conditions used.

2.8. **MDF/HDF scratch-wound assays**

MDF or HDF monolayer migration assays were performed by scratching plated cells to mimic cell migration during wound repair. *In vitro* scratch assay has been widely used and were prepared as previously described with minor changes (Oberringer et al., 2008). Briefly, this involved counting and then evenly seeding cells at equal density (see section 2.7.4) and allowing them to grow until close to full confluence (about 80-90%). Cells were then serum starved (see section 2.7.5), exposed to the desired culture conditions, the scratch wound made with a 1ml pipette tip and then allowed to migrate over time. Scratched areas were measured over time and migration expressed as change in area of the scratch.

2.9. **Fibroblast-populated collagen lattice contraction assays**

Fibroblast can differentiate to express contractile actin isoform (αSMA) which then generates the force required for contraction during wound closure (Darby et al., 1990, Gabbiani et al., 1971). Several growth factors can influence fibroblast differentiation but the main regulator of myofibroblast phenotype is TGFβ1 (Desmouliere et al., 1993). In order to investigate changes in function of this contractile phenotype, fibroblasts were seeded into 3 dimensional collagen lattices as previously described with minor changes (Sumiyoshi et al., 2003, Masterson et al., 2004, Montesano and Orci, 1988). Briefly, cells
were detached with trypsin, counted and diluted in collagen (2.1mg/ml) to approximately 1 x 10^5 cell/ml. Lattices were prepared on ice using the following volumetric equations provided in manufacturer’s instructions with minor changes (Gibco-Invitrogen, Aus).

\[
V1 = \frac{(\text{Final conc. of collagen}) \times (\text{Total Volume} \ (V))}{\text{Initial conc. of collagen}}
\]

\[
V2 = \frac{\text{Total volume} \ (V)}{10}
\]

\[
V3 = V1 \times 0.025
\]

\[
V4 = (V) - (V1 + V2 + V3)
\]

*Where:*

\[
V = \text{Total required volume of collagen lattice}
\]

\[
V1 = \text{Volume of collagen required}
\]

\[
V2 = \text{Volume of 10X DMEM required}
\]

\[
V3 = \text{Volume of 1M NaOH required}
\]

\[
V4 = \text{Volume of cell suspension required to give desired cell density}
\]

Reagents were mixed under sterile conditions. 1 ml aliquots per lattice were added to 12 well plates. Lattices were then allowed to polymerize in standard cell culture conditions (37°C, 5% CO₂, 21% O₂, humidified) for 30 minute after which growth medium was added to wells. Cells were allowed to recover and grown in lattices for 1 day during which time some tension develops in the lattice. Lattices were then subjected to desired experimental conditions, released from the edge and bottom of the well using a needle. Lattices were returned to experimental conditions and allowed to contract for 24 hours.
after which they were fixed in formalin (10% buffered formalin). Plates were scanned and area of lattices calculated to scale. Contraction was defined the change in area (pixels$^2$) before (0 hour) and after lattice release (48 hours).
Figure 2-4. **Schematic of collagen contraction lattice protocol.** Cells seeded at equal density into lattices which are then allowed to polymerize and gain tension, after which they are incubated in experimental conditions for varying times and then detached (released) from well walls and allowed to contract.
2.10. Protein extraction, SDS gel electrophoresis and Western blotting

To investigate changes in protein expression between groups, protein from cells was extracted by detergent (Triton-x100) cell lysis, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and transferred onto polyvinylidene fluoride (PVDF) or nitrocellulose membranes and then probed for specific proteins using primary antibody as per standard SDS-page protocol and manufacturer’s recommendations.

2.10.1. Cell lysis and protein content determination

Briefly, after experimental treatments, cells were washed in Tris-buffer (TBS) (150mM NaCl, 10mM Tris-HCl) and lysed using ice cold lysis buffer solution (1% Triton-x100 and proteinase inhibitor diluted in TBS). Lysates were centrifuged at 12000x g for 10 minutes at 4°C. Supernatants were collected into separate tubes and the protein content (mg/ml) measured using a bicinchoninic acid assay (BCA assay) kit (Bio-Rad, Aus). Absorbance values of the colorimetric reactions were determined using a micro plate reader. Unknown protein values were extrapolated from the equation of a bovine serum albumin standard curve (standard concentrations were 2, 1, 0.5, 0.25 and 0.125 μg/μl).

Equation:
\[ Y = MX + C \]

Where:
- \( Y \) = absorbance value of protein
- \( X \) = protein value in μg/μl or mg/ml
- \( M \) = gradient or slope
- \( C \) = Y intercept
2.10.2. Protein sample preparation

Unless otherwise stated, proteins were loaded at a normalised mass of 10µg per well. The final protein samples comprised of 3 parts; cell lysate (10µg), 1 part loading buffer (4x Invitrogen, Aus) and reducing agent (0.1mM dithiothreitol). The final volume of the protein sample loaded (VL) was determined by following formula:

\[ VL = VS + SB + R \]

Where:

\( VL \) = final loading sample preparation volume per well
\( VS \) = volume protein sample containing desired protein mass (10µg)
\( SB \) = volume 4X sample buffer required (1/4 of protein volume)
\( R \) = volume of reducing agent (1M DTT) required to give final concentration of 0.1mM DTT

Protein samples were denatured at 90°C for 10 minutes and cooled on ice before loading.

2.10.3. SDS PAGE and PVDF/ nitrocellulose membrane transfer

Denatured protein samples were loaded on SDS PAGE gels (10% acrylamide) and separated at 160 to 200 Volts direct current for 45 to 50 minutes in ice-cold running buffer (0.025M Tris glycine and 0.1% SDS). Proteins were transferred on to either PVDF (Bio-Rad, Aus) or nitrocellulose (Bio-Rad, Aus) membranes that were electrolyte soaked (0.025M Tris & glycine and 20% methanol) using a semi-dry transfer cell (Bio-Rad laboratories, USA) at 19 volts direct current for 75 minutes. Protein transfer was confirmed by aqueous protein staining solution (0.05 % Ponceau red S).

2.10.4. Immunoblotting and protein detection

Non-specific protein binding sites on membranes were blocked (60 minutes in 5% non fat powdered milk diluted in 0.01M Tris Base Saline buffer, 0.05% (v/v) Tween 20, pH 7.6 (TBST)). Primary antibodies (diluted in TBST) (see Table 2-2 and Chapter 4) were added
onto the membrane and incubated overnight (about 16 hours) at 4°C. Membranes were washed in TBST for 30 minutes. HRP-conjugated secondary antibodies (diluted 1:1000 in TBST) were added to membranes for 60 minutes at room temperature and washed off thoroughly in TBST. Protein bands on membranes were visualised via peroxidase-catalysed chemiluminescence reaction detected using a Chem-Doc® unit (Bio-Rad, Aus). Protein band density was analysed using Quantity One® densitometry analysis software.

To control for errors in loading, β-actin was used as a housekeeping protein, detected using anti-β-actin antibody (Sigma, Aus). All protein expression values were collected as a ratio of the housekeeper optical density, except for phosphor-Smad2 which was expressed as the ratio of total Smad2 (see Chapter 4).

### 2.11. Flow Cytometry

Flow cytometry analysis (FAC) technique was used to distinguish multiple parameters of individual cells or cell populations in samples based on the scatter of light passed through individual cells, as previously described with minor changes (Wang et al., 2008b). Briefly, the forward scatter was used to generate data about the size of the cells while the side scatter was used to generate information about the internal components of the cells, for example granularity. In combination with appropriate excitation and emission filters, FAC was used to detect fluorescence intensity in a specific population of cells using side and forward scatter analysis. Thus in this thesis the technique was adopted for immunophenotyping, the use of fluorescently-labelled antibodies to determine differences in expression of specific proteins within cell samples. The difference in the expression of Smad2, phosphor-Smad2, PCNA, αSMA, COL1, Siah2, TGFRI and HIF-1α in dermal fibroblasts after specific treatments was analysed by this technique (see Chapters 3 and 4).
2.11.1. Sample preparation

Briefly, cells were counted, starved and incubated in the desired experimental conditions (see section 2.7.4 to 2.7.5). After which cells for FAC were enzymatically detached using trypsin (0.05% trypsin, 2% BSA in PBS), washed and centrifuged (300x $g$ for 5 minutes). Cells were fixed with formalin (2% formalin, 2% BSA in PBS) for 20 mins and permeabilized by 20 mins incubation in saponin (0.05% saponin, 2% BSA in PBS). Cells were pelleted by centrifugation at 300x $g$ for 5 minutes and the supernatant discarded.

2.11.2. Immuno-labelling for FAC

Cells were resuspended in the primary antibody (diluted in 2% BSA in PBS) of interest and incubated at room temperature for 30 minutes. Primary antibodies were washed off with PBS. Cells were centrifuged at 300x $g$, PBS was then discarded and cells were resuspended in the appropriate secondary antibody (diluted in 2% BSA in PBS) and incubated for 30 minutes at room temperature. Cells were washed in PBS and centrifuged at 300x $g$ (washing steps repeated twice). Cells were then resuspended in PBS containing 2% BSA. Negative controls (incubation in secondary antibody only) and unstained cells were prepared for each experimental condition.

2.11.3. FAC of samples and data collection

Cells were collected at a high flow rate (up to 70 cells per second). Forward and side scatter characteristics (i.e size and granularity) of cells were used for their preliminary identification and appropriate gates were further used to localise cells and exclude cellular aggregates and debris. Fluorescence intensity of negative controls and stained samples were analysed for at least 2,000-10,000 gated events per sample. Mean fluorescence intensity of samples was analysed using Weasel ™ FAC software (Walter and Eliza Hall Institute, Aus). Mean fluorescence intensity of negative controls (secondary antibody labelled only) was used to subtract background fluorescence of test samples.
2.12. Human dermal fibroblast - Siah protein inhibition model

Briefly, transient transfection of HDFs with a Phyllopod expressing vector was used to investigate the effect of inhibiting Siah proteins in an in vitro human fibroblast model. Phyllopod (PHYL) is a drosophila melanogaster protein which has inhibitory effects on both Siah 1 and 2 activity (Moller et al., 2008). PHYL expressing vectors have been successfully transfected in mammalian cells and demonstrated inhibition of Siah expression and of proteins downstream of the Siah (and HIF1) pathway (Moller et al., 2008). PHYL expression vectors were introduced into cells to compare and validate the effects of Siah2 gene deletion on mouse primary fibroblast cultures (see Chapter 3), with the major difference that PHYL inhibits both Siah1 and Siah2. The PHYL expression vector was constructed and kindly donated by Andreas Moller (Moller et al., 2008), a collaborator at Peter MacCallum Cancer Centre. Transient transfection of HDF cells with PHYL was performed using previously described protocols and following the manufacturer’s transfection guidelines with minor modifications (Tandara et al., 2006) (Invitrogen, USA).

2.12.1. Generation of competent E.coli

E.coli colonies were grown in fast shaking Luria broth (LB) cultured overnight at 37°C. Colonies were used to inoculate 100-fold dilution of LB containing MgSO$_4$ (20mM) and grown at 37°C for 5 to 6 hours. Cells were immediately chilled and centrifuged. The pellet was resuspended in ice cold calcium chloride (CaCl$_2$) (0.1M) and swirled on ice (30mins). Cells were centrifuged and resuspended in CaCl$_2$/ Glycerol (0.1M /15%v/v).

2.12.2. Transformation of competent E.coli

Plasmid complementary dinucleotide acids (pcDNA) (10ng) was added to competent cells and mixed gently before heat shocking at (42°C) for 40 to 60 seconds. Cells were
returned to ice for 2 mins before addition of 3ml of LB medium without antibiotic and then incubated at 37°C for 45 minutes.

2.12.3. Selection and growth of Transformed E.coli

To select for transformed cells, aliquots of cells were streaked onto antibiotic (100μg/ml Ampicillin) containing LB plates and incubated overnight at 37°C. Ampicillin resistant E.coli colonies were selected by wire loop and then grown in liquid cultures of LB containing selection antibiotic overnight at 37°C with agitation.

2.12.4. Purification of cDNA Plasmid

Plasmids were purified using Midi and Maxi Pure Link ™ prep kits (Invitrogen, Aus) as per manufacturer’s guidelines. Briefly, cells were lysed and supernatants washed and filtered through a series of column filters. Plasmid quality and quantity was determined using a UV spectrophotometer in line with manufactures guidelines (Invitrogen, Aus). Quality of pcDNA was determined by comparing absorbance of plasmid preps at 260 and 280 nm. High quality preps had an absorbance ratio (260/280nm) between 2.0 to 1.8. Concentration of pcDNA was determined using the following equation:

\[ X = A_{260} \times 50\mu l/ml \]

Where:

\[ X = \text{pcDNA concentration} \]

\[ A_{260} = \text{Absorbance of pcDNA at 260nm} \]

\[ 50\mu l/ml = \text{Concentration of double stranded DNA when } A_{260} \text{ equals 1.0} \]
2.12.5. Lipofectamine 2000™ Transfection

Transient transfection of HDF cells was performed using Lipofectamine 2000™ with reference to the previously described methods (Tandara et al., 2006) and manufacturer’s instructions albeit with slight modifications. HDFs were grown to about 70 to 80% confluence. Cells were incubated for 5 to 6 hours in transfection solution containing Lipofectamine 2000™ (1.0 µl/cm²) and plasmid (0.35 µg/cm²) diluted in low serum medium. Following transfection, cells were washed with PBS and allowed to recover under normal cell culture conditions for 24 hours. The cells were then passaged (1 in 4 to 5) and allowed to grow under normal cell culture conditions until about 70% confluent (about 3 days post-transfection). Please refer to Chapter 5 for specific experimental procedures performed post-transfection.

2.12.6. Transfection efficiency

Positive transfection was determined by analysis of HA-expression using a rabbit polyclonal Anti-HA antibody (Abcam, USA) as previously described (Moller et al., 2008). Briefly, Phyllopod expression vectors contained a HA incorporating sequence that allowed Phyllopod proteins to be tagged when expressed in transfected cells (Moller et al., 2008). After the described transfection time, cells were fixed and permeabilized by addition of 100% methanol for 10 minutes and then hydrated. Primary anti-HA antibody (1:200) was added and incubated for 30 minutes in line with manufacturer’s guidelines (Abcam, USA). Secondary antibody, Anti-rabbit IgG Alexa-Fluora ®488 (1:300, Molecular Probes, Invitrogen) was used to probe primary IgG. Images of protein expression were taken using an Olympus BX-50 fluorescent microscope and compared to that of empty or scrambled vector-transfected cells.
2.13. Vascular endothelial growth factor (VEGF) ELISA

Dermal fibroblasts increase expression of VEGF in hypoxia conditions (Steinbrech et al., 1999). VEGF is a well described downstream target of hypoxia through HIF1 inducible pathways (Semenza, 1999, Forsythe et al., 1996) and therefore is also a downstream target of Siah activity (Moller et al., 2008, Nakayama et al., 2004, Shah et al., 2009). To investigate the impact of Siah inhibition on fibroblast release of VEGF, a VEGF ELISA was used to measure HDF VEGF secretion. VEGF ELISA was performed according to manufacturer’s instructions (PeproTech, USA). Briefly, anti-VEGF capture antibody (0.2µg/ml anti-VEGF in PBS with 2% BSA in) was coated onto a high-binding 96-well plate for 24 hours and washed off thoroughly. Non-specific binding was blocked with bovine serum albumin (2% BSA in PBS). Samples were bound to capture VEGF for 2 hours then washed off thoroughly. Bound samples were then probed with biotinylated anti-VEGF (0.2µg/ml) for 2 hrs and again washed thoroughly. Avidin conjugated with HRP (1: 2000 dilution in PBS with 2% BSA)) was added to detect the secondary antibody and the excess washed off. Absorbance readings of colorimetric reactions of TMB (3, 3’, 5, 5’-tetramethyl-benzidine) catalysed by HRP (Clear to Blue) were used to measure VEGF concentration using a plate reader. The amount of VEGF captured from samples was calculated using a VEGF standard curve with concentrations from 0.06 to 2ng/ml.

2.14. Statistical analysis

Statistical analysis was performed using two way analysis of variance (ANOVA) and Bonferroni’s correction for multiple comparisons. In addition, Mann-Whitney’s nonparametric test (U-test) was performed for planned comparisons. Results were expressed as the mean ± standard error of the mean (S.E.M) for atleast 3-10 separate observations. A P value of less than 0.05 was considered statistically significant (*).
Chapter 3

Deficiency of Siah2 and its effect on murine cutaneous wound healing
3.1. Introduction

Wound healing in the skin is a complex and well orchestrated process involving the interaction of numerous cell types as healing progresses through phases involving inflammation, proliferation and remodelling (Clark, 1996). Infiltration by inflammatory cells debrides the wound of damaged tissue, minimises microorganism accumulation and initiates the beginning of the proliferative phase through the release of soluble factors that are chemotactic for mesenchymal cells, in particular keratinocytes, endothelial cells and fibroblasts (Leibovich and Ross, 1975, Mirza et al., 2009). The proliferative phase seals the wound site through rapid re-epithelialisation by keratinocytes from the wound edge and restores tissue architecture through the deposition of extracellular matrix by fibroblasts, while simultaneously restoring vasculature through endothelial cell proliferation and differentiation (Singer and Clark, 1999). In adult skin, the generation of granulation tissue and its eventual remodelling as wound maturation occurs, results in a scar with reduced functionality compared to the original tissue. Impairment of any of these processes results in delayed or aberrant healing. Abnormal wounds clinically present mostly as fibroproliferative lesions and chronic ischaemic wounds and are a significant burden to patients and healthcare systems (Sen et al., 2009). Due to loss of function associated with scarred tissue and the difficulty in healing chronic wounds, there is considerable interest in the factors involved in the regulation of normal healing.

Following injury, vascular damage often occurs resulting in hypoxia which may be further sustained by the rapid influx of inflammatory and mesenchymal cells with high metabolic demands for oxygen (Remensnyder and Majno, 1968). Detection of hypoxia during wound healing has been demonstrated convincingly both immunochemically and by direct measurement of oxygen pressure (Lokmic et al., 2006, Haroon et al., 2000, Knighton et al., 1983). Acute hypoxic tissue gradients are important for stimulating adequate wound healing angiogenesis as well as enhanced matrix
deposition and re-epithelialisation (Knighton et al., 1981, O'Toole et al., 1997, Ridgway et al., 2005, Siddiqui et al., 1996). Slight changes in systemic and cellular oxygen concentrations induce tightly regulated response pathways that modulate cell function in hypoxic conditions. Most of these responses occur through the induction of the HIF-1 transcription factor which is increased during wound healing and regulates many processes needed to carry out tissue repair processes during wound ischaemia. HIF-1 transcriptionally up-regulates expression of metabolic proteins (Glut-1), adhesion proteins (integrins), soluble factors (PAI-1, thrombospondin-1, CTGF, TGFβ, and VEGF) and matrix components (collagens and fibronectin) which enhances the healing process (Elson et al., 2000, Zhang et al., 2003b, Distler et al., 2007, Higgins et al., 2004). For these reasons, HIF-1 is viewed as a positive regulator of wound healing and when over-expressed, it has been hypothesised to be an important factor in tissue fibrosis. The relevance of HIF-1 is effectively demonstrated in numerous in vitro systems and in HIF-1 deficient animal models which are shown to have impaired wound healing (Distler et al., 2007, Tang et al., 2004, Zhang et al., 2010b). Furthermore, HIF-1 deficiency has been linked to defective hypoxic responses in diabetic- and age- related chronic wounds where healing is impaired (Botusan et al., 2008, Distler et al., 2007, Liu et al., 2008, Loh et al., 2009, Thangarajah et al., 2009).

HIF-1 is negatively- and positively-regulated by several proteins. Briefly, HIF-1 is a heterodimer of alpha and beta subunits (Wang et al., 1995). Both subunits are constitutively expressed, however tight regulation of HIF-1 activity occurs primarily through post-translational modification of HIF-1α subunit (Wang et al., 1995). Under normal physiological oxygen HIF-1α is tagged for proteosomal degradation by pVHL, a process that requires HIF-1α hydroxylation by HIF-hydroxylases (PHDs and FIH) and oxygen co-activator (Kamura et al., 2000, Bruick and McKnight, 2001, Mahon et al., 2001). In hypoxia conditions HIF-hydroxylases are reduced which allows HIF-1α to escape ubiquitination machinery thus increasing its abundance (Bruick and McKnight, 2001, Mahon et al., 2001).
Siah proteins are a family of E3 ubiquitin ligases that tag HIF-hydroxylases for degradation during hypoxia and increase HIF-1α abundance (Nakayama et al., 2004, Fukuba et al., 2007). In this context, Siah knockout mice have delayed and abrogated responses to hypoxic conditions (Nakayama et al., 2004). The importance of Siah induced degradation of PHD in the regulation of HIF-1, has been well defined in hypoxia driven pathological conditions such as tumourgenesis and metastasis (Shah et al., 2009, Qi et al., 2008, Moller et al., 2008). However, the role of Siah in wound healing has not been previously studied. We hypothesise that Siah, through its regulatory effects on PHD activity and thus downstream HIF-1α availability, will have an effect on early wound healing, where hypoxia is prevalent requiring adaptive response through HIF-1 for cellular responses to hypoxia. The aims of this chapter are to cutaneously wound Siah2 homozygous knockout mice (Siah2−/−) mice and to compare their early wound healing events to that of matched Siah2 wildtype mice (Wt).

3.2. Results

3.2.1. Macroscopic and histological wound size and re-epithelialisation

To evaluate the physiological role of endogenous Siah2 in wound healing, Siah2−/− and Wt mice were subjected to full thickness excisional wounds on shaved dorsal skin using a 5mm punch biopsy. The wounds were allowed to heal by secondary intention then collected for histological and immunochemical analysis with particular focus on days 6 and 9, where hypoxic wound conditions have previously been described (Lokmic et al., 2006, Haroon et al., 2000). Figure 3-1 shows a series of typical macroscopic and histological images from excisional wounds of Wt versus Siah2−/− mice. Macroscopic wound closure of Siah2−/− mice is delayed and wounds appeared to be gaping and raw at day 4 and 6 compared to Wt wounds. This macroscopic difference was indistinguishable from the Wt mice at day 9 (Figure 3-1a). The macroscopic observations were also supported with histological analysis of granulation tissue area (mm²) (Figure 3-1b and
Figure 3-1c). Wound areas in Siah2$^{-/-}$ mice were significantly larger at days 6 (5.30 ± 0.83mm$^2$, P<0.001, n=10) and 9 (2.97 ± 0.28mm$^2$, P<0.01, n=8) in comparison to Wt mice at days 6 (2.17 ± 0.23mm$^2$, n=10) and 9 (0.76 ± 0.13mm$^2$, n=8) (Figure 3-1c). Although wounds in Siah2$^{-/-}$ were still larger at day 12, they had substantially contracted and were not significantly different (1.87 ± 0.17 mm$^2$, P = 0.70, n=3) to Wt mice (1.07 ± 0.33 mm$^2$, n=3).

The percentage of re-epithelialisation (% re-epithelialisation) was also analysed histologically by measuring the distance covered by re-epithelising sheet compared to diameter of wound (Figure 3-1d). At day 6, % re-epithelialisation in Siah2$^{-/-}$ wounds was significantly delayed (66.33 ± 9.28%, P<0.001, n=10) compared to Wt mice (99.18 ± 0.82%, n=10). % re-epithelialisation at day 9 was mostly complete and undistinguishable between Siah2$^{-/-}$ (85.6 ± 5.92%, P = 0.506, n=8) and Wt wounds (91.03 ± 6.06%, n=8). Siah2$^{-/-}$ wounds at day 6 also appeared to have more residual provisional matrix (fibrin clot) compared to Wt mice in the central regions of the wounds indicative of delayed debridement or invasion and replacement with granulation matrix (Figure 3.1b). By day 9 normal granulation features appeared to be formed in Siah2$^{-/-}$ but not Wt wounds. No overt signs of infection or chronic inflammation were observed in any of the analysed wounds. These results suggest that Siah2 plays an important role in promoting wound closure and re-epithelialisation.
Chapter 3
Figure 3-1. Macroscopic and histological analysis of wound closure in Siah2⁻/⁻ and Wt mice. (a) Qualitative macroscopic comparison of Siah2⁻/⁻ and Wt wounds at day 0, 4, 6 and 9 post injury (b) H&E sections of Wt and Siah2⁻/⁻ wounds at 6, 9 and 12 days (c) histological analysis of average wound area (mm²) and (d) percentage (%) re-epithelialisation calculated using these H&E sections. Key: F=Fibrin clot, E=epithelial layer, D=unwounded dermis, G=newly formed granulation tissue. Each data point represents a mean ± SEM (day 6 n=10, day 9 n=8 and day 12; n=3). Mann-Whitney’s U-test analysis was used to determine statistical significance. *** P<0.001, ** P<0.01. (Macroscopic of images, (a) 1 small bar =1 mm) (Magnification, (b) = x4)

3.2.2. Delayed collagen deposition in Siah2⁻/⁻ wounds

Replacement of provisional matrix (fibronectin rich fibrin) with collagenous matrix is critical to the establishment of granulation tissue and restoration of tissue integrity during wound healing. To investigate the impact of Siah2 deficiency on collagen deposition, sections were stained with picrosirius red and analysed under polarized light (Figure 3-2) which shows mature and immature collagen fibres. As shown in Figure 3-2a,c newly synthesised granulation collagen fibres appeared fine and demonstrated a weak green-yellow birefringence, while increasingly mature granulation tissue collagen fibres were thicker and demonstrated a bright orange-yellowish birefringence (Constantine and Mowry, 1968). Areas lacking in collagen deposition appeared dark-grey (without birefringence). At day 6, Siah2⁻/⁻ granulation tissue contained deposits of fine collagen fibres (green-yellow) in peripheral regions of the granulation tissue and reduced collagen deposition (dark-grey) in central regions of the granulation tissue while Wt wounds demonstrated homogenous deposition of fine collagen fibres (green-yellow birefringence) throughout the granulation tissue (Figure 3-2 a, c). By day 9, Siah2⁻/⁻ wounds appeared to have matured collagen fibres (yellow birefringence) in the peripheral regions of the granulation tissue and some areas of fine collagen fibres in the central areas, while Wt wounds appeared to have a homogenous distribution of mature collagen fibres (yellow birefringence) throughout the granulation tissue (Figure 3-2 b).
The percentage of picrosirius red birefringence was analysed (Figure 3.2d). At day 6 Siah2\(-/-\) wounds had significantly less collagen in the granulation tissue (30.73 ± 4.46%, p<0.01, n=10) compared to Wt wounds (54.75 ± 5.83%, n=10). At day 9, the percentage collagen deposited in Siah2\(-/-\) granulation tissue was still less (68.5 ± 5.39%, p = 0.165, n=8) but not significantly different to Wt wounds (80.2 ± 4.16%, n=8). These results suggest that the downstream effects of Siah2 are important in enhancing deposition of collagenous matrix during normal wound healing.
Figure 3-2 Picrosirius red staining and analysis of collagen deposition in Siah2/− and Wt wounds. (a)(c) Birefringence of picrosirius red in Wt and Siah2/− sections at day 6 and (b) day 9 under polarised light (areas of low birefringence are indicated by white arrows). Analysis of % picrosirius red stain in Siah2/− sections at day 6 and 9 compared to Wt (d). Each data point represents a mean ± SEM (day 6, n=10; day 9, n=8). Mann-Whitney’s U-test analysis was used to determine statistical significance. ** P<0.01. Magnification, (a), (b) = x4 and (c) = x20)
3.2.3. Proliferation of cells in granulation tissue of Siah2\(^{-/-}\) wounds

Increased proliferation and rapid migration of mesenchymal cells, from unwounded skin to replace damaged tissue, is a hallmark of wound repair and regeneration. To examine the impact of Siah2 on cell proliferation, sections were immunostained with an antibody directed against PCNA. Figure 3-3a shows typical PCNA stained sections of 6, 9 and 12 day wounds from Wt and Siah2\(^{-/-}\) mice. During the earlier phases of wound healing (day 6) PCNA stained cells in Siah2\(^{-/-}\) and Wt wounds were localised on re-epithelialising basal keratinocytes and the peripheral regions of the wounds (wound margins). Mesenchymal cells also appeared to be proliferating and migrating from the unwounded dermis. By day 9 and 12 the proliferating cells were detectable almost homogeneously throughout the granulation tissue of both Siah2\(^{-/-}\) and Wt wounds. Analysis of the percentage area of PCNA staining (Figure 3-3b) showed that Siah2\(^{-/-}\) (3.25 ± 0.24%, n=10) and Wt (3.56 ± 0.35%, n=10) wounds had similar levels of proliferation during the early phases of healing at day 6. Surprisingly, at day 9 the percentage of PCNA positive cells was significantly higher in Siah2\(^{-/-}\) wounds (5.37 ± 0.33%, P<0.05, n=8) in comparison to the Wt wounds (4.02 ± 0.36%, n=8) indicating a slight increase in the rate of proliferation. At day 12 Siah2\(^{-/-}\) wounds (2.89 ± 0.437 %, n=3) had similar levels of PCNA positive cells in comparison to the Wt wounds (3.05 ± 0.82%, n=3). The results indicate that Siah2 does not appear to impact overall cell proliferation during the early phase of wound healing but might instead modulate cell migration. Surprisingly, the absence of Siah2 causes a transient increase in the rate of proliferation, seen at day 9 post-injury, which may be suggestive of a compensatory mechanism by which impairment of cell migration is overcome.
Figure 3-3. Cell proliferation activity in wounds of Siah2−/− and Wt wounds assessed by immunohistochemistry using PCNA. (a) PCNA immunostained wound sections at day 6, 9 and 12 and (b) analysis of percentage (%) PCNA staining in wounds sections of Siah2−/− compared to Wt mice. Each data point represents a mean ± SEM (day 6, n=10; day 9, n=8 and day 12, n=3). Mann-Whitney’s U-test analysis was used to determine statistical significance. * P<0.05. Magnification, (a) = ×10. Scale, 1 bar =100 µm. Annotation: black arrows indicate wound edge, E= epithelial layer, G= granulation tissue, L= leading epithelial edge and P= provisional matrix.
3.2.4. Reduced macrophage infiltration into Siah2<sup>-/-</sup> wounds

In the inflammatory phase of wound healing, macrophages play a vital role in infection control, tissue debridement and initiation of the proliferative phase through the release of paracrine factors (Mirza et al., 2009). Hypoxia induces macrophage invasion, activation and the release of cytokine release (Hempel et al., 1996, Scannell, 1996, Albina et al., 1995). HIF-1 stabilisation is critical for myeloid cell mediated inflammation and function during tissue hypoxia (Cramer et al., 2003). To determine the role of Siah2 on macrophage invasion, sections were immunostained with anti-F4/80 antibody. Figure 3-4a shows typical sections of F4/80 immunostained Wt and Siah2<sup>-/-</sup> wounds at day 6 and 9 post-injury. In the earlier phases of healing at day 6, F4/80 positive cells in Siah2<sup>-/-</sup> wounds were mostly present in the peripheral regions of the granulation tissue. In contrast, F4/80 positive cells in Wt wounds appeared to be homogeneously distributed throughout the granulation tissue. At day 9, F4/80 positive cells in Siah2<sup>-/-</sup> wounds were increasingly detectable in central and peripheral areas of the granulation tissue but appeared to be sparse in the central regions compared to Wt. Analysis of percentage F4/80 staining in the granulation tissue was performed using). At day 6, Siah2<sup>-/-</sup> wounds had a significantly lower percentage of F4/80 staining (1.98 ± 0.35%, P<0.01, n=10) than the Wt wounds (4.51 ± 0.82%, n=10) (Figure 3-4b). At day 9, Siah2<sup>-/-</sup> wounds continued to have a significantly lower percentage of F4/80 positive cells (2.15 ± 0.18%, P<0.01, n=8) than the Wt wounds (3.09 ± 0.18%, n=8). The results demonstrate the importance of Siah2 and HIF-1 in regulating wound inflammatory phase. Regulation of macrophage infiltration may have important implications on granulation tissue levels of macrophage derived soluble factors (cytokines and growth factors) that impact on proliferative processes such as re-epithelialisation, matrix synthesis and angiogenesis.
Figure 3-4 Macrophage infiltration of Siah2\(^{-/-}\) and Wt wounds. (a) F4/80 immunostained sections of wounds at day 6 and 9 and (b) analysis of percentage F4/80 staining in the granulation tissue of Siah2\(^{-/-}\) compared to Wt wounds. Mann-Whitney’s \(U\)-test analysis was used to determine statistical significance. ** \(P<0.01\). Magnification, (a) = x10
3.2.5. Myofibroblast profile and wound contraction in Siah2\(^{-/-}\) wounds

Transient expression of αSMA in the granulation tissue during fibroblast-myofibroblast transition is a hallmark of normal wound healing and is synonymous with wound contraction (Darby et al., 1990). The presence of myofibroblasts is also associated with increased production of collagen (Schmid et al., 1998). To investigate the effect of Siah2 on fibroblast to myofibroblast transition wound sections were immunostained with anti-αSMA for immunohistochemistry. Figure 3-5 shows typical images of αSMA immunostained Wt and Siah2\(^{-/-}\) wounds at day 6 and 9 post-injury. At day 6, Siah2\(^{-/-}\) wounds had a smaller peripheral area of αSMA positive cells and fewer αSMA positive cells leading into the central regions of the granulation tissue compared to Wt. At day 9, αSMA stained cells in granulation tissue of Siah2\(^{-/-}\) were less pronounced in the central area and mostly concentrated in peripheral areas compared to Wt wounds. Analysis of the percentage area of αSMA in granulation tissue was performed using image analysis software. At day 6, Siah2\(^{-/-}\) wounds had significantly less αSMA positive cells (14.8 ± 2.87%, P<0.001, n=10) in comparison to Wt wounds (36.5 ± 2.61%, n=10) (Figure 3.5e). αSMA levels in Siah2\(^{-/-}\) wounds remained significantly lower up to day 9 albeit only marginally (34.4 ± 5.27%, P<0.05, n=8) when compared to Wt wounds (52.9 ± 4.54%, n=8). The results suggest that the downstream effects of Siah2 activity are important for normal functionality of fibroblast during wound healing including matrix synthesis, differentiation and contraction.
Figure 3-5  Presence of myofibroblasts in Siah2−/− and Wt wounds. (a-d) αSMA stained and scanned sections of wounds at day 6 and 9 and (e) analysis of percentage α-SMA in the granulation tissue of Siah2−/− wounds compared to Wt wounds. Each data point represents a mean ± SEM (day 6 n=10, day 9 n=8). Mann-Whitney’s U-test analysis was used to determine statistical significance. *** P<0.001, * P<0.05
3.2.6. Vessel distribution in Siah2⁻/⁻ wounds

Angiogenesis in a critical physiological process in wound healing that involves growth of new vessels from damaged pre-existing vessels and possibly from circulating precursor cells. Blood vessels function as conduits for the delivery of oxygen and nutrients to the repairing tissue. To investigate the impact of Siah2 deficiency on angiogenesis, vessels were counted and vascularity (vessels counts/area) analysed in Siah2⁻/⁻ and Wt wounds. CD31 immunostaining was used to localise vessels in wound sections and gain a qualitative view of vessel distribution. However, due to difficulties associated with non-specific staining on paraffin embedded sections, CD31 immunostaining was only used to generate representative images of wound angiogenesis. H&E stained sections were instead used to count vessels and analyse vascularity. Additionally, CD31 staining and in fact other vessel markers tested, tended to have heterogeneous staining patterns with some micro-vessels detected and some not and for this reason, vessel profiles were counted. Overall granulation tissue vascularity (vessels counts/area) was analysed using H&E stained sections (Figure 3-6e). At both day 6 (0.29 ± 0.03 counts/μm², P<0.05, n=10) and 9 (0.23 ± 0.03 counts/μm², P<0.05, n=8) post-injury Siah2⁻/⁻ wounds had significantly less vascularity in the granulation tissue in comparison to Wt wounds (0.37 ± 0.02 counts/μm², n=10 and 0.31 ± 0.03 counts/μm², n=8, respectively).

Further analysis of central granulation tissue vascularity was performed (Figure 3-6d). At 6 days post-injury Siah2⁻/⁻ wounds (0.18 ± 0.04 counts/μm², P<0.01, n=10) had significantly less vascular density in central regions of granulation tissue in comparison to the Wt wounds (0.39 ± 0.05 counts/μm², n=10). At 9 days post-injury, Siah2⁻/⁻ wounds (0.12 ± 0.04 counts/μm², P= 0.08, n=8) had less vascularity in the central granulation, albeit not significantly when compared to Wt wounds (0.26 ± 0.06 counts/μm², n=8). The results demonstrate the importance of Siah2 activity in wound healing angiogenesis. These results also support other studies that show a role for Siah2 in regulation of pathological angiogenesis, mostly in cancers (Nakayama et al., 2004, Moller et al., 2008, Qi et al., 2008).
Figure 3-6. Vessel distribution in Siah2$^{-/-}$ and Wt wounds. (a-d) CD31 immunostained and scanned sections of Wt and Siah2$^{-/-}$ wounds at day 6 and 9 are shown (leading edge of newly formed vessels indicated by black arrows and yellow dashed lines). (e) Analysis of vessel counts per area (µm) in whole wounds and (f) wound centres using H&E sections at high magnification (x 20). Each data point represents a mean ± SEM (day 6 n=10, day 9 n=8). Mann-Whitney’s U-test analysis was used to determine statistical significance. ** P<0.01, * P<0.05
3.2.7. Glut-1 expression and HIF-1 transcriptional activity in Siah2\(^{-/-}\) wounds

Glucose transporter-1 has been used as a surrogate marker of hypoxia by virtue of its regulation by HIF-1. As cells become hypoxic and switch to anaerobic respiration for energy, they up-regulate Glut-1 (Young and Moller, 2010). Spatio-temporal expression of HIF-1 and its downstream targets (Glut-1 and PGK-1) have been well described during wound healing (Elson et al., 2000). To confirm the role of Siah2 on HIF-1 transcriptional activity, Glut-1 immunostaining was performed on wound sections, with particular emphasis of day 6 and 9 sections where hypoxia is reported to be prevalent (Lokmic et al., 2006) (Figure 3-7). At day 6 post-injury, Siah2\(^{-/-}\) wounds showed positive but faint Glut-1 staining along the cell membranes of re-epithelialising basal keratinocytes compared to strong staining in Wt keratinocytes (Figure 3-7a). By day 9 post-injury, both Siah2\(^{-/-}\) and Wt wounds had relatively low Glut-1 staining around the membranes of scattered basal keratinocytes (Figure 3-7b) suggesting that hypoxia had reduced by this time. Glut-1 expression is a marker for hypoxia and increased HIF-1-regulated transcriptional activity. Therefore, the results indicate that HIF-1 activity and normal responses to wound hypoxia may be highly dependant on Siah2 activity.
Figure 3-7 Activation of hypoxia induced signalling in Siah2−/− and Wt wounds using Glucose transporter 1 (Glut-1) as a marker. (a) Qualitative Glut-1 immunostained wound sections at day 6 and (b) day 9 post-injury. Magnification, (a) = x20 and (b) = x40. Scale in (a), 1 bar = 100µm.
3.2.8. Apoptosis in Siah2\textsuperscript{\textminus/\textminus} wounds

As the wound closes, myofibroblastic and vascular cells undergo programmed cell death which eventually causes a loss in cellularity and scar maturation (Darby et al., 1990, Desmouliere et al., 1995). To test if Siah2 had any effect on the onset or level of apoptosis, TUNEL staining was performed. Figure 3-8 (a-c) shows typical sections of Siah2\textsuperscript{\textminus/\textminus} and Wt wounds at day 6, 9 and 12 post-injury. In the earlier phases of healing (day 6) TUNEL staining was barely detected in both Siah2\textsuperscript{\textminus/\textminus} and Wt wounds. Apoptotic nuclei were more pronounced in both Siah2\textsuperscript{\textminus/\textminus} and Wt wounds at days 9 and 12 post-injury. The results suggest no obvious effect of Siah2 on the rate apoptosis.
Figure 3-8. Visualisation of apoptotic nuclei in the granulation tissue. (a-c) TUNEL stained Siah2⁻/⁻ and Wt wounds at days 6, 9 and 12. Black arrows show TUNEL stained apoptotic nuclei in the granulation tissue. Magnification of (a), (b) and (c) = x20.
3.3. Discussion

3.3.1. Hypoxia through HIF-1 regulates wound healing

Wound healing consists of three overlapping phases: inflammation, proliferation, and maturation and remodelling (Clark, 1996). These phases are regulated by complex signalling pathways that may be activated or deactivated by an array of soluble factors secreted by inflammatory and mesenchymal cells. These highly orchestrated events may be subject to several abnormalities. These abnormalities can occur at different phases of wound repair and lead to different types of abnormal wound healing with significant burden to the patient and health care system (Darby and Hewitson, 2007, Sen et al., 2009).

Acute tissue hypoxia is a characteristic microenvironment of injured tissues owing to vessel disruption and the high metabolic demands of wound repair cells (Remensnyder and Majno, 1968, Haroon et al., 2000, Lokmic et al., 2006). Low oxygen tension provides a strong biological stimulus for chemotaxis of tissue repair cells particularly inflammatory and mesenchymal cells. In normal healing acute hypoxic gradients are important stimuli for inflammation, re-epithelialisation, matrix synthesis and angiogenesis (Knighton et al., 1981, Knighton et al., 1983, O'Toole et al., 1997, Ridgway et al., 2005, Falanga et al., 2002, Steinbrech et al., 1999). Therefore, abnormal responses to hypoxia present an important mechanism by which wound healing may be impaired or overstimulated.

Cellular adaptive responses to hypoxic conditions are mostly regulated by HIF-1, a master regulator of oxygen homeostasis (Semenza, 1998). HIF-1 directly and indirectly controls the transcription and activation of hundreds of genes that can enhance cell function during low oxygen tension. The relevance of HIF-1 in the wound healing milieu has been demonstrated in several transgenic and pathologically HIF-1-deficient cutaneous wound studies, most of which are characterised by delayed wound healing (Loh et al., 2009, Botusan et al., 2008, Tang et al., 2004, Zhang et al., 2010b).
Additionally, abnormal accumulation of HIF-1 is associated with the pathogenesis of, and predisposition to fibroproliferative disorders (Distler et al., 2007, Zhang et al., 2003b, Wipff et al., 2009). Therefore, targeting HIF-1 and its regulatory factors may have important clinical implications in the treatment and management of abnormal wounds.

HIF-1 transcriptional activity is tightly regulated through several proteins which control post-translational availability of the HIF-1α subunit. Siah proteins are a family of E3 ubiquitin ligases that tag HIF-prolyl hydroxylases for degradation during hypoxia and thus positively regulate HIF-1α availability and the expression of hypoxia inducible genes (Nakayama et al., 2004, Fukuba et al., 2007). The relevance of Siah-HIF-1 axis in hypoxia driven pathophysiology has been demonstrated in several mice cancer models using xenografts including prostate, breast and lung (Ahmed et al., 2008, Moller et al., 2008, Qi et al., 2008). Here, the role of Siah2 in normal healing of an excisional skin wound is described for the first time.

### 3.3.2. Delayed wound healing in Siah2/− mice

Inflammation acts as a protective attempt to remove microbes and injurious debris during wound healing. In addition, inflammatory cells in particular macrophages are widely appreciated as important regulators of cytokines and growth factors during wound healing (Leibovich and Ross, 1975). These released factors are critical for normal wound healing, so much so, that selective and specific ablation of skin macrophages results in severe impairment to multiple phases of adult cutaneous wound healing including re-epithelialisation, angiogenesis, formation of granulation tissue and wound contraction (Mirza et al., 2009). In contrast, an alternative study has reported normal wound healing kinetics using young PU-null mice, which are genetically incapable of raising an immune response due to a lack of macrophage progenitor lineages and functioning neutrophils, and that healing in these mice results in embryonic-like scarless wound repair (Martin et al., 2003). A similar study has yet to be conducted in adult animals where a reliance on immune responses would be more likely.
Macrophages typically accumulate in hypoxic areas, characteristic of most injured tissue and pathological lesions (Lewis et al., 1999, Bosco et al., 2008). Hypoxia is a feature of inflammation and has a profound effect on a several myeloid cells properties including cytokine expression, phagocytosis, cell surface maker expression, migration and cell survival (Lewis et al., 1999). It is now well appreciated that HIF-1 directly regulates genes associated with survival and myeloid cell function in the inflamed microenvironment (Cramer et al., 2003). Importantly, HIF-1 regulates myeloid cell aggregation, metabolism, motility, invasiveness, cytokine release and phagocytic potential during inflammation (Cramer et al., 2003). In cutaneous wound healing, the relevance of HIF-1 in inflammation is demonstrated by considerable delays in myeloid cell infiltration and cytokine release in HIF-1α deficient mouse models (Botusan et al., 2008, Zhang et al., 2010b).

This study provides evidence of a role of the Siah2-HIF-1 axis in macrophage invasion during wound healing. Herein, Siah2−/− mice show reduced wound macrophage numbers particularly at six days post injury when compared to Wt mice, as determined by F4/80 immunostaining (Figure 3-4). The finding is indicative of attenuated macrophage invasion in the absence of Siah2 and correlates to a delayed or abrogated inflammatory response during wound hypoxia. This finding is consistent with the role of HIF-1 in myeloid cell function during tissue inflammation and the positive regulation of HIF-1 availability by Siah2 (Cramer et al., 2003, Zhang et al., 2010b, Nakayama et al., 2004). Macrophages are critical to the debridement of apoptotic cells and damaged tissue, which are important stimuli for the secretion of several important cytokines (TGFβ1 and VEGF) (Peters et al., 2005, Mirza et al., 2009). Therefore secondary to reduced HIF-1 availability, Siah2−/− wounds with reduced macrophages are likely to be deficient in macrophage-derived cytokines.
Following inflammation, rapid restoration of the epithelial barrier helps to seal the wounded area from the external environment and prevents further infiltration by microorganisms (Clark et al., 1982a). Furthermore, keratinocytes are an important source of paracrine factors that regulate the spatio-temporal function of other mesenchymal cells including fibroblasts and endothelial cells (Werner et al., 2007). Successful re-epithelialisation relies on rapid proliferation, differentiation and migration of keratinocytes from the edges of the wound to cover a mostly fibronectin-rich provisional matrix (Clark et al., 1982a).

Re-epithelialisation of the wound requires adaptive responses to acute hypoxia, as migration of keratinocytes proceeds over an avascular provisional matrix. During wound re-epithelialisation HIF-1α and the expression of its downstream targets (Glut-1, phosphoglycerate kinase (PGK)-1, and VEGF are transiently increased correlating to the presence of tissue hypoxia and an adaptive response to the hypoxic stimulus (Elson et al., 2000, Haroon et al., 2000, Lokmic et al., 2006). Increased expression of Glut-1 and PGK-1 would be necessary for the provision of metabolic energy required for increased cell migration and proliferation which may suggest that rapid re-epithelialisation is partly hypoxia-driven. In vitro, hypoxia enhances keratinocyte migration on connective tissue (fibronectin and collagen), a process associated with increased integrin and MMP expression (O’Toole et al., 1997, Ridgway et al., 2005). Additionally, given that HIF-1α inhibition or deficiency dramatically delays keratinocyte migration (Fitsialos et al., 2008, Xia et al., 2001), it is likely that HIF-1 plays a central role in directly or indirectly regulating integrins and MMPs. Furthermore, HIF-1 suppresses expression of the cell to cell adhesion protein, E-cadherin, thus allowing keratinocytes to switch to a migratory phenotype (Grzesiak and Pierschbacher, 1995, Esteban et al., 2006).

The Siah2-HIF-1 axis appears to be relevant in the regulation wound re-epithelialisation. Herein, Siah2−/− mice show delayed wound re-epithelialisation (Figure 3-1) and reduced Glut-1 expression in the basal keratinocytes of the re-epithelialising sheet (Figure 3-7) at
days six post-injury in comparison to Wt mice. Rapid re-epithelialisation is a high energy
demanding event requiring up-regulation of metabolic proteins. Reduced Glut-1
expression would therefore partly explain the delay in epithelial regeneration in Siah2^{-/-}
wounds. Furthermore, because Glut-1 is a well defined downstream marker of HIF-1
transcriptional activity, it is conceivable that other HIF-1 regulated processes are
reduced in the Siah2^{-/-} wound milieu that may have contributing effects to the delay in
closing the epithelial defect. For, example reduction in macrophage invasion in the
absence Siah2 would correlate to a reduction in macrophage-derived cytokines.
Macrophage derived cytokines including FGF2, KGF, TGFβ1 and TGFα and these are
viewed as important in stimulating re-epithelialisation (Gailit et al., 1994, Li et al., 2004,
Li et al., 2006). Additionally, HIF-1 deficiency in the absence of Siah2, may have a
negative bearing on modulation of migratory proteins, such as regulation of cell to cell
adhesion proteins (e-cadherin), cell matrix receptor (integrins) and MMPs, which are
normally modulated by hypoxic stimuli to enhance migration (O’Toole et al., 1997,
Ridgway et al., 2005, Esteban et al., 2006).

In addition to epithelial regeneration, the restoration of tissue integrity and vasculature
are fundamental functions of the granulation tissue during wound healing. Following
tissue insult, fibroblasts infiltrate the provisional matrix from local mesenchymal
connective tissue and bone marrow-derived circulating progenitor lineages in response
to released inflammatory factors (Leibovich and Ross, 1975, Leibovich and Ross, 1976,
Abe et al., 2001). As fibroblasts migrate into the wound they replace provisional matrix
with granulation tissue matrix (mostly type I and III collagen) whilst simultaneously
releasing proteolytic factors and their inhibitors ensuring organised formation of
granulation tissue through remodelling (Salo et al., 1994, Darby et al., 2002).
Furthermore, autocrine and paracrine factors released by activated fibroblasts take over
from overlapping inflammatory cell-derived factors becoming the central regulators of
subsequent cell proliferation and tissue remodelling events including angiogenesis,
matrix synthesis/remodelling and wound contraction (Denton et al., 2009). With
increasing tension generated by matrix remodelling, mechano-perception via cell-matrix junctions (fibronexi and focal adhesions) become activated causing fibroblasts to further differentiate into myofibroblasts characterized by expression of α-SMA (Tomasek et al., 2002, Hinz et al., 2003, Hinz and Gabbiani, 2003a, Hinz and Gabbiani, 2003b). In addition to tension, TGFβ and the fibronectin splice variant ED-A fibronectin are also required for this phenotypic change (Desmouliere et al., 1993, Serini et al., 1998). The expression of αSMA in the granulation tissue is transient and correlates to a contracting phase of healing (Gabbiani et al., 1971, Darby et al., 1990). Furthermore, the presence of myofibroblasts is synonymous with increased production of collagenous matrix (Schmid et al., 1998).

Over the years the role of HIF-1 and hypoxia on fibroblast function has been well defined. In culture acute hypoxia (0 to 72 hours) there is enhancement of several fibroblast functions including migration, proliferation and production of collagenous matrix through up-regulation of TGFβ1 (Falanga and Kirsner, 1993, Falanga et al., 1991, Falanga et al., 2002, Steinbrech et al., 1999). Accumulating evidence, points to a strong correlation between HIF-1 activity and enhanced TGFβ signalling by dermal fibroblasts. Increased HIF-1 signalling enhances fibroblast expression of matrix proteins (collagens and fibronectin), growth factors (TGFβ, CTGF, and VEGF) and TGFβ1 activating factors (thrombospondin-1) (Distler et al., 2007, Higgins et al., 2004). The relevance of HIF-1 in the regulation of these factors during hypoxia has been convincingly tested using HIF-1 depleted or inhibited fibroblasts (Distler et al., 2007, Higgins et al., 2004). The evidence suggests that HIF-1 is important in the regulation of dermal fibroblast function during angiogenesis and granulation tissue formation, events that occur in relatively hypoxic conditions (Lokmic et al., 2006). Indeed recent cutaneous burn wound healing in HIF-1α heterozygous null mice showed a significant reduction in wound vascularization, perfusion and granulation tissue contraction (Zhang et al., 2010b).
Consistent with other HIF-1 depletion studies (Distler et al., 2007), the findings of the current study demonstrate the importance of hypoxia signalling in fibroblast function in vivo. In our study, Siah2^{-/-} mice show delays in collagen deposition six days post injury in comparison to Wt mice, as determined by picrosirius red staining (Figure 3-2). Acute hypoxia increases collagen production by dermal fibroblast in a manner that is most likely TGFβ-dependant (Falanga et al., 2002, Siddiqui et al., 1996). The type I collagen gene has not been shown to contain a HRE and therefore HIF-1 does not directly regulate its transcription (Falanga et al., 2002). Instead, HIF-1 regulates the expression and activation of factors which can directly augment collagen production by dermal fibroblast in hypoxia including procollagen hydroxylase, TGFβ, CTGF and the TGFβ activating protein thrombospondin-1 (Hofbauer et al., 2003, Distler et al., 2007). Therefore by virtue of HIF-1 deficiency it is conceivable that matrix deposition enhancing events during hypoxia are defective in Siah2^{-/-} wounds. Furthermore reduced macrophage invasion in Siah2^{-/-} may also restrict the levels of paracrine cytokine expression and secretion required for normal fibroblast function and matrix synthesis. Collectively, this would explain the simultaneous delay in αSMA expression seen in the granulation tissue of Siah2^{-/-} mice (versus Wt) (Figure 3-5), a process that is matrix and cytokine dependent. Consequently, the delay in αSMA expression and collagen deposition suggests impairment in wound contraction (Figure 3-1).

Blood vessels function as conduits for the delivery of oxygen and nutrients in all physiological and pathological states (Clark, 1996). Following full-thickness cutaneous injury, vessels are generally disrupted and neovascularization of the newly formed granulation tissue is essential for successful wound healing (Remensnyder and Majno, 1968). Hypoxic gradients are mandatory stimuli for achieving good angiogenesis during wound healing (Knighton et al., 1981). Hypoxia is well known to induce the up-regulation of major angiogenic factors, the recruitment of inflammatory and progenitor endothelial cells as well as the migration of fibroblasts and keratinocytes which coordinate the angiogenic effort (Steinbrech et al., 1999, Cramer et al., 2003, Berse et

Expression of virtually all critical angiogenic growth factors required for local activation of vascular cells during angiogenesis is induced by HIF-1, including FGF-2, VEGF, stromal derived factor 1 (SDF-1), Ang 2, PDGF and TGFβ (Forsythe et al., 1996, Loh et al., 2009, Distler et al., 2007, Semenza, 2010). The released factors also serve as homing signals for mobilization and recruitment of circulating angiogenic cells (CACs) (progenitor endothelial cells and bone marrow derived myeloid cells) from distant sites such as bone marrow and vessels from other tissue (Botusan et al., 2008, Loh et al., 2009, Zhang et al., 2010b).

Impairment of HIF-1-dependent responses to hypoxia is a major factor contributing to the impaired vascular responses to wound ischaemia. For example, defective HIF-1 signalling has been identified in ageing- and diabetes-related impaired angiogenesis during wound healing in animal models, and correction by constitutive expression or stabilization of HIF-1 significantly improves the angiogenic defect and accelerates wound healing (Botusan et al., 2008, Liu et al., 2008). Conversely, prolonged hypoxia and over stimulation of HIF-1 associated responses are viewed as an important mechanism involved in elaborate angiogenesis that could drive pathological conditions. High levels of HIF-1 and its downstream targets (such as VEGF) are reported in a variety of pathological wound healing or fibroproliferative disorders including keloid lesions, arthritis, pulmonary fibrosis and liver cirrhosis (Distler, 2003, Zhang et al., 2006, Tzouvelekis et al., 2007, Bozova and Elpek, 2007). Consequently, targeting HIF-1 signalling is viewed as an important candidate therapeutic target for the treatment of fibrotic diseases (Distler et al., 2007, Moon et al., 2009) Furthermore, impaired angiogenesis and reduced mobilisation of endothelial progenitor cells and has been demonstrated in wound healing models of HIF-1 heterozygous null and conditional HIF-1 deficient (endothelial) mice (Tang et al., 2004, Zhang et al., 2010b). These studies
confirm that acute hypoxia responses mediated through HIF-1 are required for good angiogenic responses during wound healing.

The findings of this present study demonstrate the relevance of Siah2 in the regulation of angiogenesis during wound healing. Herein, Siah2−/− mice showed a delay and overall reduction in vascularity (Counts/area) at day six and nine post injury (Figure 3-6(e)). Furthermore, analysis of central regions of the granulation tissue revealed the greatest difference in vascularity between Siah2−/− and Wt mice, indicating that angiogenic responses in Siah2−/− wounds were especially impaired towards the central likely most hypoxic regions of the granulation tissue, since these are most distant from blood supply (Figure 3-6(f)). The findings of delayed angiogenesis are consistent with results in other HIF-1 deficient wound healing models where reduced angiogenic factors such as VEGF and SDF-1 are a major contributing factor (Tang et al., 2004, Zhang et al., 2010b). VEGF is arguably the most potent and important angiogenic growth factor and is an important downstream target of HIF-1. In this context Siah2−/− mice have previously been shown to have reduced VEGF expression in hypoxia (Nakayama et al., 2004). This would partly explain the delay in angiogenesis seen in the present study. Furthermore, paracrine sources of VEGF such as macrophages, fibroblasts and keratinocytes are fewer or delayed in invasion of Siah2−/− wounds. Additionally, rapid and organised capillary formation by endothelial cells is highly dependent on the presence of collagen (Montesano et al., 1983). Therefore reduced collagen deposition by fibroblasts/myofibroblast may also contribute to rate limiting of the angiogenic process in Siah2−/− mice. Additionally, it is likely that lack of macrophages seen in the Siah2−/− wounds could also in part be due to the reduced number blood vessels which may have potentially prevented a steady influx of these cells into the site of injury.

Siah2 has also been described to regulate pathophysiological events such as angiogenesis through HIF-1-independent mechanisms (Qi et al., 2008). Mammalian Sprouty (Spry) gene expression is rapidly induced upon activation of the FGF receptor
signalling pathway in multiple cell types, including cells of mesenchymal and epithelial origin, where it functions as a negative regulator of FGF mediated signaling (Nadeau et al., 2007). Siah2 negatively regulates Spry 1 and 2 (tumour repressor protein) by tagging it for proteosomal degradation (Nadeau et al., 2007). Endogenous Spry2 has recently been shown to function as a negative regulator of angiogenesis in the late phase of wound healing (Wietecha et al., 2011) suggesting that its activity is tightly regulated during the early phases of healing. Additionally, wounds treated with the dominant-negative Spry2 exhibit a moderate increase in vascularity (Wietecha et al., 2011). It is therefore conceivable that Siah2 regulates endogenous Spry activity in the early phases of wound healing when rapid and organized angiogenesis is critical.

Interestingly, despite the delay in major wound healing parameters observed in Siah2-/- wounds, proliferation as determined by PCNA staining did not reveal any significant difference at day six (versus Wt) (Figure 4-3). And even more surprising, was the significant increase in proliferation observed in the Siah2-/- nine day wounds (Figure 4-3). The reasons for this observation are not clear, but raise the possibility of compensatory mechanism(s) in the wound leading to the recovery of Siah2-/- mice. Siah E3 ubiquitin ligases tag a range of proteins for proteosomal degradation including TNF-receptor associated factor 2 (TRAF2), synphilin-1, N-cor, m-cyb, Sprouty and β-catenin (Liu et al., 2001, Matsuzawa and Reed, 2001, Hu et al., 1997, Hu and Fearon, 1999, Li et al., 2002, Nadeau et al., 2007). Reduced regulation of one or more of these proteins in Siah2-/- wounds may be a possible explanation for the increase in proliferation. Deregulated β-catenin expression for example, has previously been described to promote cell hyperproliferation during wound healing (Cheon et al., 2006). Furthermore m-cyb functions as a promoter of hematopoietic progenitor cells proliferation during normal murine fetal hepatic hematopoiesis and mesenchymal cell proliferation during skin wound healing (Mucenski et al., 1991, Kopecki et al., 2007). Additionally, functional redundancies between Siah1 and Siah2 isoforms have previously been described (Frew et al., 2003) and cannot be ruled out as a possible factor for the increase in proliferation
seen in Siah2\textsuperscript{−/−} mice. Unfortunately, lethality in Siah1/2 double knockout transgenic mice has restricted investigations in absolute Siah deficient models (Frew et al., 2003).

Remodelling is characterised by contraction of the granulation tissue, degradation of collagen and a significant loss in cellularity in the wound bed, the net result of which is a markedly less cellular and less vascular scar (Darby et al., 2002, Gabbiani et al., 1971, Desmouliere et al., 1995). Myofibroblasts expressing contractile actin (αSMA) align parallel to the direction of mechanical tension by adhering to collagen through focal adhesion proteins and integrins (Darby et al., 1990, Tomasek et al., 2002, Tomasek et al., 1992, Hinz et al., 2003). Myofibroblasts also make fibronexus connections to each other (Singer et al., 1985). TGFβ1 in combination with the splice variant fibronectin (ED-A FN) and mechanical stress stimulate contraction of αSMA bundles which significantly reduces the granulation tissue area (Desmouliere et al., 1993, Serini et al., 1998). Following contraction αSMA positive myofibroblasts and vascular numbers in the granulation tissue are reduced by the induction of apoptosis late in wound healing when re-epithelialisation has occurred (Desmouliere et al., 1995).

In this study the role of Siah2 in tissue contraction and remodelling was considered through analysis of αSMA expression (Figure 3-5) and localisation of apoptotic nuclei (Figure 3-8). Siah2\textsuperscript{−/−} wounds show reduced αSMA expression in granulation tissue correlating with the delay in wound contraction (Figure 3-1), suggesting a possible delay in granulation tissue remodelling. Paradoxically, no overt differences in apoptosis were visible following localization of apoptotic nuclei using TUNEL immunostaining. The phenotypic difference in rate of healing as analysed by granulation tissue size (Figure 3-1) would demand a difference in the rate of cellular loss as the wounds contract at different rates. The inability of TUNEL staining to differentiate between necrotic and apoptotic nuclei may be a factor in the lack of difference between Siah2\textsuperscript{−/−} and Wt wounds or the possibility that small differences observed at any one timepoint may have significant effects on the rate of cell loss and these were not picked up using the
TUNEL technique. Therefore other apoptotic makers such as activated caspase-3 staining may be required to draw a conclusion on the effect of Siah2 in wound remodelling.

3.3.3. Clinical implications

The results of this study may have several important clinical implications. Firstly, genetic or pharmacological strategies to increase Siah levels may be beneficial in increasing HIF-1α levels in tissue. Increased HIF-1α has been shown to promote vascularisation and improve healing in ischaemic, excisional and burn wounds (Botusan et al., 2008, Liu et al., 2008). This is especially important for specific groups such as the aged and diabetic subjects who may be prone to HIF-1 deficiency in hypoxia (Catrina et al., 2004, Botusan et al., 2008, Liu et al., 2008, Loh et al., 2009) The use of HIF-hydroxylase (PHD) inhibitors to improve wound healing has also been demonstrated in cutaneous wound healing models in animals (Botusan et al., 2008). PHD inhibitors are now in clinical trials for the treatment of several ischaemia-related pathologies such as chronic wounds, anaemia, ischaemic heart disease, stroke, cancer, and pulmonary hypertension (Smith and Talbot, 2010). This strongly supports the use of Siah or a pharmacological mimetic in the treatment of ischaemic wounds. Accelerated healing by Siah over-expression may also be beneficial in preventing scar formation, as slower healing wounds tend to scar excessively (Cubison et al., 2006). Furthermore, Siah may be relevant in enhancing skin graft “take rates”, granulation tissue formation and epithelialisation, which have recently been demonstrated to be dependent on myeloid cell HIF-1 activity (Vural et al., 2010).

Conversely, inhibition of Siah using specific pharmacological inhibitors may prove beneficial in the treatment of fibroproliferative disorders, where HIF-1α is over-expressed or expressed chronically and thought to promote fibrosis (Distler et al., 2007, Zhang et al., 2006). This may be especially important for patients with increased susceptibility to fibrotic disorders owing to HIF-1A gene polymorphisms (Wipff et al.,
At present, menadione (a Vitamin K derivative) is the only pharmacological inhibitor of Siah2 (Shah et al., 2009). Menadione topical creams are currently in trials for treatment or prevention of EGFR inhibitor-induced skin rashes (Li and Perez-Soler, 2009). Based on the results of the current study, experiments to test effect of menadione in a wound healing scenario would seem desirable.

### 3.4. Conclusion

In summary, hypoxia is an important part of the microenvironment in early wound healing. Adaptive responses to hypoxia are controlled by HIF-1, which is positively regulated by Siah2. This study demonstrates for the first time a role for Siah2 in wound healing. Cutaneous wound healing of Siah2-deficient mice results in delayed recruitments of macrophages, delayed migration of vessel cells, delayed fibroblast–myofibroblast transformation and reduced extracellular matrix deposition during the early phase of wound healing. Modulation of Siah may therefore have important clinical implications in the treatment of abnormal wounds.

### 3.5. Specific experimental methods:

Mentioned here are summarised methods including only relevant details pertaining to this chapter. For specific details of individual methods please refer to the general methods and materials chapter (Chapter 2).

#### 3.5.1. Wound preparation, extraction and embedding

42 mice of C57 strain (genotype; 21 Siah2−/− and 21 Siah2+/+ (Wt)) were kindly provided by our collaborator Dr A Møller of Peter MacCallum Institute. Animals were age (10 ± 2 weeks) and sex matched (male only) anaesthetised, shaved, the skin disinfected and wounded (5mm radius full-thickness using a sterile punch biopsy) on the upper dorsum. Wounds were dressed and allowed to heal by secondary intention. Hypoxia in wound
healing is evident between days 3-10 post injury (Lokmic et al., 2006) and therefore animals were sacrificed and wounds were extracted mostly after 6 days (n=10) and 9 days (n=8) and to a lesser extent at 12 days (n=3). Wounds were then trimmed, fixed (4% paraformaldehyde) and embedded in paraffin. Histological analysis was performed on serial sections from the central portion of the wound.

3.5.2. Histological staining

Sections were stained with haematoxylin and eosin (H&E) and picrosirius red (F3B4). H&E section were used to measure wound size (Cross section area (mm$^2$)), % re-epithelialisation and to estimate vascular density (vessel counts /μm$^2$). Polarised light viewing of picrosirius red staining was used to determine percentage area of deposited collagen within the granulation tissue.

3.5.3. Immunohistochemistry and TUNEL staining

Immunohistostaining was performed by overnight incubation with primary mouse monoclonal anti- α SMA (1: 200 ) (Dako), rabbit polyclonal anti- CD31 (1: 100) (Abcam), mouse monoclonal anti - PCNA (1: 250), rabbit polyclonal anti- Glut-1(1: 400) or rat monoclonal anti – F4/80 (1: 50) (Abcam). Dual linked anti Mouse/ Rabbit IgG – Horse radish peroxidase (HRP) conjugated secondary antibody and a DAB kit (Dako) were used to visualise probed antigens. A biotinylated anti rat IgG secondary (Vector) and Vector ABC DAB kit (Vector) were used to visualise rat anti F4/80 staining.

Terminal deoxynucleotidyl transferase mediated X-dUTP nick end labelling (TUNEL) staining kit (Promega, DeadEnd colorimetric kit) was used to detect DNA fragmentation (apoptotic nuclei). Immunohistochemistry and TUNEL sections were lightly counterstained with haematoxylin.
3.5.4. Statistical analysis

Results are expressed as the mean ± standard error of mean (S.E.M) for 3 to 10 separate observations. Statistical analysis was determined by Mann-Whitney’s U-test analysis. P values between compared groups (Siah2−/− vs. Wt) less than or equal to 0.05 were considered to be statistically significant.
Deficiency of Siah2 and its effect on mouse dermal fibroblast function
4.1. Introduction

The ability of dermal fibroblasts to migrate, proliferate, differentiate, contract and deposit collagenous matrix during wound healing is critical to events that lead to the formation/remodelling of granulation tissue, angiogenesis and the eventual restoration of tissue integrity to damaged tissue (Ross and Benditt, 1962, Gabbiani et al., 1971, Darby et al., 1990, Denton et al., 2009). Acute tissue hypoxia is suggested to play an important role in modulating several fibroblast functions that promote wound healing (Siddiqui et al., 1996, Steinbrech et al., 1999, Falanga et al., 2002). Cell function in hypoxia is thought to be highly reliant on adaptive responses mediated through HIF-1 pathways (Semenza, 1998), and may therefore be subject to regulation by Siah which positively regulates HIF-1α abundance during hypoxia (Nakayama et al., 2004). In the previous chapter (Chapter 3), the relevance of Siah2 was defined in early wound healing and delayed healing was observed in Siah2−/− mice. In the current chapter further characterisation of the effect of Siah2-deficiency on fibroblast function was studied *in vitro* using primary adult dermal fibroblasts derived from Siah2−/− mice as the primary cell model. We hypothesized that Siah would have a regulatory role during dermal fibroblast function in hypoxia. Additionally, the effect of Siah on dermal fibroblast response to growth factors such as TGFβ1 has not yet been tested in normoxia or hypoxia. Thus, the aim was to test the role of Siah2 in cell migration, proliferation, matrix synthesis, myofibroblast transition and cell contraction in three basic culture conditions that may be of relevance to wound healing; culture conditions supplemented with a growth factor (TGFβ1), hypoxic culture conditions alone and hypoxic conditions supplemented with TGFβ1. Furthermore, the impact of Siah2 on TGFβ1 signalling transduction in the three culture conditions was also tested.
4.2. Results

4.2.1. Reduced HIF-1α levels in hypoxic Siah2−/− dermal fibroblasts

Siah2−/− embryonic fibroblasts show relatively normal behaviour in culture in terms of cell cycle, proliferation and survival (Frew et al., 2002). However, when challenged with hypoxic conditions, Siah2−/− show reduced availability of HIF-1α and expression of HIF-1 target genes (Nakayama et al., 2004, Fukuba et al., 2007). To confirm that Siah2−/− mouse dermal fibroblasts (MDFs) derived from adult skin were impaired in their ability to stabilise HIF-1α during hypoxia, western blot analysis of HIF-1α protein levels was determined in Wt and Siah2−/− MDF cells incubated in hypoxia (1% O2) over time (0 to 48 hours) under normal serum conditions (5%FBS)(Figure 4-1). Qualitatively, Wt MDFs increased HIF-1α abundance over time in hypoxia, with a peak in HIF-1α stabilization observed after 8 hours in hypoxia, after which HIF-1α protein levels gradually reduced but remained relatively stable for up to 48 hours, which was in keeping with other studies that show parabolic increases in HIF-1α expression in different tissue and a return to baseline levels over time in hypoxia (Stroka et al., 2001). In contrast HIF-1α was dramatically reduced and mostly undetectable in Siah2−/− MDFs using western blot analysis. These findings are in line with previous studies that have demonstrated the regulatory effects of Siah on HIF-1-prolyl hydroxylases (PHDs) and the role of Siah2 as a positive regulator of HIF-1α abundance in hypoxia (Nakayama et al., 2004)

4.2.2. Reduced collagen expression in Siah2−/− dermal fibroblasts

Low oxygen tension stimulates collagen synthesis and COL1A1 transcription through the actions of TGFβ1 (Falanga et al., 2002) and a HIF-1α dependent mechanism (Distler et al., 2007). To investigate if the Siah-HIF1 axis had any regulatory effects on collagen synthesis, western blot analysis of COL1A1 protein levels was determined in Wt and Siah2−/− MDF cells incubated in hypoxia (1% O2) over time (0 to 48 hours) in normal serum conditions (5% FBS) (Figure 4-1). Protein levels (COL1A1) were analysed as arbitrary units derived from the ratio of COL1A1 and house keeper (β-actin) optical
density readings. β-actin readings appeared to be relatively strong and manifested with some non-specific binding around 30 to 50 Kilo Daltons, possibly due to high proteins loads (20ug), but appear to be consistent across all bands and of relatively of similar intensity. Densitomery readings at 42 kilo Daltons only were used as house keepers. Wt showed an upward trend in COL1A1 expression with increasing time in hypoxia between 0 (0.39 ± 0.23), 8 (0.48 ± 0.21), 24 (0.66 ± 0.06) and 48 hours in hypoxia (0.88 ± 0.08). In contrast, collagen protein levels in Siah2−/− MDFs appeared to decrease with increasing duration of hypoxia (0.53± 0.11, 0.44 ± 0.18 and 0.145 ± 0.04 at 0, 8 and 24 hours of hypoxia respectively) with the reduction becoming significant after 48 hours in hypoxia (0.09 ± 0.02, P<0.01) when compared to the Wt cells (0.66 ± 0.06). These findings were suggestive of the regulatory effects of hypoxia on collagen expression (Distler et al., 2007) and show that Siah2, presumably through HIF-1-regulated genes, may be involved in the regulation of collagen synthesis during hypoxia.
Figure 4-1. HIF-1α and COL1A1 protein levels in Wt and Siah2−/− MDFs over time in hypoxia (0-48 hours). Western blot analysis of (a) HIF-1α and (b) COL1A1 protein levels in normalized protein amounts (20µg and 10µg respectively) of Wt and Siah2−/− MDF lysates after 0 to 48 hours in hypoxia (1%O₂). β-actin loading control blots are also shown. (c) Analysis of COL1A1 protein levels in Wt (black bars) and Siah2−/− (grey bars), where arbitrary units represent the ratio of optical densities of COL1A1 to that of β-actin loading control. Each point represents the mean ± SEM of at least three independent experiments. Bonferroni’s two-way ANOVA was performed to determine significance. **P<0.01 (Molecular weight (Mw), HIF-1α, COL1A1 and β-actin ≈ 120, 90-140, and 42 kilo-Daltons (kD) respectively)
4.2.3. Reduced TGFβ1 mediated COL1A1 expression in Siah2\textsuperscript{−/−} dermal fibroblast

Exogenous TGFβ1 causes marked and rapid increase of collagen synthesis by dermal fibroblasts \textit{in vitro} and inflammatory cell and mesenchymal cell release of TGFβ1 is important in regulating the formation of granulation tissue during wound healing and pathological fibrosis \textit{in vivo} (Roberts et al., 1986). To examine whether Siah2 had any role in the regulation of collagen synthesis in response to exogenous TGFβ, Wt and Siah2\textsuperscript{−/−} MDFs were serum starved then treated with TGFβ1 (5ng/ml), hypoxia or a combination of the two for 48 hours under low serum conditions (2% FBS). Western blot analysis was used to determine COL1A1 protein levels. Protein expression is represented as arbitrary units derived from the ratio of optical densities of COL1A1 to that of β-actin. In normoxic untreated culture conditions COL1A1 levels were similar between Siah2\textsuperscript{−/−} and Wt MDFs (1.42 ± 0.13 and 1.43 ± 0.05, respectively). Conversely, Siah2\textsuperscript{−/−} MDFs (1.32 ± 0.16, P<0.001) showed a failure to increase COL1A1 in normoxic conditions when supplemented with TGFβ1 compared to Wt MDFs (2.23 ± 0.21) suggesting a reduced responsiveness to the growth factor. Interestingly no significant differences were observed between Siah2\textsuperscript{−/−} and Wt MDFs in hypoxia (1.61 ± 0.16 and 1.30 ± 0.04 respectively) or hypoxic conditions supplemented with TGFβ1 (1.01 ± 0.01 and 1.30 ± 0.04 respectively). This was somewhat contradictory to preliminary observations made above (\textbf{Figure 4-1}) where differences in COL1A1 protein levels between Wt and Siah2\textsuperscript{−/−} MDFs were observed with increasing time in hypoxia. The reasons for this difference are not clear but may be reflective of different effects of the different serum levels (2% and 5% FBS) and varying ability of MDFs cells to adapt in response to hypoxic conditions in reduced versus normal serum conditions. The inability of Siah2\textsuperscript{−/−} MDFs to increase collagen in normoxic conditions may still be relevant to Siah2 having a role in fibrogenesis and is suggestive of a role of Siah2 to regulation collagen beyond the hypoxic milieu.
Figure 4-2. Effect of Siah2 deficiency on TGFβ1-induced COL1A1 expression in normoxia and hypoxia. Western blot analysis of COL1A1 and β-actin using normalized protein mass (10µg) from Siah2−/− and Wt MDFs after 48 hours in TGFβ1 (5ng/ml) (N+), hypoxia (1%O2) (H) or both (hypoxia and TGFβ1) (H+). Control untreated Wt and Siah2−/− MDFs (N) are also shown. Collagen (COL1A1) protein levels in Siah2−/− (grey bars) and Wt (black bars) are analysed as arbitrary units (ratio of COL1A1 and β-actin optical density). Each point represents the mean ± SEM of at least three independent experiments. Bonferroni’s two-way Anova was performed to determine significance. *** P<0.001 (Molecular weight (Mw), COL1A1 and β-actin ≈ 90-140 and 42 kilo-Daltons (kD) respectively)
4.2.4. **Normal proliferation in Siah2<sup>−/−</sup> dermal fibroblasts**

Hypoxia has previously been demonstrated to be a modulator of proliferation and expansion of adult and neonatal human dermal fibroblasts seeded as single cells (Falanga and Kirsner, 1993, Oberringer et al., 2008). To investigate the effect of acute hypoxia and Siah-HIF-1 axis on cell proliferation, Siah2<sup>−/−</sup> MDF cells were exposed to TGFβ1 (5ng/ml), hypoxia (1%O<sub>2</sub>) or combined TGFβ1 and hypoxia for 48 hours and proliferation determined by an anti-PCNA antibody and flow cytometry analysis (Figure 4-3). Forward and side scatter characteristics of MDFs were used for their preliminary identification and appropriate gates were further used to localise MDF cells and exclude cellular aggregates and debris. Proliferative activity was evaluated by a stimulation index, which was the ratio of mean fluorescence intensity of PCNA in treated MDFs to that of untreated MDFs. PCNA stimulation index in Wt MDFs increased by 30% (1.30 ± 0.10) in TGFβ1, decreased by 7% (0.93 ± 0.07) in hypoxia and decreased 8% (0.92 ± 0.16) in hypoxic conditions supplemented with TGFβ1. In Siah2<sup>−/−</sup> MDFs, PCNA stimulation index was unchanged in TGFβ1 (0.98 ± 0.10), decreased by 15% (0.85 ± 0.08) in hypoxia, and decreased by 33% (0.67 ± 0.10) in hypoxic conditions supplemented with TGFβ1. PCNA stimulation index in Siah2<sup>−/−</sup> MDFs appeared to be unchanged in response to TGFβ1 in normoxia and decreased in hypoxia conditions when compared to Wts, however, the difference was not statistically significant. The results also fail to support the findings of other studies which have reported that hypoxia increases human dermal fibroblast proliferation (Falanga and Kirsner, 1993), but support the findings of other studies that report no significant change in proliferative activity of dermal fibroblast in hypoxia (Boraldi et al., 2007). It may be that proliferation in hypoxia as reported by Falanga and others requires high amounts serums (20% FBS) (Falanga and Kirsner, 1993), instead of low serum conditions (2% FBS) as used in our study or that longer periods of hypoxia are required to cause a difference. Overall, proliferation of MDFs appeared to be relatively unaffected by hypoxia and may be indicative of their ability to proliferate in hypoxia without the need for adaptive machinery regulated through Siah2-HIF-1 axis, within the conditions applied and time point used in this study.
Figure 4-3. Characterization of the role of Siah2 in MDF proliferative activity. (a) Flow cytometry histograms of typical PCNA expression in Wt and Siah2<sup>−/−</sup> MDFs. Overlays; black (secondary antibody only), grey shaded (untreated group), red (5ng/ml TGFβ1), blue (hypoxia (1%O<sub>2</sub>)) and yellow (combined TGFβ and hypoxia)). (b) Dot plot of FSC versus SSC of MDFs and the cells gated for analysis are shown. (c) Proliferative activity in Wt (dot filled bars) and Siah2<sup>−/−</sup> (unfilled bars) was analysed as a stimulation index of PCNA, where the stimulation index was the ratio of mean fluorescence intensity of PCNA in treated (TGFβ1 (N+), hypoxia (H) and combination of both (H+) after 48 hours) to that of untreated MDF cells (N). Each point represents mean ± standard error of the mean of three separate experiments. Bonferroni’s two way ANOVA analysis was performed to determine significance.
4.2.5. Reduced αSMA expression in Siah2⁻/⁻ dermal fibroblasts

Transient expression of αSMA in the granulation tissue during fibroblast-myofibroblast transition is a hallmark of normal wound healing and is synonymous with wound contraction (Darby et al., 1990). TGFβ1 induces αSMA expression and myofibroblast transition in quiescent and growing cultured fibroblasts (Desmouliere et al., 1993). To investigate the impact of Siah on fibroblast-myofibroblast transition in hypoxia, an anti-αSMA antibody and flow cytometry analysis was used following treatment of Siah2⁻/⁻ and Wt MDF cells with TGFβ1 (5ng/ml), hypoxia (1%O₂) or the combination of both (hypoxia and TGFβ1) for 48 hours. Forward and side scatter characteristics of MDFs were used for their preliminary identification and appropriate gates were further used to localise MDF cells and exclude cellular aggregates and debris. αSMA induction in response to treatment was evaluated by a stimulation index, which was the ratio of mean fluorescence intensity of αSMA in treated to that of untreated control cells (Figure 4-4). In normoxic conditions, TGFβ1 equally increased the stimulation index of αSMA in both Siah2⁻/⁻ (1.59 ± 0.06) and Wt MDFs (1.69 ± 0.07). However, under hypoxic conditions the stimulation index of αSMA was significantly reduced in Siah2⁻/⁻ MDFs (0.65 ± 0.04, P<0.001) when compared to Wt MDFs (1.10 ± 0.04) where αSMA induction was moderately increased. Addition of TGFβ1 to hypoxic conditions failed to increase αSMA stimulation index in Siah2⁻/⁻ MDF (1.15 ± 0.05, P<0.01), remaining significantly lower when compared to the Wt MDFs (1.51 ± 0.07) where αSMA stimulation index was markedly increased. The results indicate that Siah-HIF-1 axis may have a role in regulating maintenance of αSMA in hypoxia and the enhancement of TGFβ-stimulated αSMA induction in hypoxia. This implies that Siah2 via HIF-1 may have regulatory effects on fibroblast to myofibroblast differentiation in mouse skin fibroblasts.
Figure 4-4. Effect of Siah2 deficiency on αSMA induction in mouse dermal fibroblast.

(a.) Flow cytometry histograms of αSMA expression in Wt and Siah2⁻/⁻ MDFs. Overlays; black (secondary antibody only), grey shaded (untreated group), red (5ng/ml TGFβ1), blue (hypoxia (1%O₂)) and yellow (TGFβ1 and hypoxia combined)). (b) Dot plot of FSC versus SSC of MDFs and the cells gated for analysis are shown. (c.) αSMA induction of Wt (dot filled bars) and Siah2⁻/⁻ (unfilled bars) in response to treatment (TGFβ1 (N⁺), hypoxia (H) or both (hypoxia and TGFβ1)(H⁺) for 48 hours) was evaluated by a stimulation index, which was the ratio of mean fluorescence intensity of αSMA in treated to that of untreated controls cells (N⁻). Each point represents the mean ± SEM of at least three independent experiments. Bonferroni’s two-way ANOVA was performed (⁎⁎ P<0.01, ⁎⁎⁎ P<0.001)
4.2.6. Effect of Siah2 on TGFβ1 mediated MDF collagen lattice contraction

Wound contraction is generally associated with the presence of modified fibroblasts in granulation tissue and the transient expression of αSMA by these cells (Gabbiani et al., 1971, Darby et al., 1990) although some contraction may be αSMA-independent and due to tractional forces generated by cell attachment and movement. TGFβ, extracellular matrix content and mechanical tension are also central to this contractile process (Montesano and Orci, 1988, Arora et al., 1999, Desmouliere et al., 1993). In monolayer cultures, αSMA stimulation index in Siah2\(^{-/-}\) MDFs was found to be reduced in hypoxia (Figure 4-4). To investigate whether this had any impact on the ability of cells to contract, Siah2\(^{-/-}\) and Wt MDFs were seeded into noncompliant collagen lattices (i.e. under tension) treated with TGFβ1, hypoxia or the combination of the two for 24 hours after which lattices were released, returned to their respective experimental conditions and allowed to contract for a further 24 hours. Contraction was calculated as the change in area (mm\(^2\)) at time 0 and 48 hours after release. Siah2\(^{-/-}\) MDFs showed reduced contraction in response to TGFβ1 (60.4 ± 2.31 mm\(^2\)), hypoxia (21.8 ± 8.62 mm\(^2\)) and hypoxia plus TGFβ1 (65.2 ± 3.00 mm\(^2\)) when compared to the Wt MDFs (69.3 ± 7.20 mm\(^2\), 31.2 ± 3.00 mm\(^2\), 80.7 ± 6.86 mm\(^2\) respectively), however, the results were not significantly different. The results are not conclusive but might be reflective of moderate impairment in Siah2\(^{-/-}\) MDFs to respond effectively to stimuli from hypoxia and TGFβ1. Additionally, the results somewhat correlated to the finding of reduced αSMA expression in Siah2\(^{-/-}\) MDF cells observed in monolayer cultures (Figure 4-5). Siah2 -HIF-1 axis may have a role in regulating αSMA expression in hypoxia and to a lesser extent fibroblast contraction, which may be more dependent on additional factors such as mechanical tension, extracellular matrix content and stimuli from growth factors such as TGFβ1.
Figure 4-5. Effect of Siah2 deficiency on TGFβ1- and hypoxia-mediated MDF collagen lattice contraction. (a) Images of contracted collagen lattices (outline indicated by yellow dashed line) seeded with Wt and Siah2−/− MDFs following 48 hours of normoxic culture conditions (N) or treatment with either TGFβ1 (5ng/ml) (N+), hypoxia (1%O2) (H) or combined hypoxia and TGFβ1 (H+). (b) Analysis of contraction in Wt (black bars) and Siah2−/−(grey bars), where contraction was defined as the change in area (∆mm²) between time 0 and 48 hours after release. Each data point represents the mean ± standard error of the mean of three separate experiments (b). Bonferroni’s two way ANOVA analysis was performed to determine significance.
4.2.7. Effect of Siah2 on dermal fibroblast cell migration

Hypoxia is a strong biological stimulus to dermal fibroblasts inducing migration during wound healing and has profound additive effects to migratory stimuli from cytokines and growth factors such as TGFβ (Mogford et al., 2002). However, hypoxia has also been shown to reduce dermal fibroblast migration with and without TGFβ1 in other studies (Oberringer et al., 2008). Migration of dermal fibroblasts in hypoxia is thought to be regulated mostly through HIF-1-dependent mechanisms (Li et al., 2007). To investigate the effect of Siah2 on dermal fibroblast migration, Siah2−/− and Wt MDFs were grown to full confluence and wounded by a scratch then incubated in TGFβ1 (1ng/ml), hypoxia (1%O₂) or hypoxic conditions supplemented with TGFβ1. Migration was analyzed as the change in area (Pixels²X10000) of the wound at 0 time versus time posted treatment (16 hours). Siah2−/− MDF cells displayed a reduced (26.7 ± 4.47 Pixels², P<0.01) migratory response to TGFβ1 in normoxic conditions compared to Wt MDFs (47.7 ± 3.07 Pixels²). Here, hypoxia appeared to be mostly inhibitory to migration in both Siah2−/− (29.9 ± 4.50 Pixels²) and Wt MDFs (34.4 ± 4.37 Pixels²). And TGFβ1 was unable to attenuate the decrease in migration of hypoxic Siah2−/− (32.2 ± 3.05 Pixels²) and Wt MDFs (35.1± 6.04 Pixels²). In hypoxia Siah2−/− MDFs appeared to migrate slower when compared to Wt MDFs, however, the difference was not significant. The findings demonstrate that Siah2 may be important for the regulation migration in response to TGFβ in normoxic conditions. Additionally, Siah2 appeared to be dispensable in the regulation of migration in hypoxia where impairment to MDF migration may have occurred irrespective of adaptive responses regulated through Siah and presumably HIF-1.
Chapter 4

a.

b.
Figure 4-6: Effect of Siah2 on TGFβ and hypoxia induced mouse dermal fibroblast migration. (a) Images of MDF monolayer scratch migration assay used to determine migration capabilities of Siah2\textsuperscript{-/-} and Wt MDFs in TGFβ (1ng/ml) (N+) and hypoxia (1% O\textsubscript{2}) (H) or both (hypoxia and TGFβ1) (H+). Scratches of untreated MDF controls (N) are also shown. (b) Analysis of cell migration by Wt and Siah2\textsuperscript{-/-} MDF, where migration represents the change in area (Pixels\textsuperscript{2}) of the original scratch (T\textsubscript{0}) and wound area post treatment (TGFβ1, hypoxia or both (hypoxia +TGFβ1)) or control untreated conditions for 16 hours. Each point represents mean ± standard error of the mean of three separate experiments. Bonferroni’s two way ANOVA statistical analysis was performed to determine significance. **P<0.01

4.2.8. Effect of Siah2-deficiency on TGFβ1 mediated Smad phosphorylation

Cellular responsiveness to TGFβ1 is reliant on the growth factor binding to TGFβ receptors leading to phosphorylation of receptor activated Smad proteins and subsequent induction of TGFβ-regulated genes (Massague, 1998, Piek et al., 1999, Massague and Chen, 2000, Massague and Wotton, 2000). Siah2\textsuperscript{-/-} MDFs showed reduced responsiveness to TGFβ in cell migration (Figure 4-6), collagen matrix synthesis (Figure 4-2) and αSMA expression (Figure 4-4). To investigate if Siah2 had an effect on TGFβ1 signal transduction western blot analysis of phosphorylated (p-Smad2) and total (t-Smad2) Smad2 was performed on MDFs cells treated with TGFβ1 (5ng/ml), hypoxia (1%O\textsubscript{2}) or hypoxic conditions supplemented with TGFβ1, while untreated MDFs served as controls (Figure 4-7). Smad2 phosphorylation was analysed as the ratio of phosphor-Smad2 (p-Smad) and total Smad2 (Smad2) optical densities. P-Smad was undetectable in untreated MDF using western blot analysis. In normoxic conditions TGFβ1 induced equally strong levels of p-Smad2 in Siah2\textsuperscript{-/-} and Wt MDFs (ratio of 0.24 ± 0.04 and 0.25 ± 0.03 respectively). Hypoxia induced relatively low but similar levels of p-Smad in both Siah2\textsuperscript{-/-} and Wt MDFs (0.07 ± 0.05 and 0.05 ± 0.05 respectively). Hypoxic conditions supplemented with TGFβ1 induced relatively robust but lower levels of p-Smad in Siah2\textsuperscript{-/-} MDFs compared to Wt cells (ratio of 0.18 ± 0.02 and 0.24 ± 0.02 respectively), however the observed differences were not significant. The results indicate that Siah2 deficiency
had no significant effect on TGFβ or hypoxia induced Smad2 phosphorylation in MDF cells imply that its effects occurred either through non-Smad-dependent mechanism or downstream of Smad phosphorylation.
Figure 4-7. Impact of Siah2 deficiency on TGFβ1 and hypoxia induced Smad2 phosphorylation. (a) Western blot of p-Smad2, Smad2 and β-actin protein levels in normalised proteins mass (10µg) of Siah2−/− and Wt MDF cell lysates collected from control untreated cells (N) or after 48 hours in TGFβ1 (5ng/ml) (N+), hypoxia (1%O2) (H) or both (H+). (b) Analysis of Smad2 phosphorylation in Wt (black bars) and Siah2−/− (grey bars), where arbitrary units represent the ratio of optical densities of p-Smad2 to that of Smad2. (c) Analysis of Smad protein levels in Wt (black bars) and Siah2−/− (grey bars), where arbitrary units represent the ratio of optical density of Smad2 to that of β-actin loading control. Each data point represents the mean ± SEM of four independent experiments. Bonferroni’s Two Way Anova was used to analyse significance. (Molecular weight (Mw) of p-Smad2, Smad2 and β-actin ≈ 55, 55 and 42 kilo-Dalton (kD) respectively)
4.3. Discussion

4.3.1. Hypoxia through HIF-1 regulates skin fibroblast

Fibroblasts are important stromal cells that synthesize the structural components of the extra-cellular matrix. During wound healing dermal fibroblasts are central in the regulation of granulation tissue development and its eventual remodelling and also modulate the angiogenic process through the release of extracellular matrix proteins and paracrine factors that are essential for both endothelial adhesion and chemotaxis during wound healing. In addition to soluble factors and matrix proteins released during inflammation, hypoxia has emerged as an important regulator of dermal fibroblast chemotaxis, production of autocrine and paracrine factors as well as extracellular matrix which ultimately accelerate wound healing through the promotion of granulation tissue formation and angiogenesis. HIF-1-regulated pathways are important adaptive responses to hypoxia and have important regulatory effects on fibroblast functionality in skin. For example, HIF-1 up-regulates Hsp90 transcription and release which in turn modulates cell migration (Li et al., 2007); increases a number of growth factors, metalloproteinase expression and collagen hydroxylases to enhance extra-cellular matrix synthesis and remodeling (Falanga et al., 1991, Hong et al., 2006, Zhang et al., 2003b, Hofbauer et al., 2003). Additionally HIF-1 regulates expression and release of angiogenic factors to promote neovascularisation (Thangarajah et al., 2009).

HIF-1 transcription activity is controlled by several proteins that regulate posttranslational availability of the HIF-1α subunit. Siah is an important upstream regulator of HIF-1 abundance and is required for adaptive cellular responses during tissue hypoxia by virtue of its inhibitory effects on HIF specific prolyl-hydroxylases (Nakayama et al., 2004). Here the role of Siah2 in murine dermal fibroblast function in response to TGFβ and hypoxia was tested using Siah2−/− dermal fibroblasts which have been shown to be HIF-1 deficient in hypoxia (Figure 4-1).
4.3.2. Impaired dermal fibroblast function in Siah2−/− mouse dermal fibroblasts

The ability of dermal fibroblasts to migrate and proliferate into the provisional matrix from local mesenchymal connective tissue and bone marrow-derived circulating progenitor lineages is dependent on their ability to respond to growth factors (Roberts et al., 1986, Denton et al., 2009) and their interactions with a variety of released matrix proteins (Postlethwaite et al., 1978, Kleinman et al., 1982). Additionally, adaptive responses to hypoxia through HIF-1 may be required as migration proceeds through an avascular and inflamed hypoxic tissue where the demands of metabolically active by inflammatory cells are a further draw on available oxygen, particularly during early wound healing. Here the role of Siah2 deficiency in MDF cell migration (Figure 4-6) and (Figure 4-3) fibroblast proliferation in response to TGFβ and hypoxia were tested. The results of the current study do not reflect any significant effect of Siah2 deficiency in murine dermal fibroblast migration and proliferation in hypoxia. Furthermore, hypoxia in general appeared to impair migration in response to exogenous TGFβ, which was in contrast to other studies that report enhanced dermal fibroblast migration in hypoxia and additive effects of the growth factor during hypoxia, albeit that this effect was observed in human dermal fibroblasts (Mogford et al., 2002). The reasons for this difference are not very clear and may suggest an inability of the primary adult MDF cells to migrate under oxidative stress in monolayer cultures and that some differences in the migratory response to hypoxia may exist between MDF and human dermal fibroblasts. Interestingly, the results revealed that Siah2 may be important for TGFβ-induced migration in normoxic culture conditions, as demonstrated by reduced migration of Siah2−/− MDF in response to TGFβ (compared to Wt cells). This may be of relevance to wound healing where TGFβ induces rapid invasion of dermal fibroblasts (Roberts et al., 1986, Denton et al., 2009). The findings may in part explain why fibrogenesis, which mostly relies of TGFβ induced chemotaxis and matrix synthesis, appeared delayed in Siah2−/− mice wounds. However, a conclusive outcome on the impact Siah in dermal fibroblast migration in hypoxia would require further investigation in human dermal
cells where an adaptive response to hypoxia has been previously reported. This is investigated further in the following chapter (see Chapter 5).

As fibroblasts migrate into the wound, they replace the fibrin clot with type I collagen which is critical for providing tensile strength and to the restoration of tissue integrity (Darby and Hewitson, 2007). In human dermal fibroblasts cultured in hypoxia enhanced type I collagen synthesis through mechanisms dependent on HIF-1 and the up-regulation of growth factors such as TGFβ and CTGF has been reported (Falanga et al., 2002, Hong et al., 2006). Here a role for Siah2 in MDF collagen expression was tested over time in hypoxia (Figure 4-1) and in response to exogenous TGFβ (Figure 4-2). Herein, Siah2−/− MDFs cells showed reduced type I collagen expression over time in hypoxic conditions with the difference reaching significance with increasing time in hypoxia (compared to Wt MDFs) and this appeared to correlate with a reduction in HIF-1α expression or stabilization in the absence of Siah2. Surprisingly, hypoxia appeared to have a negative effect on TGFβ induced collagen expression in the primary MDFs in general under low serum conditions, which is in contrast to other studies that suggest the involvement of the growth factor in human dermal fibroblast matrix synthesis during low oxygen tension and the involvement of HIF-1 in the regulation of this process (Siddiqui et al., 1996, Falanga et al., 2002, Distler et al., 2007). The reasons for this difference are not very clear and may once again be reflective of an inability of the primary adult MDF cells to adapt well to oxidative stress in culture in comparison to human dermal fibroblasts. However, Siah2−/− MDFs showed failure to up-regulate of COL1A1 in response to TGFβ in normoxic cultures conditions suggesting the possibility that Siah2 may also play a role in the regulation of collagen expression possibly through hypoxia independent mechanisms. The involvement of Siah in matrix synthesis, particularly in hypoxia would require further clarification owing to inconsistencies of the observations made here. Inconsistency in the reported data on fibroblasts is not entirely surprising as many studies vary in the passage and thus degree of myofibroblast differentiation of the cultures, the exact source of the fibroblasts used and the culture
conditions (serum vs serum-free or low serum) that are employed. This is highlighted by a recent paper by Modarressi et al. who showed varying responses to hypoxia of fibroblasts taken from different levels of the dermis and different tissue (Modarressi et al., 2010). The role of Siah deficiency in matrix synthesis is further examined in the next chapter using primary human dermal fibroblasts.

After collagen matrix is deposited to form the granulation tissue, the appearance of myofibroblasts with contractile actin fibres (αSMA) is a hallmark of the contraction phase of wound healing (Darby et al., 1990). Fibroblast to myofibroblast differentiation and activation of contractile apparatus is dependant on several factors including growth factors (TGFβ or CTGF)(Desmouliere et al., 1993, Garrett et al., 2004), mechanical tension (Tomasek et al., 1992) and matrix composition (Serini et al., 1998). Recently published research shows that hypoxia impairs human skin fibroblast to myofibroblast differentiation and contraction depending on the duration of hypoxia and the source of fibroblasts (Modarressi et al., 2010). The effects of acute hypoxia and Siah on αSMA expression (Figure 4-4) and cell contraction (Figure 4-5) in mouse dermal fibroblast were tested here. Firstly, acute hypoxia alone did not appear to have an effect on αSMA induction or cell contraction and addition of TGFβ to hypoxic cultures up-regulated αSMA induction and stimulated contraction of MDF cells. Additionally, Siah2−/− MDFs showed reduced αSMA induction in hypoxic culture conditions compared to Wt MDFs implying that Siah may have a role in maintaining αSMA expression in hypoxia. Furthermore, Siah2−/− MDFs showed reduced αSMA induction under hypoxic conditions supplemented with TGFβ suggesting that Siah2 was at least required for TGFβ to have additive effects on the induction of αSMA in MDFs cells in hypoxic conditions. The effects of Siah2 deficiency on αSMA induction appeared to correlate with the contractile ability of MDFs, although differences in matrix contraction between Siah2−/− and Wt did not reach significance. The evidence provided here in part supports a role of Siah2 in regulation of myofibroblast differentiation in mouse skin and during wound healing.
where αSMA expression and wound contraction appeared delayed in the absence of Siah2 (Chapter 3).

Cellular responsiveness to TGFβ1 is dependent on the growth factor binding to TGFβ receptors leading to activation of Smad proteins and cytosolic mitogen activated protein kinases (MAPKs) which affect transcription of TGFβ-regulated genes (Souchelnytskyi et al., 1996, Massague, 1998, Piek et al., 1999, Massague and Chen, 2000, Massague and Wotton, 2000). Hypoxia has been reported to up-regulate TGFβ1 in human dermal fibroblast cells (Falanga et al., 1991) which then activates downstream signalling cascades including p42/p44 MAPKs (Mogford et al., 2002) and Smads (Falanga et al., 1991) to induce cell migration and extracellular matrix synthesis. Here, the effect of Siah2 deficiency on Smad phosphorylation was investigated in response to TGFβ and hypoxic stimuli (Figure 4-7). Firstly, exogenous TGFβ and hypoxia were able to induce Smad phosphorylation which supports the hypothesis that some responses in hypoxia may in part be TGFβ-Smad driven (Falanga and Kirsner, 1993). However, the results showed no significant differences in the level of Smad phosphorylation between Siah2−/− and Wt MDFs cells in response to TGFβ, hypoxia or the combination of the two stimuli.

Given that TGFβ induced migratory and matrix synthesis responses are reduced in Siah2−/− MDFs cells in normoxic conditions, it may be conceivable that Siah has regulatory effects on TGFβ responses downstream of Smad or via non-Smad dependent pathways in a fashion independent of the hypoxia signalling machinery (HIF-1). The requirement of Ras signalling in the enhancement of TGFβ-Smad dependent responses may be a potential explanation for this result. Siah1 and Siah2 are highly conserved downstream components of the RAS pathway that are required for mammalian RAS signal transduction (Holloway et al., 1997). Depletion or inactivation of Siah inhibits Ras activated pathways that drive transformation and tumourgenesis leading to the reduction of these events (Schmidt et al., 2007, Ahmed et al., 2008). In fibroblasts,
activation with exogenous TGFβ leads to transient activation of RAS cascades and subsequent enhancement of TGFβ-Smad responses (Leask and Abraham, 2004). Activation of Ras signalling by TGFβ induces expression of genes including CTGF, a protein associated with cell migration, proliferation, angiogenesis and matrix synthesis (Phanish et al., 2005, Secker et al., 2008). It is therefore conceivable that TGFβ-Smad activated responses are reduced in Siah deficient cells due to impairment in Ras signalling.

Furthermore, given that αSMA and COL1A1 induction in Siah2−/− MDF were reduced in hypoxia without any significant change to Smad phosphorylation, it may be conceivable that cross-talk between TGFβ and hypoxia signalling pathways downstream of Smad phosphorylation are affected in the absence of Siah, and presumably HIF-1α. This cross-talk may involve a physical interaction between HIF-1α, Smad and a co-activator protein (p300) at the promoter region of genes containing hypoxia response element (HRE) and Smad response element (SRE) sequences (Sanchez-Elsner et al., 2001). Thus far, Smad-HIF-1α complexes are demonstrated in the activation of several angiogenic genes including VEGF, erythropoietin-1 and TGFβ co-receptor, endoglin (Sanchez-Elsner et al., 2001, Sanchez-Elsner et al., 2004, Sanchez-Elsner et al., 2002). However, a variety of genes which contain SRE and HRE sequences may potentially be regulated through TGFβ-hypoxia cross-talk, a good example being the CTGF which can be up-regulated through synergetic effects of hypoxia and TGFβ1 regulated pathways. (Hong et al., 2006, Higgins et al., 2004). CTGF is an important regulator of αSMA expression, cell contraction and matrix synthesis by dermal fibroblasts (Frazier et al., 1996, Garrett et al., 2004).

Finally, although the results of this study show some differences between Siah2−/− and Wt MDFs and point to a potential role of Siah in the regulation of dermal fibroblast cell functionality, some of the results are not consistent with established roles of HIF-1 and hypoxia in the regulation of dermal fibroblasts functions, particularly in human skin. The
reasons for these differences are not very clear and may be indicative an inability of primary cells to adapt well to oxidative stress in culture conditions. Furthermore, adult primary MDFs, particularly from Siah2−/− mice, proved difficult to establish in culture, often displaying premature senescence and early differentiation at low passage number. Therefore it may be difficult to rule out the impact of problematic or inconsistent culture conditions in our experimental observations. Furthermore others have pointed out differences in the behaviour of hypoxic fibroblasts even from different regions of the dermis, different tissue (Modarressi et al., 2010), different culture conditions (Falanga and Kirsner, 1993, Boraldi et al., 2007, Oberringer et al., 2008) and therefore differentiation status of the cells are often used, making interpretation of data from fibroblast hypoxic cultures in the literature problematic. Additionally, most studies reporting effects of hypoxia on dermal fibroblasts have mostly being reported in adult human dermal fibroblasts with little evidence of similar effects on adult murine dermal fibroblast cultures as far as we know. To examine the role of Siah proteins in human fibroblasts, we then used the drosophila protein phyllopod in cell culture experiments. Thus, in the next chapter the role of Siah was investigated in healthy adult human dermal fibroblasts which in contrast to mouse dermal cells appeared to thrive in normoxic and hypoxic cultures condition with low serum without any overt or reportable signs of stress or early senescence (Chapter 5).

4.4. Conclusion

In summary, this study confirms that Siah is a positive regulator of HIF-1α stabilisation in hypoxia (Nakayama et al., 2004). Reduced HIF-1α may therefore partly explain reduced COL1A1 and αSMA induction in the absence of Siah2 implying that Siah2 may in part regulate granulation formation and myofibroblast differentiation during tissue hypoxia. Siah2 may also have a regulatory role in TGFβ1-induced COL1A1 expression and cell migration beyond that observed in a hypoxic milieu. Siah2 deficiency does not appear to have a significant effect on cell proliferation and TGFβ1 induced Smad phosphorylation. Therefore the regulatory effects of Siah on dermal fibroblast cell functionality are likely
to occur downstream of Smad phosphorylation or via non Smad dependent pathways possibly through Ras mediated mechanisms or HIF-1-Smad cross talk as discussed above. Overall, Siah2 through HIF-1 and possibly other mechanisms appears to be a positive regulator of dermal fibroblast cell function which may in part explain why early wound healing is delayed in Siah2\(^{-/-}\) mice (Chapter 3).

4.5. **Specific experimental methods in this chapter.**

Mentioned here are summarized methods including only relevant details pertaining to this chapter. For the specific of individual methods, please refer to the general and methods chapter (Chapter 2)

4.5.1. **Fibroblast isolation and culture**

Primary MDFs were isolated from the tails of age- (8-12 week) and sex- (male only) matched wild type (Wt) and Siah2 homozygous mutant (Siah2\(^{-/-}\)) mice using previously described enzymatic digestion methods with minor modifications. Tails were collected and dermis was isolated using dispase (10mg/ml) (Gibco). Cells were isolated in collagenase solution (dispase (4mg/ml)/collagenase (Gibco) (3mg/ml)) and cultured in Dulbecco Modified Eagle media (DMEM) supplemented with 5% Fetal calf serum (FBS) in standard culture conditions (5% CO\(_2\), 21% O\(_2\) in humidified chamber). MDFs were used between 2\(^{nd}\) to 4\(^{th}\) passages only, as viability, growth and normal morphology of cells dramatically declined after the 4\(^{th}\) passage.

4.5.2. **Analysis of protein expression by western blotting**

Protein expression was determined by means of SDS-PAGE and western blot analysis. Briefly, Siah2\(^{-/-}\) and Wt MDFs cells were seeded into flasks (25cm\(^2\)) at normalized cell density and grown until 70-80 % confluent. Cells were subjected to experimental conditions after which protein from cells was extracted by detergent (Triton-x100) based cell lysis and protein content measured using a bicinchoninic acid assay (BCA assay) kit. Protein samples were mixed with loading buffer and reducing agent and then
denatured at 90°C for 10 minutes and cooled on ice before loading. Denatured protein samples were loaded on SDS PAGE gels (10% acrylamide) and separated at 160 to 200 Volts direct current for 45 to 50 minutes in ice-cold running buffer (0.025M Tris glycine and 0.1% SDS). Proteins were transferred on to either PVDF (Bio-Rad, Aus) or nitrocellulose (Bio-Rad, Aus) membranes that were electrolyte soaked (0.025M Tris & glycine and 20% methanol) using a semi-dry transfer cell (Bio-Rad laboratories, USA) at 19 volts direct current for 75 minutes. Primary antibodies were added onto the membrane and incubated overnight (approximately 16 hours) at 4°C. Membranes were washed in TBST (0.01M Tris Base Saline buffer, 0.05% (v/v) Tween 20, pH 7.6) for 30 minutes. HRP-conjugated secondary antibodies (1:1000) were added to membranes for 60 minutes at room temperature and washed off thoroughly in TBST. Protein bands on membranes were visualised via peroxidase -catalysed chemiluminescence reaction detected using a Chem-Doc ® unit (Bio-Rad). Protein band density was analysed by densitometry.

4.5.2.1. Preliminary hypoxia treatment

To determine the effect of Siah2 on HIF-1α stabilisation and type I collagen over time in hypoxia, Wt and Siah2/− MDFs at matching passages, confluence (80%) and normal serum (5% FBS) were incubated in hypoxia (1% O₂, 5% CO₂ humidified) for increasing length of time (0 to 48 hours). Protein from cells was extracted and proteins levels determined by western blot analysis using anti-COL1A1 (1:500) and anti-HIF-1α (1:500) in normalised amounts of loaded proteins (10µg and 20 µg per well, respectively).

4.5.2.2. Hypoxia and TGFβ1 treatment

To determine whether Siah2 had any effect on TGFβ1 or hypoxia induced MDF functionality (extracellular matrix expression and TGFβ1 signalling transduction) MDFs were treated with TGFβ1 (5ng/ml), hypoxia (1% O₂, 5% CO₂ humidified) or the combination of hypoxia supplemented with TGFβ1 for a period of 48 hours. Protein from cells was extracted and protein levels determined by western blot analysis using
anti-COL1A1 (1:500), anti-Smad2 (1:1000) and anti-p-Smad (1:1000) using normalised amounts of loaded proteins (10µg per well).

4.5.3. Analysis of protein expression by flow cytometry

Protein expression was also determined by means of immuno-labelling and subsequent flow cytometry. Briefly, Siah2<sup>−/−</sup> and Wt MDFs cells were seeded into flasks (25cm<sup>2</sup>) at normalized cell density and grown until 70-80% confluent. Cells were serum starved for 6 hours and later supplemented with low serum media (2% FBS) with or without TGFβ1 (5ng/ml). Cells were incubated in normoxia or hypoxia for 48 hours. Cells were detached by trypsin, fixed in formalin and permeabilized. Immuno-labelling of cells with mouse monoclonal anti-αSMA-FITC conjugated (1:250) and mouse monoclonal anti-PCNA (1:200). Fluorescently conjugated secondary anti-rabbit and anti-mouse antibodies were used to probe their respective primary antibodies (collagen I and Smads). The fluorescence intensity of at least 2000-5000 cells per sample was measured using a flow cytometer and analyzed by weasel cytometry software (Walter and Eliza Hall Institute, Aus)

4.5.4. Collagen lattice contraction assay

To determine if Siah2 deficiency had any impact on fibroblast contractile properties Siah2<sup>−/−</sup> and Wt MDFs were counted and seeded into collagen (2.1mg/ml) at normalized density (2 x 10<sup>5</sup>cell/ml). Lattices were allowed to polymerize and then recover for 24 hours in normal cell culture conditions (5% FBS). Cells were serum starved (2% FBS) for 6 hour and later supplemented with either with low serum media or TGFβ1 (5ng/ml). Cells were incubated in normoxia or hypoxia for up 24 hours. Lattices were released and returned to their respective experimental conditions for a further 24 hours. The area of lattices was measured and contraction calculated as change in area (pixels<sup>2</sup>).
4.5.5. **Migration of murine dermal fibroblasts**

MDFs cells were seeded into 6 well plates at normalized cell density and grown until 80-90% confluent. Cells were then serum starved for 6 hours and scratched using a micropipette tip (1 ml) and then supplemented either with low serum medium or low serum medium with added TGFβ1 (1ng/ml). Cells were incubated in normoxia or hypoxia overnight (16 hours). Migration was determined by measuring the difference in the area of the gap at time 0 versus time post-wounding.
Chapter 5

Transient inhibition of Siah and its effect on human fibroblast function
5.1. Introduction

It is now well established that during hypoxia, HIF-1α escapes proteosomal degradation through the inhibitory effects of Siah ubiquitin ligases on PHD and FIH activity (Nakayama et al., 2004, Fukuba et al., 2007). In this context, Siah deficiency thus results in reduced HIF-1α availability and transcription of HIF-1 inducible genes (Nakayama et al., 2004). Previous chapters (3 and 4) have investigated the role of Siah2 in wound healing \textit{in vivo} and \textit{in vitro}, using a murine Siah2\textsuperscript{-/-} transgenic mouse model and cells derived from these animals. In this chapter human dermal fibroblasts were transiently transfected with phyllopod expressing vectors (PHYL) to investigate the potential effects of reduced Siah-HIF-1 mediated signalling on human dermal fibroblast function \textit{in vitro}. PHYL has the added advantaged of inhibiting both Siah 1 and 2, potentially giving a more complete inhibition of Hif-1α stabilization.

Phyllopod is a \textit{drosophila melanogaster} protein that controls drosophila photoreceptor cell fate via the ras activated pathway during development (Dickson et al., 1995). The protein binds with high affinity to the substrate binding domain (SBD) of SINA, a drosophila homologue of mammalian Siah (Li et al., 2002). PHYL promotes degradation of Tramtrack, through SINA-induced E3 ligase activity by acting as an adaptor protein between SINA and Tramtrack (Li et al., 2002). PHYL mammalian homologues do not, as far as we know, exist and therefore it can be introduced in mammalian cells systems to inhibit Siah activity and subsequent ubiquitination of its substrates including HIF-prolyl-hydroxylases (Moller et al., 2008). Consequently, PHYL transfected mammalian cells show reduced HIF-1α stabilization and reduced transcription of HIF-1 inducible genes during hypoxia (Moller et al., 2008).
5.2. Results

5.2.1. Phyllopod expression in transfected human dermal fibroblasts

To verify successful transfection of cells with a plasmid containing Phyllopod, HA-labelled Phyllopod (PHYL) transfected human dermal fibroblasts (HDFs) were stained with an anti-HA antibody and detected by immunofluorescence. HA-labelled cells showed a perinuclear distribution of the stain when compared to control HDFs (transfected with empty pcDNA plasmid which showed only low background staining (Figure 5-1a).
Figure 5-1. Detection of phyllopod expression in human dermal fibroblasts by immunofluorescence. Visualisation of HDFs transfected with empty pcDNA vector (a) and PHYL-HA expressing pcDNA Vector (b) with rabbit anti-HA (1:300) and anti-rabbit IgG-Alexa-fluor 488®. White arrows show perinuclear staining of HA-labelled cells (Magnification, x40).
5.2.2. Reduced HIF-1α in phyllopod transfected human dermal fibroblasts

In mammalian cells, PHYL binds to the substrate binding domain of Siah proteins (both Siah1 and Siah2) impairing their ability to tag HIF-hydroxylases (PHDs and FIH) for proteosomal degradation, thus preventing the stabilisation of HIF-1α during hypoxia (Moller et al., 2008, Khurana et al., 2006). To investigate whether downstream Siah activity had been inhibited following PHYL transfection, HIF-1α expression was determined in HDFs (Mock and PHYL transfected cells) by flow cytometry following treatment cells with TGFβ1 (5ng/ml), hypoxia (1%O₂) or the combination of the two (Figure 5-2). Forward and side scatter characteristics of HDFs were used for their preliminary identification and appropriate gates were further used to localise HDF cells and exclude cellular aggregates and debris. HIF-1α induction or stabilisation was evaluated by a stimulation index, which was the ratio of mean fluorescence intensity of HIF-1α in treated HDFs to that of untreated HDFs. HIF-1α stimulation index in control HDFs (Mock) remained relatively unchanged in TGFβ1 (5ng/ml) and hypoxia alone (1% O₂) treated cells (1.04 ± 0.02 and 1.01 ± 0.07, respectively) but decreased by 15% (0.85 ± 0.10) in combined hypoxia and TGFβ1. In PHYL transfected HDFs, HIF-1α stimulation index decreased by 11% (0.892 ± 0.08) in TGFβ1 treatment alone, decreased by 48% (0.52 ± 0.07) in hypoxia and decreased by 54% (0.46 ± 0.12) in TGFβ1 plus hypoxia. The stimulation index of HIF-1α in PHYL transfected HDFs was significantly reduced in hypoxia (p<0.01) and in hypoxia plus TGFβ1 (P<0.05) conditions when compared the mock HDFs. These results demonstrate the inhibitory effect of PHYL on HIF-1α availability in hypoxia by virtue of impairing Siah ubiquitin ligase activity, as previously described (Khurana et al., 2006, Moller et al., 2008, Qi et al., 2008) and its implications on hypoxia-induced HDF function was further tested below.
Figure 5-2. Effect of Phyllopod on HIF-1α induction or stabilisation in human dermal fibroblasts in response to hypoxia. (a.) Flow cytometry histograms of HIF-1α expression in mock and PHYL transfected HDFs. Overlays; black (secondary antibody only) grey shaded (untreated group), red (5ng/ml TGFβ1), blue (hypoxia (1%O₂) and yellow (TGFβ1 and hypoxia combined). (b.) Dot plot of FSC versus SSC of HDFs and the cells gated for analysis are shown. (c.) Analysis of HIF-1 induction or stabilisation in Mock (dotted bars) and PHYL transfected cells using a stimulation index, representing the ratio of mean fluorescence intensity of HIF-1α in treated cells (TGFβ1 (N+), hypoxia (H) or combination of both (H+) after 48 hours) to that untreated (N) cells. Each point represents mean ± standard error of the mean of three separate experiments (c). Bonferroni’s two way ANOVA analysis performed to determine significance. ** P<0.01, *P<0.05
5.2.3. Reduced VEGF expression in Phyllopod transfected human dermal fibroblasts

Activation of VEGF gene expression during hypoxia is transcriptionally regulated through HIF-1 (Forsythe et al., 1996). In HDF cells the production of VEGF in hypoxia has been reported to also require stimulation from TGFβ (Berse et al., 1999). To investigate the effects of Siah on TGFβ induced VEGF release by HDF, cell culture media was collected from PHYL and mock transfected cells and analysed using a human VEGF ELISA (Figure 5-3). In comparison to the untreated group (0.13 ± 0.03ng/ml) VEGF release in mock transfected HDFs was increased by 46% (0.19 ± 0.02ng/ml) in TGFβ1 (5 ng/ml), decreased by 47% (0.07± 0.03 ng/ml) in hypoxia and increased by 261% (0.47 ± 0.04 ng/ml) after combined treatment with both hypoxia and TGFβ1. In comparison to the respective untreated group (0.20 ± 0.02 ng/ml), VEGF release in PHYL transfected cells decreased by 30% (0.14 ± 0.05 ng/ml) in TGFβ1 treated cells, decreased by 65% (0.07 ng/ml) in hypoxia and increased by only 50 % (0.30 ± 0.04 ng/ml) following treatment with both TGFβ1 and hypoxia. In normoxia, VEGF release following TGFβ1 treatment was not significantly different between PHYL and mock transfected HDFs. However, following combined treatment with TGFβ1 and hypoxia, a significant reduction (p<0.01) in VEGF release was observed in PHYL transfected cells (versus mock transfected control HDFs). The results demonstrate that the Siah and HIF-1α axis is important for TGFβ induced VEGF release in hypoxic HDFs.
Figure 5-3: Effect of Phyllopod on TGFβ1 induced VEGF release by human dermal fibroblasts. Analysis of VEGF production and release in mock and phyllopod (PHYL) transfected human dermal fibroblasts (HDFs) after 48 hours treatment in TGFβ1 (N+), hypoxia (1% O₂) (H) and hypoxia plus TGFβ1 (H+) as well untreated controls (N). Each point represents mean ± standard error mean of three separate experiments. Bonferroni’s Two way ANOVA analysis performed ** P<0.01.

5.2.4. Reduced COL1A1 expression in phyllopod transfected human dermal fibroblasts

Increased expression and release of type I collagen is a hallmark response of human dermal fibroblasts during acute hypoxia (Siddiqui et al., 1996, Steinbrech et al., 1999, Falanga et al., 2002). This response is reliant on the up-regulation of TGFβ which can also be regulated through HIF-1 (Falanga et al., 2002, Distler et al., 2007). To determine whether Siah-HIF-1 axis inhibition by PHYL alters extracellular matrix synthesis by HDFs, flow cytometry using an anti-COL1A1 antibody was completed (Figure 5-4). Forward and side scatter characteristics of HDFs were used for their preliminary identification and
appropriate gates were further used to localise HDF cells and exclude cellular aggregates and debris. COL1A1 induction was evaluated by a stimulation index, which was the ratio of mean fluorescence intensity of COL1A1 in treated HDFs to that of untreated HDFs. The stimulation index of COL1A1 in mock transfected HDFs was increased by 98% after TGFβ1 (5 ng/ml) (1.98 ± 0.28), increased by 50% after hypoxia (1.50 ± 0.17) and increased by 128% in TGFβ1 treatment plus hypoxia (2.28 ± 0.59). The stimulation index of COL1A1 in PHYL transfected fibroblasts was increased by 49% in TGFβ1 (1.49 ± 0.15), reduced by 22% in hypoxia (0.78 ± 0.15) and increased by 32% after TGFβ1 plus hypoxia (1.32 ± 0.15). TGFβ1 alone did not induce a significant change in the stimulation index of COL1A1 in PHYL transfected HDFs (compared to mock transfected fibroblasts). However significant reductions in stimulation index of COL1A1 were observed in hypoxia (p<0.01) and hypoxia plus TGFβ1 treated PHYL transfected HDFs (versus mock cells). The results reflect the role of Siah-HIF-1 axis in the stimulation of collagenous matrix synthesis under hypoxia conditions, a process that is described to be TGFβ-dependent and important for angiogenesis and granulation tissue formation (Steinbrech et al., 1999, Falanga et al., 2002). The findings are also in line with other studies that have demonstrated the importance of HIF-1 in the regulation of TGFβ-induced matrix expression and synthesis by HDFs during hypoxia (Distler et al., 2007).
Figure 5-4. Effect of phyllopod on induction of COL1A1 in human dermal fibroblast. (a.) Flow cytometry histogram of COL1A1 expression in mock and PHYL transfected HDFs. Overlays; black (secondary antibody only), grey shaded (untreated group), red (5ng/ml TGFβ1), blue (hypoxia (1%O₂)) and yellow (TGFβ1 and hypoxia combined). (b.) Dot plot of FSC versus SSC of HDFs and the cells gated for analysis are shown. (c.) Analysis of COL1A1 induction in Mock (dotted bars) and PHYL transfected cells (non dotted bars) using a stimulation index, representing the ratio of mean fluorescence intensity of COL1A1 in treated cells (TGFβ1 (N+), hypoxia (H) or combination of both (H+) after 48 hours) to that untreated (N) cells.. Each point represents mean ± standard error of the mean of six separate experiments. Bonferroni’s two way ANOVA analysis was performed**P<0.01, *P<0.05
5.2.5. Normal αSMA expression and myofibroblast transformation in phyllopod transfected human dermal fibroblasts

Transient expression of αSMA in the granulation tissue during fibroblast-myofibroblast transition is a hallmark of normal wound healing and is synonymous with wound contraction (Darby et al., 1990). TGFβ1 induces αSMA expression and myofibroblast transition in quiescent and growing cultured fibroblasts (Desmouliere et al., 1993). To investigate the impact of Siah-deficiency on fibroblast-myofibroblast transition in human skin fibroblasts during hypoxia, an anti αSMA antibody was used and flow cytometry analysis completed (Figure 5-5). Forward and side scatter characteristics of HDFs were used for their preliminary identification and appropriate gates were further used to localise HDF cells and exclude cellular aggregates and debris. αSMA induction was evaluated by a stimulation index, which was the ratio of mean fluorescence intensity of αSMA in treated HDFs to that of untreated HDFs. In the TGFβ1 (5 ng/ml) treated group, αSMA stimulation index was increased by 182% (2.82 ± 0.31) in mock and by 191% (2.91 ± 0.66) in PHYL transfected HDFs. In hypoxia alone, αSMA stimulation index in mock HDFs was increased by 47% (1.47 ± 0.81) but remained mostly unaffected in PHYL transfected cells (1.00 ± 0.301). In the hypoxia plus TGFβ1 group, the αSMA stimulation index increased by 65% (1.65 ± 0.26) in mock and by 32% (1.32 ± 0.18) in PHYL transfected cells. Overall, no significant differences in the pattern of αSMA induction were observed between the different treatment groups following PHYL transfection. Interestingly, hypoxia appeared to have an overall inhibitory effect on TGFβ-induced αSMA expression in both PHYL and mock transfected HDFs. The result demonstrates that Siah-HIF-1 axis may be dispensable in the regulation of αSMA expression in human skin fibroblasts. Furthermore, the findings are similar to those of recently published research which shows that hypoxia impairs human skin fibroblast to myofibroblast differentiation depending on the duration of hypoxia and the source of fibroblasts (Modarressi et al., 2010).
Figure 5-5. Effect of Phyllopod on αSMA induction in human dermal fibroblasts. (a.) Flow cytometry histograms of αSMA expression in mock and PHYL transfected HDFs are shown. Overlays; black (secondary antibody only), grey shaded (untreated group), red (5 ng/ml TGFβ1), blue (hypoxia 1%O₂) and yellow (TGFβ1 and hypoxia combined). (b.) Dot plot of FSC versus SSC of HDFs and the cells gated for analysis are shown. Analysis of αSMA induction in Mock (dotted bars) and PHYL transfected cells using a stimulation index, representing the ratio of mean fluorescence intensity of αSMA in treated cells (TGFβ1 (N+), hypoxia (H) or combination of both (H+) after 48 hours) to that untreated (N) cells. Each point represents mean ± standard error mean of six separate samples. Bonferroni’s two way ANOVA analysis performed to determine significance.
5.2.6. Collagen lattice contraction by PHYL transfected human dermal fibroblasts

Wound contraction is associated with the presence of modified fibroblasts in the granulation tissue and the transient expression of αSMA by these cells (Gabbiani et al., 1971, Darby et al., 1990). TGFβ, extracellular matrix content and mechanical tension are also central to this contractile process (Montesano and Orci, 1988, Arora et al., 1999, Desmouliere et al., 1993). Hypoxia (chronic) has been associated with impaired αSMA expression and reduced dermal fibroblast contraction (Modarressi et al., 2010) (Figure 5-5). The role of acute hypoxia and Siah-HIF-1 axis in HDF-mediated contraction of collagen lattices has not yet described. To investigate this PHYL-transfected HDFs were seeded into three dimensional collagen lattices and exposed to TGFβ1, hypoxia or a combination of both for 48 hours (Figure 5-6). Contraction was calculated as the change in area between time 0 and 48 hours. Untreated mock and PHYL transfected HDFs showed similar levels of contraction (60.8 ± 18.5 mm$^2$ and 63.1 ± 15.6 mm$^2$, respectively). PHYL transfected HDFs showed some reduction in contractile response following treatment with TGFβ (77.9 ± 25.3 mm$^2$), hypoxia (51.6 ± 14.03 mm$^2$) and hypoxia plus TGFβ1 (72.4 ± 25.6 mm$^2$) when compared to mock transfected HDFs exposed to identical conditions (96.0± 16.4 mm$^2$, 69.5 ± 9.75 mm$^2$ and 96.9 ± 15.7 mm$^2$, respectively) however the observations were not statistically significant due to the high variability in results. Overall the results do not clearly support any role for Siah-HIF-1 axis in the HDF contraction process.
Figure 5-6. Effect of phyllopod on TGFβ1-induced dermal fibroblast contraction. (a.) Images of contracted collagen lattices (outline indicated by yellow dashed line) seeded with mock and PHYL transfected HDFs following 48 hours of normoxia alone (N) or treatment with either TGFβ1 (5ng/ml) (N+), hypoxia (1%O₂) (H) or combined hypoxia and TGFβ1 (H+) are shown. (b.) Analysis of contraction in mock (black bars) and PHLY transfected HDFs(grey bars), where contraction was calculated as change in area (Δmm²) between time 0 and 48 hours. Each data point represents a mean ± standard error of the mean of six separate samples. Bonferroni’s two way ANOVA analysis was performed to determine significance.
5.2.7. Normal HDF proliferation in hypoxic conditions

Hypoxia has previously been demonstrated as a potent stimulus for the proliferation and expansion of human adult and neonatal dermal fibroblasts seeded as single cells (Falanga and Kirsner, 1993). To investigate the effect of acute hypoxia and Siah-HIF-1 axis on HDF proliferation, PHYL transfected cells were exposed to TGFβ (5ng/ml), hypoxia (1% O₂) or both for 48 hours and proliferative activity determined by an anti PCNA antibody and flow cytometry analysis (Figure 5-7). Forward and side scatter characteristics of HDFs were used for their preliminary identification and appropriate gates were further used to localise HDF cells and exclude cellular aggregates and debris. Proliferative activity was evaluated by a stimulation index, which was the ratio of mean fluorescence intensity of PCNA in treated HDFs to that of untreated HDFs PCNA stimulation index in both mock and PHYL transfected HDFs appeared not to be altered by TGFβ (0.91 ± 0.01 and 1.01 ± 0.10 respectively), hypoxia (1.03 ± 0.04 and 1.08 ± 0.16, respectively) or combined treatment with TGFβ1 and hypoxia (1.04 ± 0.09 and 1.37± 0.02, respectively). Furthermore, no significant differences were observed between PHYL and mock transfected cells in all treatment groups. This study may support the findings of other groups who demonstrate little or no effect of hypoxia on the regulation of dermal fibroblast proliferation, an indication of their ability to survive and proliferate under hypoxic conditions (Boraldi et al., 2007). However an inability of PCNA to detect small proliferative differences following TGFβ treatment (where we have seen increases in mouse fibroblast proliferation) raises some questions about the sensitivity of the assay in determining proliferation activity in HDF monolayer cultures where cell doubling is perpetual until full confluence is achieved. To this end, another proliferation assay that detects fewer cells and is more strictly an S phase marker, such as bromodeoxyuridine incorporation might be more useful and more able to detect small changes in proliferation rate.
Figure 5-7. Effect of Phyllopod on proliferation activity of human dermal fibroblasts in hypoxic conditions. (a.) Flow cytometry histograms of typical PCNA expression in mock and PHYL transfected HDFs are shown. Overlays represent black (secondary antibody only), grey shaded (untreated group), red (5 ng/ml TGFβ1), blue (hypoxia (1%O_2)) and yellow (combined TGFβ and hypoxia). (b.) Dot plot of FSC versus SSC of HDFs and the cells gated for analysis are shown. (c.) Analysis of proliferative activity in Mock (dotted bars) and PHYL transfected cells (non dotted bars) using a stimulation index, representing the ratio of mean fluorescence intensity of PCNA in treated cells (TGFβ1 (N+), hypoxia (H) or combination of both (H+) after 48 hours) to that of untreated (N) cells. Each point represents mean ± standard error of the mean of three separate experiments. Bonferroni’s two way ANOVA analysis was performed to determine significance.
5.2.8. Phyllopod impairs migration of human dermal fibroblasts

Migration of fibroblasts during ischaemic conditions is considered to be essential during wound healing. Hypoxia increases the migratory index of human dermal fibroblasts compared to normoxia and has combined effects in the presence of TGFβ (Mogford et al., 2002). To investigate the role of Siah-HIF-1 axis on HDF migration, confluent PYHL transfected HDFs monolayer cultures were wounded by a scratch and allowed to migrate/heal in the presence of TGFβ (0.5 ng/ml) and/or hypoxic conditions overnight (16 hours) and compared to mock transfected HDFs exposed to the same conditions. Fibroblast migration was determined as the change in area (Pixels$^2 \times 1000$) of the original scratch to that of the remaining gap after exposure to experimental conditions (Figure 5-8). Under untreated normoxic culture conditions, PHYL and mock transfected HDFs showed no significant difference in migration (24.1 ± 0.98 and 20.0 ± 2.38 Pixels$^2$). However, PHYL transfected HDF showed a significantly reduced response to TGFβ1 induced migration under normoxic culture conditions (15.2 ± 1.34 pixels$^2$, P<0.01) compared to mock cells which showed increased migration (31.6 ± 2.36 pixels$^2$). Furthermore, PHYL transfected cells showed significantly reduced migration under both hypoxia (15.2 ± 2.44 pixels$^2$, P<0.05) and TGFβ supplemented hypoxia conditions (18.5 ± 2.89 pixels$^2$, P<0.01) compared to migration in mock transfected cells where increases in migration were observed (27.1 ± 3.48 pixels$^2$ and 36.1 ± 4.25 pixels$^2$ respectively). These results suggest that Siah plays an important role in hypoxia-induced migration, as well as in TGFβ-induced migration under both normoxic and hypoxic conditions.
Chapter 5

(a) Images showing cell migration over time with different treatments. The top row is labeled as $T_0$, followed by $N+$, $H$, and $H+$. The images on the left are labeled as Mock, and the images on the right as PHYL.

(b) Bar graph showing migration index for different treatments. The treatments Include N, N+, H, and H+. The graph includes error bars and statistical significance symbols: * for $P<0.05$ and ** for $P<0.01$. The legend indicates that black bars represent Mock and gray bars represent PHYL.
Figure 5-8. Effect of Phyllopod on TGFβ- and hypoxia-induced human dermal fibroblast migration. (a.) Images of cell monolayer scratch migration assay were used to determine migration capabilities of PHYL transfected HDFs (versus mock transfected cells) in TGFβ (N+) and hypoxia (H) or hypoxic conditions supplemented with TGFβ (H+). Scratched untreated cells served as experimental controls (N). (b.) Analysis of migratory of mock (black bar) and PHYL (grey bars) transfected HDFs, where cell migration was determined through change in pixel area ($\text{Pixels}^2$) of the original scratch at 0 hours ($T_0$) and wound area post exposure to experimental or control conditions for 16 hours. Each point represents mean ± standard error of the mean of three separate experiments. Bonferroni’s two way ANOVA statistical analysis was performed to determine significance. *$P<0.05$, **$P<0.01$

5.2.9. Effect of phyllopod on TGFβ1 signalling cascade

Cellular responsiveness to TGFβ1 is reliant on the growth factor binding to its receptor TGFRII followed by recruitment of TGFRI leading to phosphorylation of receptor activated Smad proteins and subsequent induction of TGFβ regulated genes (Massague, 1998, Piek et al., 1999, Massague and Chen, 2000, Massague and Wotton, 2000). TGFβ and its downstream signalling cascades induce important fibroblasts functions during hypoxic conditions including cell migration, extracellular matrix synthesis, and VEGF release (Berse et al., 1999, Mogford et al., 2002, Falanga et al., 2002). To investigate the potential role of Siah on TGFβ-signalling cascade TGFβR1 expression (Figure 5-9) and phosphorylation of Smad2 (Figure 5-10) in PHYL transfected HDFs was determined using respective primary antibodies (anti-TGFR1 and anti-Smad2 and anti-phospho Smad2), detection was then performed using flow cytometry. Forward and side scatter characteristics of HDFs were used for their preliminary identification and appropriate gates were further used to localise HDF cells and exclude cellular aggregates and debris. TGFR1, p-Smad2 and Smad2 induction was evaluated by a stimulation index, which was the ratio of mean fluorescence intensity of the respective proteins in treated HDFs to that of untreated HDFs. Under normoxic conditions, TGFβ induced a similar increase in
TGFR1 stimulation index in PHYL and mock transfected cells (1.21 ± 0.11 and 1.35 ± 0.13 respectively). However in hypoxia alone, PHYL transfected cells (0.72 ± 0.10) showed a reduction in TGFR1 stimulation index compared to mock transfected HDFs (0.98 ± 0.09), albeit that the difference observed was not significant. Under hypoxic conditions supplemented with TGFβ, PHYL transfected cells showed a significant reduction in the TGFR1 stimulation index (1.11 ± 0.08, P<0.05) compared to mock transfected cells where TGFβ and hypoxia appeared to synergistically increase the TGFR1 stimulation index (1.50 ± 0.13). The result implies a slight impairment in TGFR1 induction in PHYL transfected cells during hypoxic conditions. Downstream of TGFR1, p-Smad2 stimulation index (Figure 5-10) appeared to be reduced in PHYL transfected HDFs following treatment of cells in TGFβ (1.22 ± 0.08), hypoxia (1.22 ± 0.10) and hypoxic conditions supplemented with TGFβ (0.85 ± 0.21) compared to mock transfected cells (1.60 ± 0.16, 1.67 ± 0.19 and 1.33 ± 0.25 respectively). The stimulation index of total Smad2 was also analysed and appeared to be unchanged between PHYL and mock transfected cells across all treatment groups (Figure 5-10).
Figure 5-9. Effect of phyllopod on TGFR1 induction in human dermal fibroblasts. (a.) Flow cytometry histograms of TGFR1 expression in mock and PHYL transfected HDFs. Overlays represent black (secondary antibody only), grey shaded (untreated), red (5ng/ml TGFβ1), blue (hypoxia (1%O₂) and yellow (combined TGFβ1 and hypoxia). (b.) Dot plot of FSC versus SSC of HDFs and the cells gated for analysis are shown. (c.) Analysis of TGFR1 induction in Mock (dotted bars) and PHYL transfected cells (non dotted bars) using a stimulation index, representing the ratio of mean fluorescence intensity of TGFR1 in treated cells (TGFβ1 (N+), hypoxia (H) or combination of both (H+) after 48 hours) to that untreated (N) cells. Each point represents mean ± standard error of the mean of three separate experiments. Bonferroni’s two way ANOVA analysis was performed to determine significance. *P<0.05
Figure 5-10. Effect of phyllopod on phosphorylated Smad2 (phospho-Smad2) induction. (a) Flow cytometry histograms of phosphorylated Smad2 expression in mock and PHYL transfected HDFs are shown. Overlays represent black (secondary antibody only), grey shaded (untreated), red (5 ng/ml TGFβ1), blue (hypoxia (1%O₂)) and yellow (combined TGFβ1 and hypoxia). (b) Dot plot of FSC versus SSC of HDFs and the cells gated for analysis are shown. (c.) Analysis of P-Smad2 and (d.) Smad2 induction in Mock and PHYL transfected cells using a stimulation index, representing the ratio of mean fluorescence intensity of P-Smad2 and Smad2 in treated cells (TGFβ1 (N+), hypoxia (H) or combination of both (H+) after 48 hours) to that untreated (N) cells. Each point represents mean ± standard error of the mean of three separate experiments. Bonferroni’s two way ANOVA analysis was performed to determine significance.
5.3. Discussion

5.3.1. Hypoxia through HIF-1 regulates human skin fibroblasts

Hypoxic microenvironments stimulate chemotaxis and various functions of several cell types that modulate important events involved in healing including inflammation, re-epithelialisation, formation of granulation tissue and angiogenesis (Knighton et al., 1983, O'Toole et al., 1997, Knighton et al., 1981, Falanga et al., 2002). Dermal fibroblasts are central in the regulation of granulation tissue and its eventual remodelling and modulate the angiogenic process through the release of extracellular matrix and paracrine factors that are required for both endothelial adhesion and chemotaxis during wound healing. In addition to soluble factors and matrix proteins released during inflammation, hypoxia has emerged as an important regulator of dermal fibroblast functionality during wound healing with the prevailing view that hypoxia promotes fibroblast chemotaxis, production of autocrine and paracrine factors as well as extracellular matrix which ultimately accelerate wound healing, through the promotion of granulation tissue formation and angiogenesis. (Falanga and Kirsner, 1993, Falanga et al., 2002, Siddiqui et al., 1996, Steinbrech et al., 1999, Berse et al., 1999, Hong et al., 2006). HIF-1 regulated pathways are essential for cellular adaptive responses to hypoxia and have important regulatory effects on HDF functionality.

HIF-1 transcriptional activity is tightly regulated through several proteins which control post-translational availability of the HIF-1α subunit. Siah proteins are E3 ubiquitin ligases that tag HIF-prolyl hydroxylases for degradation during hypoxia and thus positively regulate HIF-1α availability and the expression of hypoxia inducible genes (Nakayama et al., 2004, Fukuba et al., 2007). In previous chapters (3 and 4) the relevance of the Siah-HIF-1 axis in normal cutaneous wounding was demonstrated in both in vivo and in vitro models using Siah2 knockout transgenic murine models of wounds or cultured fibroblasts. The studies provided evidence that Siah is important in the regulation of angiogenesis and granulation tissue formation and possibly in the regulation of
fibroblast function during hypoxia. Here, the role of Siah in human fibroblast function is described for the first time by using transfection of Phyllopod (PHYL) to otherwise healthy human dermal fibroblasts (Figure 5-1). PHYL protein has the added advantage of inhibiting both Siah 1 and 2 activity (Moller et al., 2008).

5.3.2. Siah inhibition impairs important human dermal fibroblast function.

In Chapter 3, HIF-1α protein stabilization in wild type dermal fibroblasts (Siah2+/+)

appeared to be parabolic over time in hypoxia, peaking after about 8 hours in hypoxia

and gradually returning to baseline levels with increasing time in hypoxia. In contrast

Siah2−/− knockout cells showed reduced HIF-1α stabilization over time in hypoxia where

expression remained below baseline levels (up to 48 hours), demonstrating that Siah2

was required for increased stabilization and maintenance of HIF-1α abundance in

hypoxia (Figure 4-1). Here, evidence is provided to show that PHYL abrogates HIF-1

availability in hypoxia (Figure 5-2) and that this has important downstream effects on

HDF cell functionality during hypoxic conditions. Importantly, Siah inhibition impairs

TGFβ induced responsiveness in normoxic conditions, suggesting that Siah may also

have an important role in regulation of fibroblast functionality beyond the hypoxic

milieu.

VEGF in arguably the most important angiogenic factor during wound healing. Its

expression is up-regulated in hypoxic cells as part of feedback mechanisms that lead

eventually to angiogenesis and restoration of oxygen levels. HIF-1 is essential in the

regulation and activation of VEGF transcription in hypoxic cells (Forsythe et al., 1996). In

addition to hypoxia, enhanced VEGF expression by endothelial cells, macrophages and

fibroblasts requires simultaneous activation by cytokines particularly, TGFβ1 (Sanchez-

Elsner et al., 2001, Jeon et al., 2007, Berse et al., 1999). Evidence suggests that an

important cross-talk between TGFβ and hypoxia induced pathways exist, likely involving

a physical interaction between Smad and HIF1α proteins at the promoter region of the

VEGF gene, which is required to enhance VEGF expression and release by hypoxic cells

(Sanchez-Elsner et al., 2001, Jeon et al., 2007).
Here, the role of Siah-HIF-1 axis on TGFβ induced VEGF release by HDFs was investigated (Figure 5-3). The results showed that VEGF release in response to TGFβ is reduced in hypoxic PHYL transfected cells (versus mock HDFs). The finding demonstrates that Siah may be important in regulation of enhanced VEGF release in hypoxia, presumably through regulation of HIF-1α, which is central to the TGFβ and hypoxia pathway crosstalk (Sanchez-Elsner et al., 2001). Additionally because VEGF release by hypoxic macrophages and endothelial cells requires induction by TGFβ in a similar fashion (Jeon et al., 2007, Sanchez-Elsner et al., 2001), it is conceivable that Siah-HIF-1 would have a similar regulatory role in VEGF secretion by these cells. The finding have important implications on the regulation of angiogenesis during early wound healing, a phase where TGFβ signalling by dermal fibroblasts (Denton et al., 2009) and hypoxic gradients are demonstrated to be mandatory for good angiogenesis (Knighton et al., 1981). Additionally, the evidence provided here may in part explain why wound angiogenesis appeared delayed in Siah2 knockout mice (Chapter 3).

Fibroblasts rapidly migrate into the provisional matrix from local mesenchymal connective tissue cells and bone marrow derived circulating progenitor lineages in response to inflammatory released factors where they form and resolve the granulation tissue. (Leibovich and Ross, 1975, Abe et al., 2001)(Salo et al., 1994, Darby et al., 2002). Cell migration is the result of repeated cycles of cytoskeletal-mediated protrusion and polarization, formation of adhesive contacts, cell contraction and retraction at the trailing edge (Lauffenburger and Horwitz, 1996). While granulation tissue formation and wound resolution are the result of matrix deposition/remodelling and myofibroblast transition/contraction followed by loss of cells via apoptosis (Gabbiani et al., 1971, Darby et al., 1990, Tomasek et al., 2002, Hinz et al., 2003, Hinz and Gabbiani, 2003a, Hinz and Gabbiani, 2003b).
Acute hypoxic microenvironments are an important regulator of skin cell motility, granulation tissue formation and remodelling, mostly through its modulation of matrices, soluble factors and their respective receptors (cytokine receptors and integrins) ((Falanga et al., 2002)(Steinbrech et al., 1999, Falanga et al., 1991, Keely et al., 2009). HDF cell motility in hypoxia is regulated mostly through HIF-1, which thus far has been demonstrated, through its regulation of heat shock protein-90, an intracellular molecular chaperone that can be released to induce autocrine and paracrine extracellular migratory responses during oxidative stress (Li et al., 2007). It is also conceivable that HIF-1 regulates cell function in HDFs cells through the up-regulation of hypoxia inducible proteins that control glucose intake (Glut-1) and metabolism (PGK-1), proteins that are highly expressed during cutaneous wound healing (Elson et al., 2000). Moreover, HIF-1 regulates the production and the release of several growth factors including CTGF and TGFβ1 (Hong et al., 2006, Distler et al., 2007), which act in an autocrine and paracrine manner to promote chemotaxis of fibroblastic cells, matrix production and wound contraction. HIF-1 also transcriptionally regulates the integrin beta 1 subunit, an integrin subunit that is required for attachment to collagenous matrix playing a role in mechano-perception and contraction by fibroblastic cells (Keely et al., 2009, Arora et al., 1999).

The current study provides evidence of the importance of hypoxia signalling in TGFβ induced matrix production by HDF cells (Figure 5-4). Here, PHYL transfected cells, which are deficient in HIF-1α (Figure 5-2), show reduced ability to induce COL1A1 in hypoxia (versus mock transfected or control cells). This is consistent with the role of HIF-1 in the regulation of endogenous TGFβ and collagenous matrix production in hypoxic tissue (Distler et al., 2007). Furthermore HIF-1 also directly regulates transcription of pro-collagen hydroxylases (Hofbauer et al., 2003), which may represent a mechanism which could modulate fibrillar collagen abundance secondary to collagen synthesis. Interestingly, exogenous TGFβ was unable to rescue COL1A1 induction in hypoxic PHYL transfected cells indicating that Siah-HIF-1α may in part be required to enhance TGFβ
induced expression of matrix proteins during hypoxia. These findings are supported by other research that has demonstrated synergetic effects of TGFβ and HIF-1α in the expression of matrix inducing genes such as CTGF, a gene which contains a HRE and SRE in the gene promoter region (Hong et al., 2006, Higgins et al., 2004). The current study suggests the possibility of cross-talk between TGFβ and hypoxia pathways in the production of matrix and provides evidence to support the potential role of Siah-HIF-1 axis in this interaction. These findings combined with those from our mouse studies suggest the Siah-HIF-1 axis may play an important role in wound healing in vivo (Chapter 3), where collagen production is important for angiogenesis and granulation tissue formation. Additionally, because endogenous collagen synthesis is required for attachment (Kleinman et al., 1982) and chemotaxis of HDF cells both in vitro and in vivo (Postlethwaite et al., 1978), the regulation of COL1A1 by Siah-HIF1 axis may also represent a mechanism by which HDF cell attachment and migration can be augmented or regulated by hypoxia.

Our study also provides evidence of the relevance of Siah proteins in the regulation of HDF cell migration (Figure 5-8). PHYL transfected HDF cells showed a reduced migratory responses to hypoxia alone, which maybe in line the role HIF-1 in the transcription of genes, including Hsp90, CTGF and matrices, which regulate migration (Hong et al., 2006, Li et al., 2007, Distler et al., 2007). PHYL transfected HDFs also show reduced migratory responses in hypoxia condition supplemented with TGFβ1, suggesting once again that Siah-HIF-1 axis may be required for co-operative responses between hypoxia and TGFβ pathways. Interestingly, PHYL transfected cells showed a reduced migratory response to TGFβ in normoxia. The reason for this finding is not clear but is suggestive of a potential role of Siah in regulation of TGFβ induced responses in both hypoxic and non-hypoxic settings. The result provides evidence to support the role of Siah in the regulation of cell migration during wound healing. This would in part explain why Siah2−/− deficient mice show marked delays in macrophage invasion, angiogenesis, re-epithelialisation and
granulation tissue formation, events that rely on cellular responses to cytokines that induce cell migration.

Transient expression and incorporation of α-SMA into stress fibres significantly augments contractile ability of fibroblastic cells and hallmarks the contraction phase of connective tissue remodelling (Darby et al., 1990, Hinz et al., 2001). Here, the role of Siah in myofibroblast transition and HDF cell contraction was tested (Figure 5-5 and Figure 5-6). The results suggest that the Siah-HIF-1 axis has no significant effect on the modulation of contraction and fibroblast to myofibroblast transition in HDFs cells in response to TGFβ or hypoxia. The results are in contrast to observations made using mouse dermal fibroblasts where Siah2 appeared to modulate αSMA induction in hypoxia (see Chapters 4) suggesting the possibility that Siah has differential regulatory effects in mouse versus human cells. Additionally, the results support recent research that show impaired αSMA expression in hypoxia (Modarressi et al., 2010), with the major difference being that reduced αSMA in our study did not impair lattice contraction in acute hypoxia. Although the reason(s) for this effect remain unclear, it is likely that monolayer cultures models fail to take into account other factors including cell-matrix interactions or applied tension which are required for cell contraction (Arora et al., 1999, Tomasek et al., 1992). Additionally, given that wound contraction can occur independently of αSMA dependent mechanisms, as seen in IL-6 ko mice which show reduced wound contraction despite early augmentation of α-SMA expression compared to wild type littermates (Gallucci et al., 2000), it seems possible to have a physiological state where αSMA expression or induction does not correlate to contraction. Furthermore, because αSMA expression is not absolute during cell contraction, and can be substituted by other isoforms such γ-smooth muscle and α-skeletal actin as seen in αSMA null mice (Schildmeyer et al., 2000), it may be tempting to postulate that contraction where αSMA is reduced may occur via a non-αSMA induced mechanisms (Hinz, 2007).
Various studies have reported different proliferative outcomes after exposing HDF cells to hypoxia. Some studies report that hypoxia inhibits dermal fibroblast cell proliferation (Oberringer et al., 2008) while others have reported enhanced proliferation (Falanga and Kirsner, 1993). Observations of no significant change to HDF proliferation in hypoxia have also been described (Boraldi et al., 2007). In our study, cell proliferation was determined by PCNA analysis, which did not reveal any significant change to cell proliferation during hypoxia in both control and PHYL transfected cells. Our results may support the finding of Boraldi et al., mentioned above. The lack of an increase in proliferation following treatment of HDFs with exogenous TGFβ1 raises some concerns about the sensitivity of PCNA to determine proliferative differences in culture conditions where proliferation may be perpetual and occurring at a high rate, as was apparent with the HDF cultures (data not shown). Therefore other proliferative assays such as Bromodeoxyuridine (BRDU) incorporation assays, which are more s-phase specific, may be required to draw a conclusion on the effect of Siah in dermal fibroblast proliferation in hypoxia.

Cellular responsiveness to growth factor, such as TGFβ is reliant on the growth factor binding to the receptors (TGFRIII) which leads to the recruitment of TGFR1 and activation of cytosolic signalling pathways and subsequent alteration of cellular processes important for wound healing including mitogenesis, migration and matrix production (Massague, 1998, Piek et al., 1999, Massague and Chen, 2000, Massague and Wotton, 2000). Hypoxia up-regulates TGFβ1 in HDF cells (Falanga et al., 1991) which activates downstream signalling cascades including p42/p44 MAPKs (Mogford et al., 2002) and Smads (Falanga et al., 1991). HIF-1 has been suggested as the likely candidate in the regulation of TGFR1 expression in hypoxia, as supported by reduced TGFβR1 expression and p42/p44 MAPK activation in aged dermal fibroblasts (Mogford et al., 2002) which are reported to be HIF-1α deficient cells (Loh et al., 2009). Furthermore, the importance of HIF-1 in the regulation of TGFβ responses in hypoxia is also supported by studies that demonstrate reduced TGFβ augmentation and matrix synthesis in HIF-1α
inhibited HDF cells (Distler et al., 2007). However, the study did not establish the effect of HIF-1 on TGFβ signalling cascades.

Here the effect of Siah-HIF-1 axis on TGFR1 expression (Figure 5-9) and downstream Smad cascades (Figure 5-10) were investigated in response to TGFβ and hypoxia stimuli. Here, a moderate correlation between HIF-1α and TGFR1 expression is seen in hypoxic dermal fibroblasts. PHYL transfect HDFs show downward trend (not significant) in TGFR1 induction in hypoxia (versus mock) and failure to enhance TGFR1 in hypoxia when supplemented with exogenous TGFβ (P<0.05, versus mock). Furthermore, downstream of TGFR1, PHYL transfected cells showed a downward trend in phosphorylated Smad2 following treatment with TGFβ and hypoxia (compared to mock cells) which might be suggestive a reduced response to TGFβ. However, it is likely that moderate reduction to TGFβ signalling cascades (TGFR1 and Smad) in PHYL transfected cells (compared to mock cells) only partially account for the significant reductions in cell migration, VEGF secretion and collagen induction. It is likely that reduced Siah activity (in PHYL transfected cells) affects other mechanisms such as cross-talks between TGFβ and hypoxia signalling pathways, that occur downstream of Smad phosphorylation.

These cross-talks involve a physical interaction between HIF-1α, Smad and a co-activator protein (p300) at promoter region of genes containing HRE and SRE sequences (Sanchez-Elsner et al., 2001). Smad-HIF-1α complexes are demonstrated in the activation of angiogenic genes including VEGF, erythropoietin-1 and TGFβ co-receptor, endoglin (Sanchez-Elsner et al., 2001, Sanchez-Elsner et al., 2004, Sanchez-Elsner et al., 2002). Furthermore, a variety of genes contain active SRE and HRE sequences and may potentially be regulated through TGFβ-hypoxia cross-talks. Examples that are relevant to wound healing and to the function of dermal fibroblasts include CTGF and PAI (Fink et al., 2002, Hong et al., 2006, Higgins et al., 2004, Zhang et al., 2003b). Such an interaction would explain why TGFβ responsiveness in hypoxia is reduced in HIF-1 deficient cells (PHYL transfected cells) without much difference to TGFβ cascade proteins.
Additionally, TGFβ and hypoxia are both demonstrated to induce increased activation of MAPKs (p38, p42/ p44 MAPKs) (Mogford et al., 2002). MAPK pathways are also critical for activation of Siah and HIF-1α proteins by oxidative stress and possibly through cytokines (Richard et al., 1999, Khurana et al., 2006). Therefore the possibility of a TGFβ-MAPK-Siah-HIF-axis would also explain the observations of reduced TGFβ responsiveness in hypoxia if Siah or HIF-1α were to be reduced. The existence of such an interaction has been demonstrated in human cytotrophoblast cells where TGF β-MAPK-HIF-1 interactions are required to induce VEGF expression (Qian et al., 2004).

Furthermore, because TGFβ induced migratory responses are reduced in PHYL transfected cells in normoxia, it is conceivable that Siah has regulatory effects on TGFβ responses downstream of Smad or via non-Smad dependant pathways in a fashion independent of the hypoxia signalling machinery. The requirement of Ras signalling in the enhancement of TGFβ-Smad dependent responses is a potential explanation for this result. Siah proteins have important regulatory effects on Ras signalling pathways downstream of the RAF/MEK/MAPK signalling cascade and depletion or inactivation of Siah inhibits Ras activated pathways as demonstrated in transformation and carcinogenesis models (Schmidt et al., 2007, Ahmed et al., 2008). In fibroblasts, activation with exogenous TGFβ leads to transient activation of RAS/MEK/MAPK cascades and subsequent enhancement of TGFβ-Smad responses (Leask and Abraham, 2004). Activation of Ras signalling by TGFβ induces genes including CTGF, a protein associated with cell migration, proliferation, matrix synthesis and angiogenesis (Phanish et al., 2005, Secker et al., 2008). It is therefore likely that reduced TGFβ-Smad responses in Siah deficient or inhibited cells could occur due to impairment in Ras signalling.

5.3.3. Implications on wound healing in human skin

This is the first study of its kind that demonstrates an important role for Siah proteins in regulation of cell function in human skin cells. The evidence provided here suggests that
Siah is required for important fibroblast functions during hypoxia including cell migration, matrix synthesis and the release of angiogenic factors such as VEGF. Furthermore, Siah appears to be important in the regulation of TGF\(\beta\)-induced responses in both the normoxic and hypoxic milieu. Taken together the evidence implies that Siah may be an important positive regulator of wound healing in human skin via its role in Hif-1 stabilization and possibly through other mechanisms as discussed above. This is further supported in previous chapters (3 and 4) where the role of Siah2 is described in mouse cutaneous wound healing models \textit{in vitro} and \textit{in vivo}, with the main difference being that Siah does not appear to alter \(\alpha\)SMA expression or thus contraction in HDF cells. The findings of this study also have important clinical implications in pathological states of wound healing associated with deficiency or excessive accumulation of HIF-1 (Discussed in section 3.3.3)

5.4. Conclusion

Transient inhibition of Siah 1 and 2 with PHYL reduced the availability of HIF-1\(\alpha\) in hypoxic HDF cells. This had significant inhibitory effects on cell migration, extracellular matrix protein expression, VEGF release and TGFR1 induction during hypoxia. Additionally, PHYL transfected cells showed reduced cell migration in response to TGF\(\beta\) in normoxic culture conditions. Overall, the results, in general, support the role of Siah as a positive regulator of wound healing in human skin cells.

5.5. Specific experimental methods for this chapter

Mentioned here are summarized methods including only relevant details pertaining to this chapter. For specific descriptions of individual methods, please refer to the general methods chapter (Chapter 2)
5.5.1. Transfection of human dermal fibroblasts with phyllopod

Briefly, E. coli cells were made competent and transformed with pcDNA using standard calcium chloride and heat shock methods. Transformed E. coli colonies were selected for using antibiotic resistance. Transformed colonies were propagated in selection medium containing antibiotic and pcDNA plasmid isolated and purified using plasmid mini-prep columns. Purity and concentration of plasmids were determined by spectrophotometry at 260/280nm in quartz cuvettes. Primary adult HDF cells were transfected with pcDNA (3.4μg) using Lipofectamine for 5-6 hours in serum free 106 media for fibroblasts culture (Invitrogen). Cells were allowed to recover under normal culture conditions for 24 hours. Expression of PHYL protein was verified by detection of HA tagged PHYL using an anti-HA antibody and anti-rabbit IgG- Alexa-fluor 488®. HA detection was compared to that of HDFs transfected with an empty pcDNA or scrambled (nonsense) vector.

5.5.2. Migration of human dermal fibroblasts

Transfected HDF cells were seeded into 6 well plates at normalized cell density and grown until 80-90% confluent. Cells were then serum starved for 6 hours and scratched using a micropipette tip (1 ml) and then supplemented either with low serum medium or low serum medium with added TGFβ1 (0.5ng/ml). Cells were incubated in normoxia or hypoxia overnight (16 hours). Migration was determined by measuring the difference in the area of the gap at time 0 versus time post-wounding.

5.5.3. Collagen lattice contraction assay

Transfected HDFs were counted and seeded into collagen I (2.1mg/ml) at normalized density (2 x 10⁵cell/ml). Lattices were allowed to polymerize and then recover for 24 hours in normal cell culture conditions. Cells were serum starved for 6 hours and later supplemented with either low serum media or medium containing TGFβ1 (2.5ng/ml). Cells were incubated in normoxia or hypoxia for up 24 hours. Lattices were released and
returned to their respective experimental conditions for a further 24 hours. Area of lattices was measured and contraction calculated as change in lattice area (pixels²).

5.5.4. Analysis of protein expression by flow cytometry

Protein expression was determined by means of immuno-labelling and flow cytometry. Briefly, transfected HDF cells were seeded into flasks (25cm²) at normalized cell density and grown until 70-80 % confluent. Cells were serum starved for 6 hours and later supplemented with either low serum media or medium containing TGFβ1 (2.5ng/ml). Cells were incubated in normoxia or hypoxia for 48 hours. Cells were detached by trypsin, fixed in formalin and permeabilized using saponin. Immuno-labelling of cells with mouse monoclonal Anti-HIF-1α (1:100), rabbit polyclonal anti-COL1A1 (1:100), mouse monoclonal anti-αSMA-FITC conjugated (1:250), mouse monoclonal anti-PCNA (1:200), rabbit polyclonal anti-HA (1:100,), rabbit polyclonal anti-phosphorylated-Smad2 (1:100), rabbit polyclonal anti-transforming growth factor-β receptor type-1 (1:100), mouse monoclonal anti-SMAD2 (1;100) and mouse monoclonal anti-Siah2 (1:100) was done to probe for these respective antigens. Fluorescent conjugated secondary anti-rabbit and anti-mouse antibodies were used to probe for respective primary antibodies (excepting αSMA which was conjugated). The fluorescence intensity of at least 2000-5000 cells per sample was measured using a flow cytometer and analyzed by weasel flow cytometry software (Walter and Eliza Hall Institute, Aus).

5.5.5. VEGF ELISA

VEGF production and release from cultured fibroblasts into the medium was measured using a human VEGF ELISA (PeproTech, USA). Briefly, this involved binding a capture VEGF antibody onto a high binding ELISA plate and adding normalized amounts of sample culture medium. Captured VEGF was detected by colorimetric horseradish peroxidase reaction after the addition of a biotinylated VEGF detection antibody and
HRP-conjugated streptavidin. A standard curve constructed using purified VEGF was used to quantify unknown samples.
Chapter 6

Conclusion and future directions
6.1. General discussion

Wound healing relies on a complex and precise sequence of events that lead via inflammation, proliferation and resolution phases to a generally imperfect tissue repair (Clark, 1996). These events are regulated by complex cellular interactions involving cytokines, extracellular matrix proteins, adhesion proteins and their respective cellular receptors that induce signalling pathways and lead to the activation and suppression of multiple genes controlling inflammation, re-epithelialisation, angiogenesis and granulation tissue formation during wound healing, as reviewed extensively in Chapter 1. Impairment of any of these processes results in delayed or aberrant healing. In adults, the generation of granulation tissue and its eventual remodelling as wound maturation occurs, results in a scar with reduced functionality compared to the original tissue (Darby and Hewitson, 2007). Because of the loss of function in scarring tissue and the difficulty in healing chronic wounds as well as the high cost of wound care (Sen et al., 2009), there is considerable interest in the factors involved in the regulation of normal and potentially pathological healing.

Following injury, vascular damage generally occurs resulting in low oxygen tension (hypoxia) which may be further sustained by the rapid influx of inflammatory and mesenchymal cells with high metabolic demands for oxygen. Slight changes in systemic and cellular oxygen concentrations induce tightly regulated response pathways that attempt to restore oxygen supply to cells and modulate cell function in hypoxic conditions. Tissue hypoxia is a common microenvironment during both normal and abnormal wound healing and has important regulatory effects on cell behaviours during inflammation, re-epithelialisation, granulation tissue formation and angiogenesis (Haroon et al., 2000, Lokmic et al., 2006, Remensnyder and Majno, 1968, Knighton et al., 1983, Knighton et al., 1981, Steinbrech et al., 1999, Ridgway et al., 2005). Most of these responses occur through the induction of HIF-1 which regulates many processes needed to carry out tissue repair processes during tissue ischaemia and indeed wound healing.
Over elaborate HIF-1 responses have been linked with fibrosis disorders of the skin and impaired HIF-1 signalling is thought to be an important mechanism contributing to impaired wound healing in diabetics and the aged (Botusan et al., 2008, Distler, 2003, Liu et al., 2008, Zhang et al., 2006, Zhang et al., 2003b). HIF-1 is thus an important therapeutic target for the treatment and management of abnormal wounds. The use of HIF-hydroxylase (PHD) inhibitors to increase HIF-1 stabilisation and improve wound healing has been demonstrated in cutaneous wound healing models in animals (Botusan et al., 2008). PHD inhibitors are now in clinical trials for the treatment of several ischaemia-related pathologies such as chronic wounds, anaemia, ischaemic heart disease, stroke, cancer, and pulmonary hypertension (Smith and Talbot, 2010).

It is now well established that during hypoxia, HIF-1α escapes proteosomal degradation through the inhibitory effects of Siah ubiquitin ligases on PHD and FIH activity (Nakayama et al., 2004, Fukuba et al., 2007), thus aim of this study was to investigate the relevance of Siah during wound healing.

### 6.1.1. Effect of Siah-deficiency or inhibition on wound healing and its clinical implications

We report here for the first time that cutaneous wound healing of Siah2-deficient mice results in delayed recruitments of macrophages, delayed migration of vessel cells, delayed fibroblast – myofibroblast transformation and reduced extracellular matrix deposition during the early phase of wound healing and that this delay is significant, albeit transient (Chapter 3). Furthermore, Siah2 knockout fibroblasts showed impaired functionality under hypoxic conditions and in interestingly showed an impaired response to exogenous TGFβ (Chapter 4), which may in part explain why wound healing is delayed in Siah2-deficient mice. Moreover, transient inhibition of Siah in human dermal fibroblasts using a transfected Siah1/2 inhibitor (phyllodopod) reduced the functionality of otherwise healthy dermal cells (Chapter 5) through, we suggest, both
HIF-1 dependent and HIF-1 independent mechanisms. We propose that reduced TGFβ1 responses in Siah deficient dermal fibroblasts beyond the hypoxic milieu may be due to impaired Ras signalling, given the importance of Ras signalling in TGFβ- Smad responses (Leask and Abraham, 2004) and regulatory effects of Siah in Ras mediated signalling (Ahmed et al., 2008). Furthermore, because of the importance of HIF-1 and Smad interactions in the synergistic expression of certain genes by hypoxia and TGFβ, specific examples including VEGF (Sanchez-Elsner et al., 2001) and possibly CTGF (Hong et al., 2006), it is likely that in the absence of Siah such cross talk may be reduced or impaired in hypoxia.

The results of this study may have several important clinical implications. Firstly, genetic or pharmacological strategies to increase Siah levels may be beneficial in increasing HIF-1α levels in tissue where this may prove beneficial. Increased HIF-1α has been shown to promote vascularisation and improve healing in ischaemic, excisional and burn wounds (Botusan et al., 2008, Liu et al., 2008). This is especially important for specific groups such as the aged and diabetic subjects who may be prone to HIF-1 deficiency in hypoxia (Catrina et al., 2004, Botusan et al., 2008, Liu et al., 2008, Loh et al., 2009) The use of Siah or a pharmacological mimetic may therefore be beneficial in the treatment of ischaemic wounds where HIF-1 induction of genes may be beneficial to the repair process may enhance healing.

Conversely, inhibition of Siah using specific pharmacological inhibitors may prove beneficial in the treatment of fibroproliferative disorders, where HIF-1α is over-expressed or expressed chronically and is now thought to promote fibrosis (Distler et al., 2007, Zhang et al., 2006). At present, menadione (a Vitamin K derivative) is the only pharmacological inhibitor of Siah2 (Shah et al., 2009). Based on the results of the current study, experiments to test the effect of menadione in a wound healing scenario would seem desirable.
6.1.2. Future Directions

In addition to this study, it is reasonable to hypothesise that a future study investigating the role of Siah in models with complete Siah 1 and 2 deficiencies may yield a more elaborate phenotype and better define the role of Siah in wound healing. Siah 1 and 2 have overlapping functions \textit{in vivo} which may only result in partial HIF-1α deficiency in Siah2\(^{-/-}\) (Nakayama et al., 2004) and thus the transient delay in wound healing observed in our study. Complete deletion of both Siah 1 and 2 genes results in more complete HIF-1α deficiency (Nakayama et al., 2004) but also causes embryonic lethality (Frew et al., 2003) which has hindered the effective study of Siah in later development and adulthood. It may be possible to overcome this problem \textit{in vivo} by adopting a model where both Siah 1 and 2 are conditionally deleted in adult mice and in specific cell populations. Cre-\textit{lox} P recombination is a technique commonly used to generate conditional knockout animals, in which genes can be inactivated in specific tissue or at a specific time (Sauer, 1998). Cre-\textit{lox} P systems has been used successfully to abrogate expression of genes in adults skin wound healing models, for example, CD11b gene which is required for macrophage specific invasion during inflammation and wound healing (Mirza et al., 2009). Recently, Cre-\textit{lox} P models have been used to generate adult HIF-1α deficient liver mice models enabling the pathological effects of HIF-1α to be studied in liver cirrhosis \textit{in vivo} (Moon et al., 2009). A similar model targeting both Siah 1 and 2 in skin tissue or specific wound healing cells including macrophages, fibroblast, keratinocytes and endothelial cells would be desirable for further defining the role of Siah in wound healing with respect to both HIF-1 and or RAS signalling pathways. Adoptive transfer of immune cells from Siah2\(^{-/-}\) mice into recipient mice with immune cells ablated could also allow further dissection of the relative roles of fibroblasts compared to immune/inflammatory cells in Siah2 deficient mice. Our data and preliminary data from our collaborator’s laboratory (Dr Möller, Peter MacCallum Cancer Institute) are certainly suggestive of a prominent role for macrophages in the Siah2 knockout phenotype.
As mentioned earlier establishing primary murine dermal fibroblasts cultures proved challenging and may have limited the ability to clearly define the role of Siah2 in these cells (see Chapter 4 section 4.3.2). This problem was mostly overcome by inhibiting Siah activity in healthy adult human dermal fibroblasts which had the additional advantage of becoming an in vitro model that was more relevant to adult human skin. Inhibition of Siah in the HDF study might also benefit from improving the transfection efficiency of Phyllopod into these cells. This might be achieved by using a cellular marker such as DAPI, along side Phyllopod TAG (HA) which could help to determine and improve the transfection co-efficient. Establishment of stable Phyllopod transfected HDF could also be attempted by incooperating. Additionally, MDF cell lines, although mostly embryonic, are readily available and well characterised in culture. The role of Siah in murine dermal cell functions could be further tested in such a model using Siah inhibitors such as Phyllopod or anti-sense Siah oligonucleotides, taking into account that some differences between embryonic and adult dermal cell behaviour are likely. Embryonic fibroblasts may produce different types of matrix proteins in hypoxia and may be less responsive to changes induced by TGFβ1 compared to adult dermal fibroblasts which predominately produce type I collagen in hypoxia and show high responsiveness to the growth factor (Scheid et al., 2000, Scheid et al., 2002, Rolfe et al., 2005). Additionally embryonic fibroblasts are generally less differentiated towards a myofibroblast phenotype (Rolfe et al., 2005).

The results of this study indicate that Siah proteins may be important in the regulation of matrix synthesis during wound healing and indeed in dermal fibroblasts during hypoxia, which could have important implications in scarring and fibrosis, thus testing the role of Siah in cutaneous and non-cutaneous scarring or fibrosis models is an important future direction. Ear models of hypertrophic scarring in the skin (Kryger et al., 2007) and bile duct ligation or carbon tetrachloride models of liver fibrosis (Moon et al., 2009) are specific examples models that could be employed to evaluate the effects of
Siah in scarring and fibrosis in vivo. Our hypothesis here would be that Siah inhibition may be protective against fibrosis either through regulation of fibroblast function or through dampening of macrophage activity and TGF\(\beta\) sensitivity. Conversely, genetic or pharmacological strategies to increase Siah levels in ischaemic wound healing models such as diabetic cutaneous healing models may be desirable to further investigate the relevance of Siah as a positive regulator of wound healing during hypoxia.

6.2. Overall Conclusion

In summary, hypoxia is an important part of the microenvironment in early wound healing. Adaptive responses to hypoxia are controlled by HIF-1, which is positively regulated by Siah. This study demonstrates for the first time a role for Siah2 in wound healing and an effect on healing of Siah2 deficiency. Cutaneous wound healing of Siah2-deficient mice results in delayed early phase wound healing. Siah through HIF-1 and possibly other mechanisms appears to be a positive regulator of dermal fibroblast cell function which may in part explain why early wound healing is delayed in Siah\(2^{-/-}\) mice. Modulation of Siah protein levels or activity may therefore have important clinical implications in the treatment of abnormal wounds and fibrotic conditions.
Chapter 7

References


Chapter 7


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