# *The Effect of a Myostatin Antagonist on the Healing of Burn Wounds in Skin.*

A thesis
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of the requirements for the degree
of
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by
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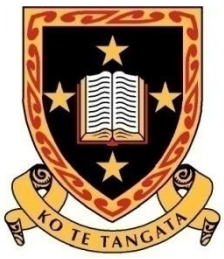


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# ABSTRACT

The body's remarkable ability to repair itself from damage is both very useful and extremely complex. The wound healing system is characterised by four overlapping phases: hemostasis, inflammation, proliferation, and remodelling. It is designed to heal rapidly and efficiently any breach in the protective barrier we call skin, in order to restore normal body function in the quickest possible time. However, pathological conditions such as keloids or hypertrophic scar may arise if events in the wound healing cascade are not coordinated properly, which can lead to loss of skin function and disfigurement. The purpose of this study was to determine whether myostatin plays a role in the healing of burn wounds in skin. Myostatin is best known for its powerful negative regulation of muscle development. Absence of myostatin results in a heavy muscling phenotype, whereas over-expression is associated with muscle wasting conditions. Recently, myostatin has been shown to be involved in muscle wound healing, where knockout of the myostatin gene resulted in improved healing, with decreased fibrotic scar tissue formation. It is noteworthy that the cell surface receptor to which myostatin binds is also present in skin. In consideration of this information the prospect of myostatin involvement in skin biology represents a gap in scientific knowledge. Therefore, the present investigation was designed to ascertain whether the effects of myostatin in muscle regeneration can also be seen in skin wound healing. To this end, a mouse burn wound model was designed to determine the efficacy of antagonising myostatin to bring about improved healing and decreased fibrosis of skin burns. Wounds of mice treated with the myostatin antagonist showed no significant difference compared to saline-treated controls in collagen content at any time point. Gene expression studies on TGF-β1, TGF-β3, decorin and fibromodulin revealed that differences between antagonist-treated and saline-treated groups were possibly masked by the effect of the burn injury but were present in uninjured skin. Similarly, uninjured skin of antagonist-treated animals exhibited a significantly higher fluid content than uninjured skin of saline-treated animals, whereas the effect was not significant in burned skin between treatments. Histological analysis revealed that antagonist-treated wounds showed evidence of decreased wound contraction which may indicate improved scar resolution and decreased risk of fibrosis. Interestingly, the gene expression results in many ways parallel those seen in foetal skin, which after injury, heals without scar. These results warrant further study into the subject area, especially to observe whether further improvements can be made by adjusting the treatment regimen.

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To the rest of the K Wing team, thanks for your friendship, it's been a privilege to work alongside you, and some of you aren't bad at touch and volleyball either.

To my friends who have prayed for me during this time, thank you for your support. And lastly, to the One who receives our prayers, and offers salvation, Jesus Christ, thank you for your creation, and your grace, which I need daily.

*"God is like the sun; you cannot look at it, but without it, you cannot look at anything else."* G.K. Chesterton.

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# ABBREVIATIONS

| | |
|---|---|
| α | alpha |
| β | beta |
| °C | degrees Celsius |
| γ | gamma |
| µg | micrograms |
| µl | micro litres |
| µM | micro molar, micro moles per litre |
| µm | micrometers |
| 18S | small ribosomal subunit in eukaryotes |
| 3' | three prime - the end of a nucleotide sequence with a terminal hydroxyl group |
| 5' | five prime - the end of a nucleotide sequence which contains a terminal phosphate group |
| A | adenine |
| $A_{260}$ | absorbance at 260 nm |
| $A_{280}$ | absorbance at 280 nm |
| ActRI | activin receptor type one |
| ActRIIA | activin receptor type two-A |
| ActRIIB | activin receptor type two-B |
| ADP | adenosine diphosphate |
| ANOVA | analysis of variance |
| APS | ammonium persulfate |
| arg | arginine |
| ATP | adenosine triphosphate |
| BLAST | basic local alignment search tool |
| BMP | bone morphogenic protein |
| bp | base pair(s) |
| C | cytosine |
| C2C12 | a murine myoblast cell line |
| C57bl | common laboratory mouse strain used in this study |
| CCL | chemokine (C-C motif) ligand |
| cDNA | complementary DNA |
| Cl- | chloride ion(s) |
| c-*myc* | member of the Myc family of transcription factors |
| $CO_2$ | carbon dioxide |

| | |
|---|---|
| COL1:COL3 | ratio of collagen type I to collagen type III |
| CSF | colony stimulating factor |
| ctrl | control |
| CTGF | connective tissue growth factor |
| Dcn | Decorin |
| DEPC | diethyl pyrocarbonate |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| DP | dermal papilla |
| DPX | dinbutyl phthalate in xylene |
| ds | double strand |
| DTT | dithiothreitol |
| *E. coli* | *Escherichia coli* |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| EtOH | ethanol |
| FGF | fibroblast growth factor |
| FLRG | Follistatin Related Gene |
| Fmod | Fibromodulin |
| g | gram(s) in weight OR a unit of force equal to the force exerted by gravity when preceded by x |
| G | guanine |
| GAG | glycosaminoglycan |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GASP-1 | growth and differentiation factor-associated serum protein-1 |
| GDF-8 | growth and differentiation factor-8 |
| H | histidine |
| h | hour(s) |
| H & E | Hematoxylin and Eosin |
| $H_2O$ | water |
| HB-EGF | heparin binding EGF-like growth factor |
| HCl | hydrochloric acid |
| HF | hair follicle |
| HIF-1 | hypoxia-inducible factor-1 |
| his | histidine |
| HIV | human immunodeficiency virus |

| | |
|---|---|
| H3.3A | histone three variant 3A |
| I | one |
| IFE | interfollicular epidermis |
| IFN | interferon |
| IGF | insulin-like growth factor |
| II | two |
| III | three |
| IL | interleukin |
| IRS | inner root sheath |
| ITP | individual time point |
| IV | four |
| K | potassium |
| K# | a type of keratin protein where # represents an identifying number |
| K+ | potassium ion(s) |
| L | litre(s) |
| LAP | latency associated peptide |
| LEF1 | lymphoid enhancer-binding factor-1 |
| LMP | low melting point |
| M | molar, moles per litre |
| mA | milli Amps |
| MB | myoblast |
| MCP-1 | monocyte chemotactic protein-1, also known as Chemokine (C-C motif) ligand 2 (CCL2) |
| mg | milligram(s) |
| $MgCl_2$ | magnesium chloride |
| Milli-Q | registered trademark for water purification systems manufactured by Millipore |
| min | minute(s) |
| MIP | macrophage inflammatory protein |
| MIS | Müllerian-inhibiting substance |
| ml | millilitres |
| mm | millimeters |
| mM | milli molar, milli moles per litre |
| MMP | matrix metalloproteinase |
| MOPS | 3-(N-morpholine)-propane-sulfonic acid |
| mRNA | messenger ribonucleic acid |
| Mstn | myostatin |

| | |
|---|---|
| Mstn-ant4 | myostatin antagonist 4 |
| N | normality of a solution |
| Na+ | sodium ion(s) |
| NaOAc | sodium acetate |
| NCBI | National Centre for Biotechnology Information http://www.ncbi.nlm.nih.gov/ |
| ng | nanograms |
| nm | nanometers |
| ORS | outer root sheath |
| p | probability |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PDGF | platelet derived growth factor |
| pET 16-B | cloning/expression vector from Novagen |
| pH | *potenz hydrogen* - hydrogen ion concentration |
| pmol | pico moles |
| pol | polymerase |
| PubMed | public citation database http://www.ncbi.nlm.nih.gov/pubmed/ |
| R | arginine |
| RNA | ribonucleic acid |
| RNAse | ribonuclease |
| rRNA | ribosomal RNA |
| RT | reverse transcriptase |
| RT-PCR | real-time polymerase chain reaction |
| s | second(s) |
| SAL | Saline |
| SC | stem cell |
| SDS | sodium dodecyl sulfate |
| SG | sebaceous gland |
| SLRP | small leucine-rich proteoglycan |
| Smad | intracellular signal transducer protein and transcription factor of TGF-β superfamily members |
| ss | single strand |
| T | thymine |
| TAE | tris-acetate-EDTA |
| *Taq* | DNA polymerase from *Thermus aquaticus* |
| TBE | tris-borate-EDTA |
| TEMED | tetramethylethylenediamine |

| | |
|---|---|
| TGF-α | transforming growth factor alpha |
| TGF-β | transforming growth factor beta |
| TIMP | tissue inhibitors of metalloproteinases |
| TNF-α | tumour necrosis factor alpha |
| tris | tris hydroxymethylaminoethane |
| TβRI | TGF-β type one receptor |
| TβRII | TGF-β type two receptor |
| TβRIII | TGF-β type three receptor |
| USFDA | United States Food and Drug Administration |
| UV | ultra-violet |
| V | five |
| V | volts (when preceded by a quantifying number) |
| VEGF | vascular endothelial growth factor |
| VII | seven |
| VIII | eight |
| vol | volume |
| Wnt | group of signalling proteins involved in embryogenesis and cancer |
| x | multiplied by |
| XI | eleven |

# 1 LITERATURE REVIEW

## Introduction

Wound healing and treatments for improving wound healing in skin have been studied since ancient times. Currently, the basic molecular pathways and biological processes of wound healing are well understood and are categorised by four overlapping phases: hemostasis, inflammation, proliferation and tissue remodelling. The final phase is characterised by scar formation, which, if not coordinated properly, can lead to pathological conditions such as keloids or hypertrophic scar, not to mention loss of skin function and disfigurement. To understand the principles of healing in skin it is necessary to understand the biological elements of skin. This review will outline the structure and developmental origin of skin, the phases of wound healing and the various key growth factors and cytokines involved, as well as a review of research that has attempted to intervene in the healing process to make improvements, or restore normal healing. This information will provide an important backdrop in anticipation of the objectives this study has been designed to achieve.

## 1.1 Skin: Structure and Function.

The skin is the largest organ in the human body and serves many essential physiological functions. These include: regulating body temperature; providing a protective barrier against the outside environment and its microbial threats; detecting sensations relating to touch, pressure, temperature, and pain; excreting water, ions, organic compounds, and heat; accomodating important immune cells; acting as a reservoir for blood, especially during exercise; and synthesising Vitamin D (Montagna 1986; Tortora and Grabowski 1996). Structurally, there are two main parts: the thin outer layer known as the epidermis, which overlays the thicker, connective tissue-rich dermis (Figure 1.1). The boundary between the dermis and subcutaneous tissue is not discrete and each extends into the other so that the skin remains anchored to the rest of the body. The skin must be highly flexible to adapt to every movement of the body and yet rigid enough to maintain form and function.

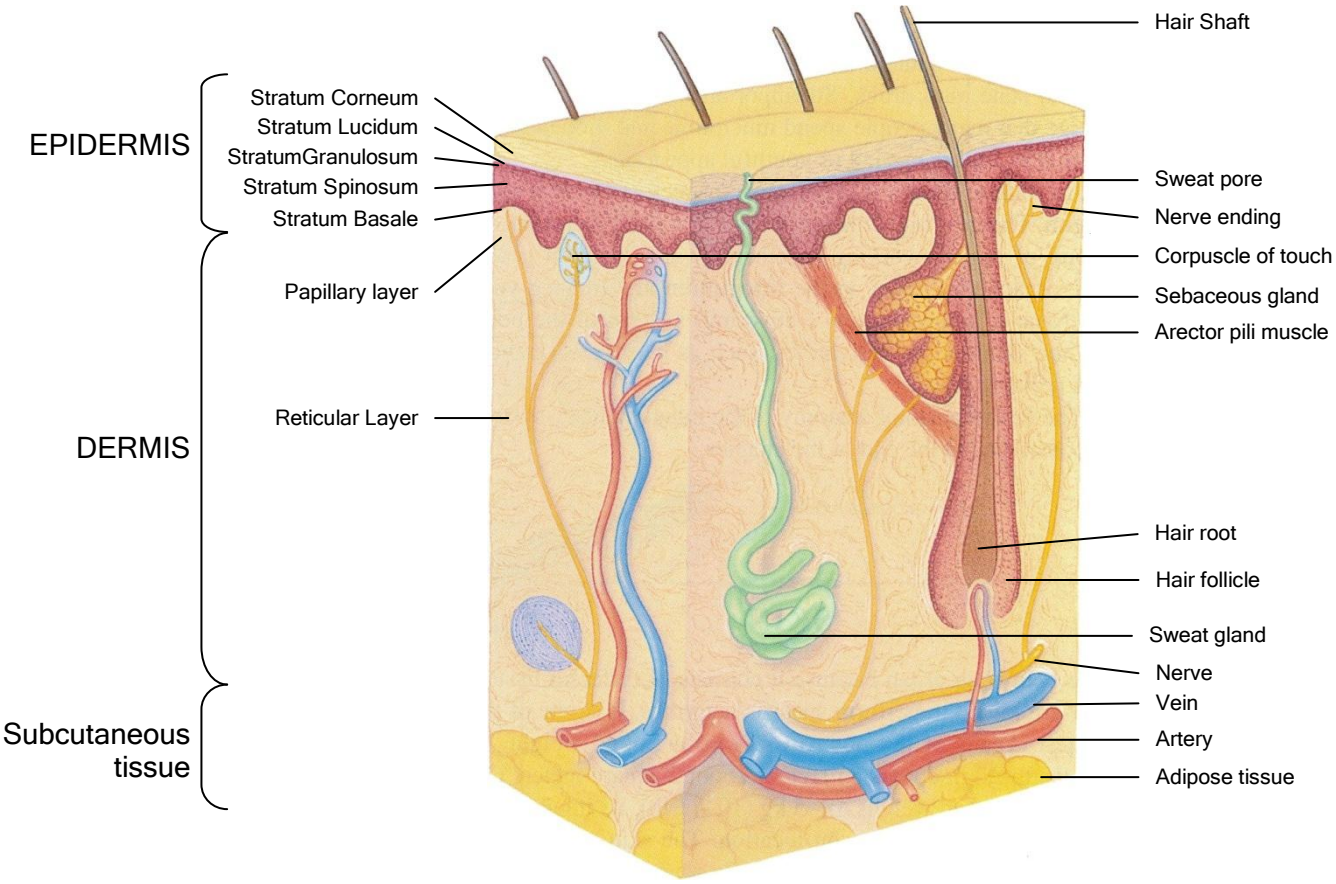



**Figure 1.1: Structure of the skin and underlying subcutaneous tissue**
(Adapted from Tortora and Grabowski 1996)

### 1.1.1 The epidermis

#### Keratinocyte stratification

The epidermis comprises the interfollicular epidermis with its adjacent structures, including the sebaceous glands and the hair follicles (Watt *et al.* 2006). The epidermis usually consists of four layers, each containing a group of keratinocytes at different stages of development (Figure 1.2). The one-cell-deep basal layer in contact with the dermis is termed the stratum basale or stratum germinativum (Montagna and Parakkal 1974). This layer contains a continuum of three types of cells: stem cells (Figure 1.3), transit-amplifying cells which have undergone a few rounds of division (Watt *et al.* 2006), and committed cells (Jones *et al.* 1995). Only cells of the basal layer are mitotically active and, as they leave the basal layer and push outward toward the skin surface, they withdraw from the cell cycle and become terminally differentiated (Blanpain and Fuchs 2006; Fuchs and Horsley 2008).

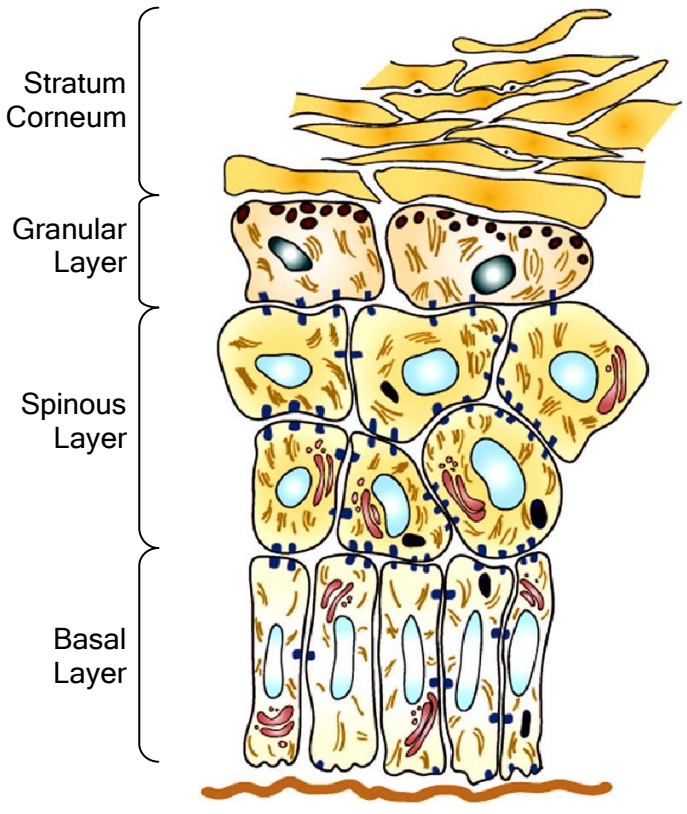



**Figure 1.2: Epidermal layers**
Epidermal cell types overlaying basement membrane showing keratin filament bundles and intercellular junctions (desmosomes) (modified from Fuchs 2008).

The next stratum is an 8-10 cell layer consisting of many-sided cells welded together at junctions known as desmosomes (Tortora and Grabowski 1996). This is the stratum spinosum and is where the process of keratinisation begins (Smith and Dale 1986). The next layer is the stratum granulosum which is a granular layer containing various sized keratohyalin (the precursor of keratin) granules (Montagna and Parakkal 1974). About 90% of epidermal cells are keratin-producing keratinocytes. Keratin provides waterproofing and protection from light, heat, microbes and many chemicals (Tortora and Grabowski 1996). Cells in the stratum granulosum show various levels of transcriptional activity (Blanpain and Fuchs 2006), as their nuclei are at various stages of degeneration (Tortora and Grabowski 1996).

The last and outermost layer is called the stratum corneum. It consists of dead, flattened keratin-filled cells which are regularly shed and replaced by those coming up from underneath. The life span of a keratinocyte is about 4 weeks (Fuchs 2007) from basal stem cell to enucleated, cornified, dead keratinocyte. These dead skin cells, also known as squames, are eventually sloughed off. In certain hairless areas of the body where the epidermis is quite thick, for example, the palms of the hands and soles of the feet, there is a fifth layer between the stratum granulosum and the outermost stratum corneum called the stratum lucidum. This layer contains three to five layers of dead cells which enclose droplets of eleidin, a substance which eventually becomes keratin (Tortora and Grabowski 1996).

## Other cells of the epidermis

Aside from keratinocytes and the various forms they take, there are other specific cell types contained within the epidermis. Almost a tenth of the epidermal cells are melanin-producing melanocytes. These cells have long slender projections, which transfer granules of melanin to keratinocytes. This functions to shield the nucleus on the inner side from mutagenic UV light (Tortora and Grabowski 1996). Melanogenesis is thought to be stimulated by DNA photodamage and its repair (Young and Agar 2005). Another type of cell known as Langerhans cells are considered to be immigrants to the epidermis but their exact origin is controversial. They are dendritic cells originally described as bone marrow-derived, antigen-presenting cells (Steinman and Cohn 1973). They are therefore an important member of the immune system and constitute the first immunological barrier against pathogenic threats from the outside environment (Merad *et al.* 2002). Recent research suggests that under normal conditions,

turnover of Langerhans cells is accounted for by the rate of division of Langerhans cells in the epidermis. However, when Langerhans cells become depleted, for example, by exposure to UV light (to which they are very sensitive), replacements are derived from either the hair follicle, which is a reservoir for epidermal Langerhans cells (Gilliam *et al.* 1998), or from blood-borne precursors known as monocytes, which respond to chemokine attractants expressed by keratinocytes (Merad *et al.* 2002; Ginhoux *et al.* 2006). A fourth type of cell is found in the basal layer, attached to keratinocytes in thickened regions of the epidermis known as tactile discs (Montagna and Parakkal 1974). Named Merkel cells, they are associated with touch sensation. They contain granules which enclose neuropeptides thought to work as neurotransmitters through which Merkel cells exert their effect on their associated sensory nerves (Moll *et al.* 2005).

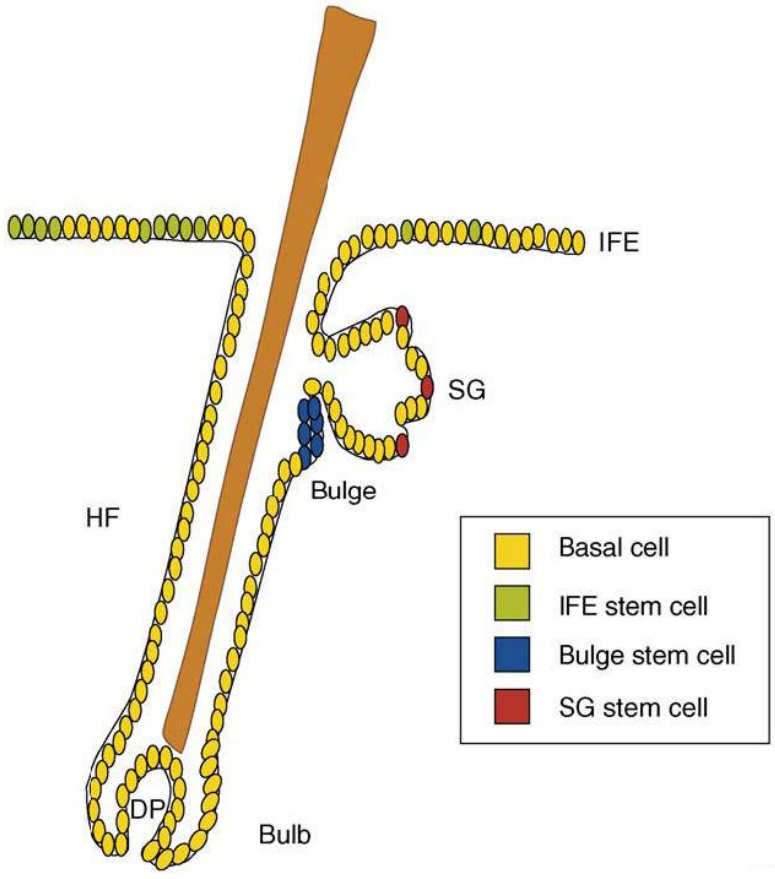



**Figure 1.3: Stem cell locations in mammalian epidermis**
There is strong evidence for the existence of stem cells in the hair follicle bulge (blue). It is unclear whether interfolicular epidermal (IFE) stem cells exist singularly or in clusters. There is potentially a third stem cell population in the sebaceous gland (red); however, it is also possible that the sebaceous gland is maintained by bulge stem cells. Abbreviations: DP, dermal papilla; HF, hair follicle; IFE, interfollicular epidermis; SG, sebaceous gland (modified from Watt *et al.* 2006).

## 1.1.2 The dermis

### General structure

The dermis provides the overall structure of the skin. It offers strength and flexibility via its extensible and elastic properties. Its texture varies depending on the area of the body.It is thin and flexible over joints, yet very thick and tough on the back (Vines 1995). Its primary constituent is known as extracellular matrix (ECM), which provides structural support and a surface for the body's cells to attach. ECM is composed of a loosely arranged mass of randomly oriented connective tissue fibres set in an unstructured gel-like ground substance. The fibrous connective tissue proteins (collagen and elastin) provide the tensile strength and elastic properties of the ECM. The upper portion of the dermis is known as the papillary region which is characterised by small, often vascularised and innervated, finger-like projections. These projections greatly increase the surface area of the basement membrane separating the dermis from the epidermis, aiding delivery of nutrients to the non-vascular epidermis (Tortora and Grabowski 1996). These projections in the papillary dermis known as dermal papillae should not be confused with dermal papilla cells at the base of the hair follicle (see Section 1.2.3 *Hair follicle morphogenesis*)

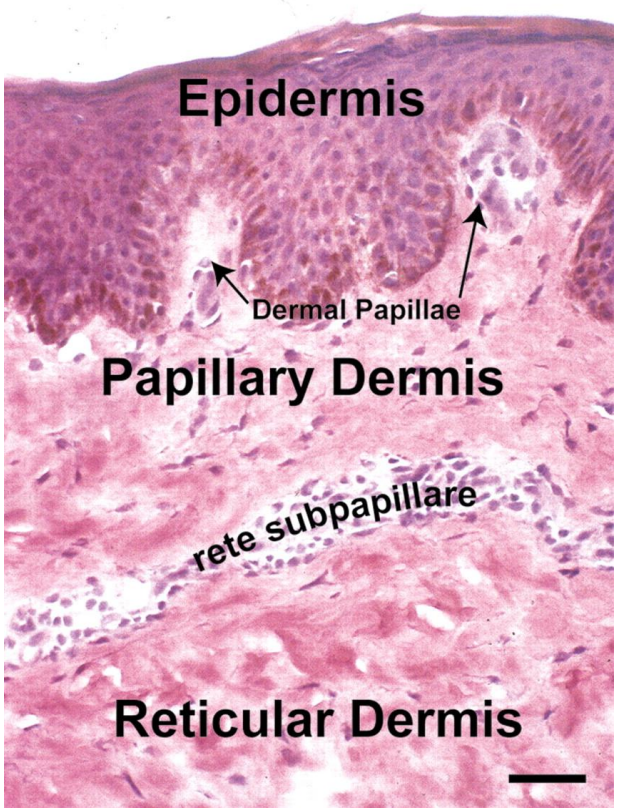



**Figure 1.4: Divisions of the dermis.**
Papillary and reticular regions of the dermis are separated by the rete subpapillare. Scale bar = 46 µm (from Sorrell and Caplan 2004).

The reticular region of the dermis is separated from the papillary dermis by a vascular plexus called the rete subpapillare (Sorrell and Caplan 2004) (see Figure 1.4 and Figure 1.5). While the reticular dermis is dense in connective tissue, there are spaces which are occupied by hair follicles, nerves, oil glands, sweat glands, and some adipose tissue (Tortora and Grabowski 1996).

## Extracellular matrix (ECM)

### *Collagens and Elastin*

While many different keratins dominate in the ever-renewing epidermis, collagens dominate the dermis. Collagens and other structural compoments of the ECM are secreted by dermal fibroblasts. Collagen is the most abundant protein in the mammalian body and many authors cite that it constitutes three quarters of total protein in skin. Collagens are glycoproteins which exist in at least 26 distinct forms (Alberts *et al.* 2002), but the primary two in the dermis are type I (80–85%) and type III (8–11%) collagens (Schultz *et al.* 2005). Collagen type I fibrils (75 nm diameter) are organised into bundles of thick fibres varying in diameter (2–10 μm), their main function being resistance to tension. Conversely, collagen type III fibrils (45 nm in diameter) exist as loosely packed fibres of more uniform diameter (0.5–1.5 μm), which form a mesh network maintaining the elastic property of skin and expansible organs (Montes 1996). Mutations affecting type I collagen cause Osteogenesis Imperfecta, wherein bones are weak and fracture easily. Mutations affecting type III collagen cause Ehlers-Danlos syndrome, characterised by fragile skin and blood vessels (Alberts *et al.* 2002).

Elastin is an extremely durable, insoluble biopolymer which exhibits little turn-over in healthy tissue (Mithieux and Weiss 2005). It comprises only a small proportion of normal human skin (2-4% dry weight) but several cutaneous diseases reveal the important structural role played by elastic fibres (Uitto 2008). A network of elastic fibres (consisting of 90% elastin) in the ECM gives skin the required resilience so that it can recoil after stretch (Mithieux and Weiss 2005). Collagen fibrils are entwined with elastic fibres to limit the extent of stretching and avoid tissue rupture (Alberts *et al.* 2002).

### *Fibronectin and Laminin*

The remaining fibrous glycoproteins of the dermis include fibronectin and laminin. Fibronectin has many different types of binding sites allowing it to link together different types of ECM cells and molecules. For example, when fibronectin molecules bind to integrin receptors on cells, signalling pathways that promote

cell attachment, migration and differentiation are stimulated (Schultz *et al.* 2005). Laminin is the fundamental protein of the basal lamina, also known as the basement membrane. The basement membrane constitutes sheets of laminin with a layer of specialised anchoring fibrils made of type VII collagen molecules, which tether it to the underlying connective tissue (Alberts *et al.* 2002). It takes this sheet-like form in almost every animal tissue (Colognato and Yurchenco 2000). In the case of skin, epidermal cells rest on the basement membrane which separates the dermis and epidermis. The basement membrane is rich in tyrosine kinase growth factors; these stimulate proliferation in the basal layer of the epidermis (Fuchs and Horsley 2008).

### *Proteoglycans and glycosaminoglycans*

The other main class of extracellular macromolecules are polysaccharide chains of the group called glycosaminoglycans (GAGs) (Alberts *et al.* 2002). These are usually found covalently linked to a core protein in the form of proteoglycans, which can have one or more GAGs attached. Proteoglycans possess a diverse array of abilities, and knockout studies have shown some to be essential to life (Iozzo 1998). They are known as tissue organisers for their influence on cell growth and the maturation of specialised tissues by modulating growth factor activities. They also act as biological filters and regulators of collagen fibrillogenesis, determining skin tensile strength (Iozzo 1998). GAGs are strongly hydrophilic. Their strong negative charge (owing to sulfate and carboxyl groups) attracts osmotically active Na+, causing large amounts of water to be drawn into their structure (Chakravarti *et al.* 1998; Schultz *et al.* 2005).This creates the gel-like consistency which pervades the extracellular spaces providing a turgor pressure, enabling the matrix to withstand compressive forces (in contrast to collagen fibrils, which resist stretching) (Alberts *et al.* 2002). Several different species of GAG have been described: chondroitin sulfate, dermatan sulfate, keratin sulfate, heparin sulfate, and hyaluronate (this is non-sulfated and not found as a proteoglycan) (Montagna and Parakkal 1974).

## Dermal cells

### *Fibroblasts*

The dominant cell type in the dermis is the fibroblast. Fibroblasts are the cells responsible for secreting most of the ECM components. Fibroblasts are present in various connective tissues and represent a heterogeneous population of cells. However, there seems to be no formal classification to define fibroblast subphenotypes (Darby and Hewitson 2007; Rinn *et al.* 2008). A large-scale gene

expression study of human fibroblasts has revealed strikingly distinct patterns of gene expression for fibroblasts from several different anatomical sites, but which were morphologically similar. It was discovered that the diversity of fibroblasts from multiple sites within an individual subject was greater than the diversity of fibroblasts at the same anatomical site between individuals (Rinn *et al.* 2008). Fibroblasts also retain a positional memory when removed (Chang *et al.* 2002; Rinn *et al.* 2008), and this is likely to be important in determining the developmental programs of various structures and organs in the body. Fibroblasts present in many tissues are normally in a relatively quiescent state (Darby and Hewitson 2007). The slow, continuous turnover of matrix macromolecules aids cell migration, where matrix metalloproteases and serine proteases cooperate to degrade matrix proteins such as collagen, laminin, and fibronectin (Alberts *et al.* 2002). Fibroblasts also help to organise the matrix: they crawl over it and pull on the collagen they have secreted, helping to compact it into sheets and draw it out into cables (Alberts *et al.* 2002). Normal adult human skin contains at least three distinct subpopulations of fibroblasts. They are: papillary dermal fibroblasts, which reside in the superficial dermis; reticular fibroblasts, which reside in the deep dermis; and fibroblasts associated with hair follicles (Sorrell and Caplan 2004). A few specialised types of fibroblasts are known such as osteoblasts in bone, chondroblasts in cartilage, and myofibroblasts in regenerating soft tissue. Fibroblasts are crucial during cutaneous wound repair and will be discussed further in this review (see *Fibroplasia,* in Section 1.3.3 below)

### Other dermal cells

Other cells of the dermis include macrophages, mast cells, adipocytes and endothelial cells. Macrophages and mast cells play key roles in the immune system response and are derived from bone marrow and blood-borne precursors known as monocytes. Macrophages are known as scavengers because of their phagocytic capability in dealing with cellular debris. They are important in the inflammatory response, but are suggested to be involved in numerous other metabolic and immunological processes (Makoto 2008). They have also been observed in the dermis at all stages of prenatal development (Van Exan and Hardy 1984). Skin mast cells are best known for their often detrimental role in allergic responses. However, this view is changing and mast cells are now appreciated for their physiologic roles in skin, where their ability to sense a wide range of danger signals suggests they are important players in innate immunity (Metz *et al.* 2008). Mast cells contain heparin, a natural anti-coagulant, and

histamine, a vasodilator. Adipocytes and endothelial cells, with their associated pericytes (Armulik *et al.* 2005) (see *Endothelial migration,* Section 1.3.3), develop to form adipose tissue and blood vessels, respectively, in the lower dermis extending into the hypodermis.

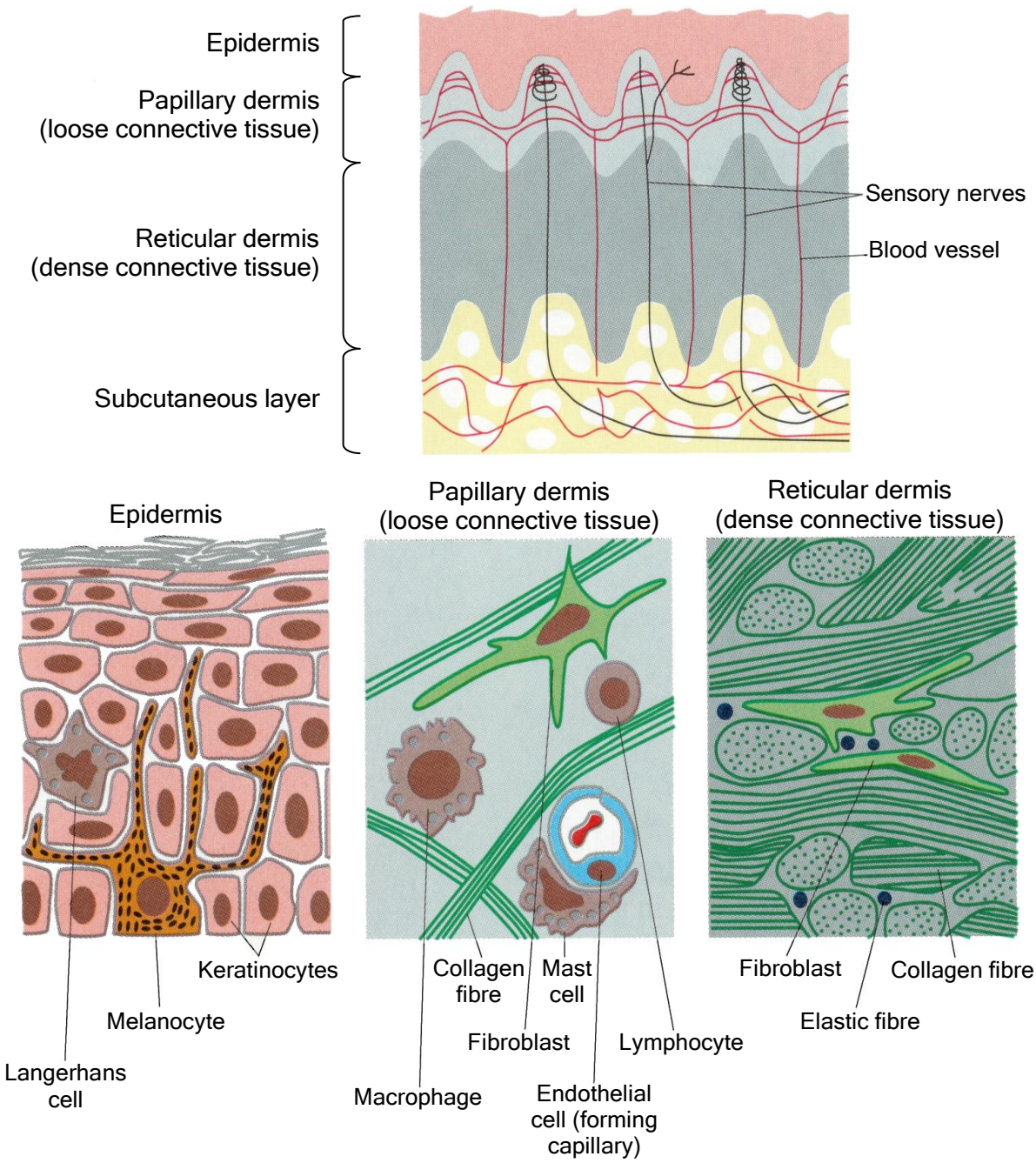



**Figure 1.5: Summary diagram for the structure and main cell types of the skin** (Adapted from Alberts *et al.* 2002).

## 1.2 Developmental Origin of Skin and its Structures

A few days after implantation, the inner cell mass of the blastocyst begins to differentiate into the three primary germ layers (Tortora and Grabowski 1996). These are termed ectoderm, mesoderm and endoderm by way of their positional relationship to each other. Shortly after gastrulation, the single layer of neuroectoderm cells that remain at the embryo surface will ultimately become the nervous system and skin epidermis. Here, Wnt signalling blocks the effect of fibroblast growth factors (FGFs) causing the cells to express bone morphogenetic proteins (BMPs), and therefore, develop into epidermis (Fuchs 2007). The single layer of ectoderm becomes two-layered with the formation of the protective periderm (Cui *et al.* 2007) over a single layer of multipotent epithelial cells (Fuchs 2007). The periderm undergoes further stratification to form intermediate layers at about the time that appendages begin to appear (Polakowska *et al.* 1994). The transient periderm is destined to be sloughed into the amniotic fluid and eventually replaced by the first fully keratinised epidermal cells that move up from the basal layer at later stages of development (Polakowska *et al.* 1994). As development continues, from the original layer of multipotent epithelial cells, a stratified epidermis, hair follicles, sebaceous glands, and sweat glands form (Blanpain and Fuchs 2006). All epidermal layers are present by the fourth month of foetal development in humans (Tortora and Grabowski 1996).

### 1.2.1 Dermal – epidermal interactions during development

Cells of the dermis derive from the somite mesoderm. Signalling from specialised mesenchymal cells within the dermis coordinates the decision to form hair follicles or sweat glands (Millar 2002; Fuchs and Horsley 2008). This has been demonstrated elegantly by several studies which place ectopic epithelial tissue over native mesenchyme, causing native epithelial structures to develop in most cases (Montagna and Parakkal 1974; Hardy 1992; Koster and Roop 2007; Rinn *et al.* 2008). The reverse is also true, as shown in a study in which dermal-sheath tissue from the scalp of an adult human male was implanted into the forearm of a genetically unrelated female. This was sufficient to form new dermal papillae and induce new hair follicles of a larger, thicker and mostly pigmented form more like the donor's scalp and not at all like the small un-pigmented hairs of the recipient's forearm (Reynolds *et al.* 1999). These studies show that the process of appendage development seems to be characterised by an initial signal from the

dermis to the epidermis with instructions to make an appendage. After this initiating signal, the epidermis replies with a chemical message of its own to which the dermis reciprocates with more specific instructions as to what the epidermis is to do next (Hardy 1992; Millar 2002) (see Figure 1.6 and Section 1.2.3 below).

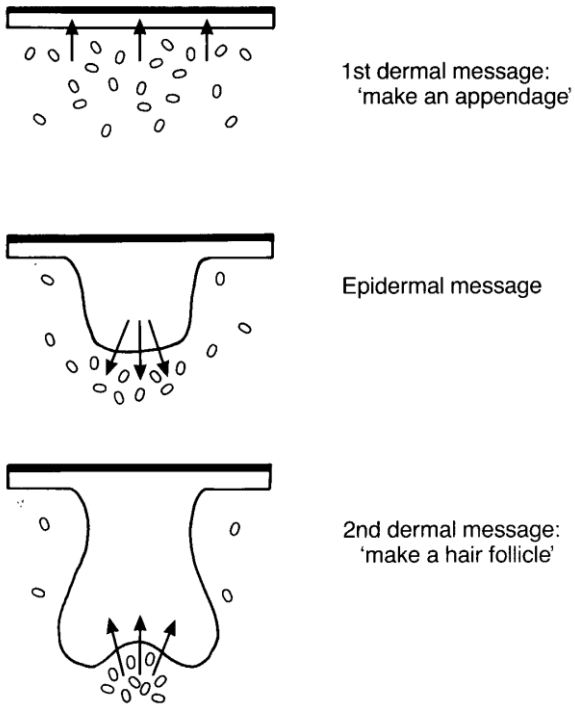



**Figure 1.6: Dermal to epidermal communication during development**
(From Hardy 1992).

Epidermis, hair follicles and sebaceous glands all have distinct stem cells which can self-renew, fully generate their respective tissues, and maintain homeostasis once the tissue is formed (Fuchs and Horsley 2008).

## 1.2.2 Epidermis morphogenesis

Epidermal morphogenesis begins in the basal layer, which maintains a stem cell population that gives rise to the more differentiated keratinocytes in higher levels of the stratified epidermis (see *Keratinocyte stratification* in Section 1.1.1). The basal layer produces, secretes, and assembles a type of extracellular matrix, comprising most of the basement membrane separating the epidermis and the dermis (Blanpain and Fuchs 2006). The basement membrane, upon which the basal layer rests, is rich in proteoglycans and other proteins that act as 'molecular sinks' for growth factors which restrict (Transforming Growth Factor (TGF)-βs) or promote (TGF-α, Epidermal Growth Factor (EGF), Insulin-like Growth Factors

(IGFs)) epidermal proliferation (Fuchs 2008). In the life of an organism, there must be a continuous supply of differentiated keratinocytes from the basal layer, as all are eventually sloughed off. Therefore, basal keratinocytes must repress the expression of genes that initiate terminal differentiation to prevent this differentiation occurring prematurely, while also maintaining the expression of genes required for proliferation (Koster and Roop 2007). The protective and anti-dehydration qualities of the epidermis are found in the properties of keratin. Keratins constitute up to 85% of a fully differentiated keratinocyte and keratin filaments are among the most stable found in nature (Fuchs 1995). Typically, the basal layer expresses keratins K5 and K14, whereas the intermediate suprabasal layers express K1 and K10 (Fuchs 1995; Blanpain and Fuchs 2006; Fuchs 2007). K6, K16 and K17 are also expressed suprabasally, but only in hyper-proliferative situations such as wound healing (Fuchs 2007). Epidermal growth and proliferation is in constant balance: if there is too little proliferation, the result will be thinning of the skin, thus compromising protection (Fuchs 2007), while too much can result in hyper-proliferative disorders, including psoriasis (Lowes *et al.* 2007) and cancers.

Mesoderm-derived cells contribute to the collagen-secreting fibroblasts of the underlying dermis (Montagna and Parakkal 1974), blood vessels, arrector pili muscles that attach to each hair follicle, subcutaneous fat cells, and the immune cells that infiltrate and reside in the skin (Blanpain and Fuchs 2006). Neural crest-derived cells contribute to melanocytes, sensory nerve endings of the skin, and the dermis of the head (Blanpain and Fuchs 2006). Two main types of dermis are present at the onset of skin morphogenesis: a superficial dense dermis and a superficial loose dermis, which will form future hair and bare skin regions respectively (Olivera-Marinez *et al.* 2004).

### 1.2.3 Hair follicle morphogenesis

As alluded to earlier, hair follicle formation occurs during embryogenesis with a series of signals sent between dermal cells and overlying epithelial cells on the surface, causing fate changes in both cell populations (Millar 2002). An initial signal originating in the dermis (1st dermal message) triggers the formation of regularly spaced thickenings in the epidermis, known as placodes (Hardy 1992; Millar 2002). As dermal cells populate the skin, they receive signals from the epithelium (epidermal message) to form ‘dermal condensates’, which are the precursors to dermal papilla (DP) cells (Fuchs 2008). Signalling candidates for this phase include Wnt family and platelet-derived growth factor (PDGF)-A

pathways (Millar 2002). The diversity of human dermal fibroblasts likely has connection with anatomic patterning of skin also (Rinn *et al.* 2008), and this is expected to contribute to the remarkable diversity of its structure and function. Following another signal (2nd dermal message) from the dermal condensate, the epithelial placode cells proliferate, down-grow, and invade the dermis, surrounding the dermal condensate, which becomes the dermal papilla of the hair follicle (Hardy 1992; Millar 2002). Sonic Hedgehog (involved in embryonic patterning of organs and nervous system) when expressed in the placode is a key player in follicle morphogenesis, together with Wnt (also involved with embryonic morphogenesis) and the BMP inhibitor Noggin (Fuchs 2008). These factors are required to form the DP from the dermal condensate (St-Jacques *et al.* 1998; Millar 2002; Levy *et al.* 2005). Lymphoid enhancer-binding factor-1 (LEF1) and stabilised β-catenin are also critical for hair follicle morphogenesis (Fuchs 2008; Fuchs and Horsley 2008) in humans. The down-growing epidermal cells become known as the hair matrix, whose daughter cells then move up the follicle, differentiating into either hair cells or inner root sheath (IRS) cells, depending upon their position (Hardy 1992). The outer layer of cells becomes the outer root sheath (ORS), which runs contiguous with the basal epidermis and is enclosed on its exterior by the basement membrane (Blanpain and Fuchs 2006). Notch-1 expression is a key factor for controlling the phenotype of keratinocytes as they leave the matrix and differentiate into the particular cell lineages of the hair follicle (Millar 2002). The final result of this process is a keratinised hair (see Figure 1.7).

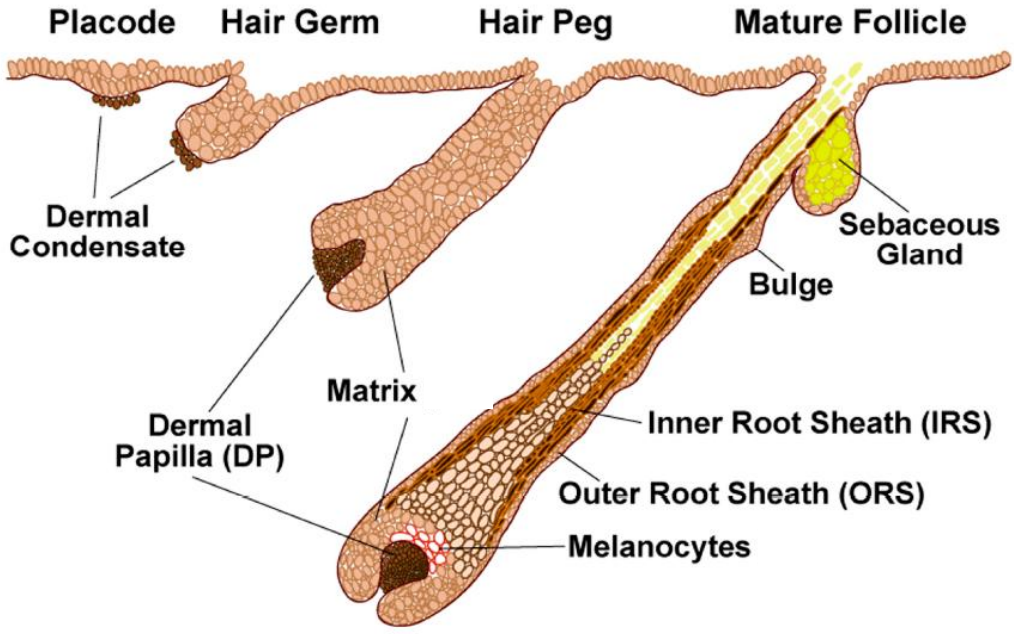



**Figure 1.7: Embryonic stages of hair follicle morphogenesis**
(From Fuchs 2008).

### 1.2.4 Sebaceous gland development

Sebaceous glands are usually located attached to the follicle above the bulge and arrector pili muscle, just below the skin surface. Together with the follicle, the sebaceous gland is known as the pilosebaceous unit. They are found all over the body except on the palms of hands or soles of the feet (Smith and Thiboutot 2008). Until recently, relatively little was known about its function as it was considered an evolutionary vestige. However, today there are at least 30 specific functions under investigation by various researchers (Zouboulis *et al.* 2008). Broadly speaking, the sebaceous gland produces lipids and sebum for lubrication and protection against bacteria. It does this via the production of terminally differentiated sebocytes, which disintegrate to release their oils in a holocrine manner (Fuchs 2007; Fuchs 2008; Smith and Thiboutot 2008). The sebaceous gland remains attached to, and develops alongside, the developing hair follicle, where at least three important pathways are involved: the Wnt signalling pathway, the c-*myc* signalling pathway, and the hedgehog signalling pathway (Smith and Thiboutot 2008).

### 1.2.5 Sweat gland development

During the third month of human embryonic development, epidermal ridges form on the palms, fingers, and soles of feet. Epidermal or 'rete' ridges occur because the undulating contour of the dermis-epidermis boundary (with underlying dermal papillae) is more pronounced in these areas (Jones *et al.* 1995; Tortora and Grabowski 1996; Blanpain and Fuchs 2006). About the same time, sweat glands begin to develop as a cord of epithelial cells growing from the epidermal ridge on the palms, fingers, and soles, and by the fifth month on the rest of the body (Sato *et al.* 1989 cited in; Morimoto and Saga 1995; and Saga 2002). The human body has 3-4 million sweat glands, of which there are two types: eccrine and apocrine sweat glands (Morimoto and Saga 1995; Saga 2002). Eccrine sweat glands cover almost the entire body surface with ducts that open directly to the skin surface, whereas apocrine glands exist mainly in the axillary and genital areas with ducts opening to hair follicles (Morimoto and Saga 1995; Saga 2002) The main function of eccrine glands is the control of body temperature. Humans have a unique ability to dissipate heat faster than any other animal (Quinton 1983). The secretory portion of the sweat gland derives from embryonic epidermal tissue which differentiates into three types of cells: clear secretory cells (rich in sodium-potassium-chloride [Na+-K+-2Cl-] co-transporters), dark secretory cells (which contain glycoprotein granules), and myoepithelial cells (for gland contraction) (Morimoto and Saga 1995; Saga 2002; Kreyden and Scheidegger 2004).

In summary, the epidermis and dermis derive from epithelial and mesenchymal progenitor tissues respectively. These tissues themselves arise from different embryonic sources; the epidermis from ectoderm and the dermis from mesodermal provenance. These tissues, with their respective gene expression programmes, interact and orchestrate the development of the skin and its structures in a highly coordinated manner. Any failure to properly develop either the dermis or the epidermis will almost certainly result in noticeable defects in appendage development.

## 1.3 Wound Healing

Tissue disruption in higher vertebrates, unlike lower vertebrates, results not in regeneration, but in a rapid repair process (Wahl 2002; Eming *et al.* 2007b) involving bleeding, inflammation, proliferation (which includes re-epithelialisation, angiogenesis, and fibroplasia), and the formation and maturation of a scar (tissue remodelling) (Yamaguchi and Yoshikawa 2001; Chodorowska and Rogus-Skorupska 2004; Diegelmann and Evans 2004; Watson 2006; Eming *et al.* 2007b). Numerous cell signalling events involving various cytokines and growth factors coordinate this extremely complex process (Li *et al.* 2007).

### 1.3.1 Healing Phase I - Hemostasis

The shortest in duration yet most critical phase in healing is hemostasis. Injury to any vascularised tissue, whether internal or external, will cause some blood loss. Therefore, immediately following injury, minimising blood loss is the primary goal. Hemostasis involves two major processes: development of a fibrin clot and coagulation (Li *et al.* 2007) which are carefully directed by a series of important cytokines and growth factors (Table 1.1).

#### Fibrin clot

Platelets accumulate at the injury site by a process involving activation, adhesion, and aggregation to form a tightly-packed plug, which is the first step in hemostasis (Li *et al.* 2007; Nurden 2007). As blood components escape and leak into the injury site, platelets come into contact with, and are activated by, exposed collagen and other ECM components. Platelets have a series of surface membrane glycoproteins that allow adhesion to collagen (Nurden 2007), fibronectin and other adhesive proteins (Li *et al.* 2007). Upon contact, platelets release clotting factors, essential growth factors and cytokines such as TGF-β and platelet-derived growth factor (PDGF) (Singer and Clark 1999; Yamaguchi and Yoshikawa 2001; Diegelmann and Evans 2004; Metcalfe and Ferguson 2007; Hantash *et al.* 2008). Platelets also release substances that promote angiogenesis, inflammation, and the immune response (Nurden *et al.* 2008). When integrin αIIbβ3 becomes activated and binds fibrinogen (cleaved by thrombin (Martin 1997)), and other adhesive proteins form bridges between platelets (Nurden 2007; Nurden *et al.* 2008), aggregation occurs and this stabilises the plug (Laurens *et al.* 2006).

## Coagulation

Coagulation is a very complex process involving a couple of different cascades (coagulation and complement cascades), which entails a number of proenzyme-to-activated-enzyme conversions resulting in the transformation of prothrombin into thrombin, which in turn converts soluble fibrinogen into insoluble fibrin (Lorenz and Longaker 2003; Li *et al.* 2007). The end result is a provisional matrix of platelets embedded in a mesh of cross-linked fibrin fibres over and through which cells such as leukocytes, keratinocytes, fibroblasts, and endothelial cells can migrate (Martin 1997; Li *et al.* 2007). The clot also functions as a reservoir of growth factors and cytokines released by degranulating platelets, which will be involved in all phases of wound healing, such as attracting circulating inflammatory cells, contributing to the process of re-epithelialisation and wound contraction, and stimulating the angiogenic response (Martin 1997; Baum and Arpey 2005; Li *et al.* 2007).

**Table 1.1: Key growth factors released during and shortly after hemostasis**

| Growth factor | Function |
|---|---|
| **PDGF** | Chemotaxis for macrophages and fibroblasts, macrophage activation, fibroblast mitogen and matrix production and angiogenesis (Yamaguchi and Yoshikawa 2001). |
| **TGF-β** | Keratinocyte migration, chemotaxis for macrophages and fibroblasts, and fibroblast matrix synthesis and remodelling (Yamaguchi and Yoshikawa 2001). Chemotaxis of neutrophils and monocytes (Wahl 2002). |
| **EGF** | Keratinocyte migration and replication (Yamaguchi and Yoshikawa 2001). |
| **Vascular Endothelial Growth Factor (VEGF)** | A vascular permeability factor; influences the extravasation of plasma proteins to create a temporary support structure (Wahl 2002) to produce localised swelling (Chodorowska and Rogus-Skorupska 2004) bind fibrin and stimulate angiogenesis (Werner and Grose 2003). |
| **Fibroblast Growth Factor-2 (basic FGF)** | Binds fibrin and stimulates angiogenesis (Werner and Grose 2003) |
| **IGF-1** | Binds fibrin and stimulates stromal cell function and proliferation (Werner and Grose 2003). |

### 1.3.2 Healing Phase II - Inflammation

On the surface, the signs of inflammation are redness, heat, swelling, and pain. Once bleeding has stopped, vasoconstriction ceases and vasodilation occurs in the capillaries allowing extravasation of serum proteins and inflammatory cells into the wound site - a highly orchestrated process involving the nervous system, cytokines, chemokines, and growth factors (Baum and Arpey 2005) (see Figure 1.8). Vasodilation is promoted via by-production of bradykinin and vascular permeability factor in the 'coagulation cascade' and C3a/C5z anaphylatoxin production in the 'complementary cascade' (Lorenz and Longaker 2003). In response, resident mast cells produce a number of mediators which promote vasodilation including histamine (Lorenz and Longaker 2003; Baum and Arpey 2005), as well as chemotactic agents for leukocytes such as Tumour Necrosis Factors (TNFs), proteases, leukotrienes, and Interleukins (IL) (Li *et al.* 2007). With the onset of vasodilation in the acute stages, polymorphonuclear cells (polymorphs) are the first to arrive at the scene of a cutaneous wound. They are recruited from circulating blood in response to molecular changes in the surface of endothelial cells lining the capillaries at the wound site (Martin 1997). As the name suggests, polymorphs are leukocytes which give the appearance of possessing a many-lobed nucleus.

#### Neutrophils

Neutrophils arrive at the wound site within minutes of injury and steadily increase in number to peak at 24-48 hours post-wounding in adult human tissue (Metcalfe and Ferguson 2007). Their primary roles are to sterilise the wounded area by ingesting foreign particles and bacteria (Singer and Clark 1999; Lorenz and Longaker 2003), and to release pro-inflammatory cytokines such as IL1α, IL1β, and TNF-α (Yamaguchi and Yoshikawa 2001; Baum and Arpey 2005). This activates growth factor expression in macrophages, keratinocytes, and fibroblasts (Hübner *et al.* 1996), and promotes diapedesis (movement of cells in to and out of circulating blood through vessel walls) (Eming *et al.* 2007b). Neutrophils also begin the process of debridement (removal of dead, damaged tissue) and phagocytosis of infectious agents by release of a large variety of antimicrobial host-defence peptides and proteases (Eming *et al.* 2007b; Steinstraesser *et al.* 2008). Later in inflammation, provided infection doesn't occur, neutrophils decline in number; macrophages then dominate (Li *et al.* 2007).

## Macrophages

Macrophages scavenge and phagocytose any foreign bodies including expended neutrophils that have previously phagocytosed foreign elements (Singer and Clark 1999). They release a myriad of factors including TGF-α, TGF-β, IGF-1, FGF, PDGF, VEGF, HB-EGF (heparin binding EGF-like growth factor) with the latter four promoting angiogenesis (Yamaguchi and Yoshikawa 2001). Although polymorphonuclear cell infiltration peaks at 24-48 hours, much is accomplished by this time with early cleansing of wound the site from cellular debris, foreign particles and bacteria (Baum and Arpey 2005). The exit of polymorphonuclear cells is facilitated by apoptosis (Darby and Hewitson 2007), macrophage phagocytosis, and via sloughed eschar (scab) (Baum and Arpey 2005). From here, the intensity of inflammation begins to subside and monocytes replace polymorphs as the major infiltrating cell type, where they subsequently transform into macrophages (Lorenz and Longaker 2003; Diegelmann and Evans 2004; Baum and Arpey 2005; Faler *et al.* 2006; Darby and Hewitson 2007). These macrophages continue the phagocytotic and proinflammatory work of the previous cohort but also participate in a more complex role in wound healing, remaining at the site for longer (days to weeks) (Baum and Arpey 2005). Monocytes and activated macrophages can bind to the ECM via cell surface integrin receptors (Hantash *et al.* 2008). In addition, macrophages release digestive enzymes such as matrix metalloproteinases (MMPs) for ECM degradation and turnover (Faler *et al.* 2006). Macrophages are the primary cells responsible for the regulation of tissue repair, and as such secrete more than 20 different cytokines and growth factors during the repair process (Lorenz and Longaker 2003). Several authors have reviewed the stimulatory effects of macrophages and their products on other cells. Some of these include: attracting fibroblasts to the wound site, for example, by release of PDGF, TGF-β (Diegelmann and Evans 2004), and fibronectin (Li *et al.* 2007); stimulating fibroblasts to produce collagen via FGF-2, IGF, and TGF-β (which also stimulates fibroblasts to differentiate into myofibroblasts); stimulating angiogenesis by release of FGF-2, VEGF-A, and TGF-β; and stimulating re-epithelialisation via release of TGF-α, FGF-2 and IGF-1 (Werner and Grose 2003; Baum and Arpey 2005). Therefore, macrophages are crucial in the progression of wound healing from inflammation to repair.

## T-lymphocytes and Eosinophils

T-lymphocytes play a significant regulatory role in wound healing. One of these roles in humans is to produce connective tissue growth factor (CTGF) which

stimulates fibroblasts to divide and produce ECM components such as collagen type I, fibronectin, and integrin $\alpha_5$ (Workalemahu *et al.* 2003). Eosinophils appear to have a role in wound healing by producing TGF-α which promotes re-epithelialisation by keratinocytes (Baum and Arpey 2005).

The final stages of inflammation usher in the onset of re-epithelialisation, which coincides with the migration of fibroblasts and endothelial cells, and the formation of granulation tissue (Metcalfe and Ferguson 2007).

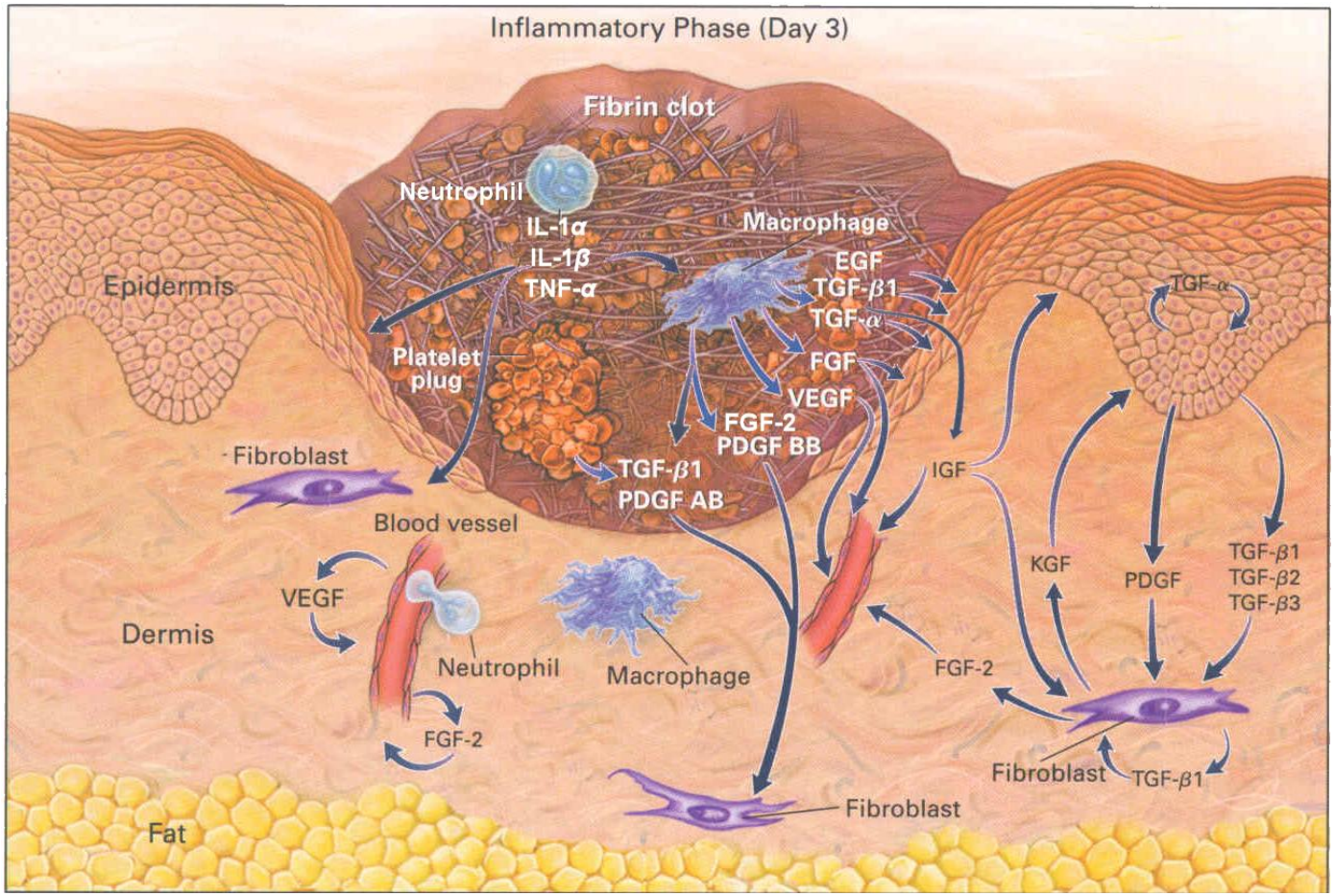



**Figure 1.8: Inflammatory phase after approximately 3 days**
Growth factors and cytokines at play in the inflammatory phase with macrophages directing much of the traffic. Illustration has been modified from the original by Singer and Clark (1999) to incorporate recent findings.

### 1.3.3 Healing Phase III – Proliferation

The proliferative phase is characterised by the formation of a new epithelium at the wound surface and granulation tissue deep in the wound (Chodorowska and Rogus-Skorupska 2004). It is orchestrated throughout by cytokines produced by platelets, macrophages, fibroblasts and keratinocytes. Re-epithelialisation, granulation tissue formation, and angiogenesis all occur simultaneously in the proliferative phase, but each is discussed individually below.

#### Re-epithelialisation

Within hours of wounding, the epidermis thickens and keratinocytes undergo morphological changes (Lorenz and Longaker 2003). Immature keratinocytes dissolve their attachment points (hemidesmosomes) to the basal membrane (via MMP-9 secretion which degrades collagen IV and laminins (Li *et al.* 2007)) and to each other (desmosomes) releasing themselves and migrating across the wound bed in response to chemo-attractants (Martin 1997; Singer and Clark 1999; Wahl 2002). Leading-edge keratinocytes must express new integrins in order to grasp and crawl over the provisional wound matrix and underlying dermis (Martin 1997). Migration is aided by adhesion glycoproteins such as tenascin and fibronectin which act like 'railroad tracks' (Lorenz and Longaker 2003). Migrating cells originate from the basal layer, from suprabasal cells, or a large contribution may come from stumps of hair follicles left intact after wounding (Martin 1997; Yamaguchi and Yoshikawa 2001; Ito and Cotsarelis 2008). It has been demonstrated that stem cells extracted from the hair follicle bulge can be induced to differentiate into hair follicle and sebaceous cells, which supports the multipotential capacity of cutaneous stem cells (Roh and Lyle 2006). In order to cut a path along the wound margin between the fibrin clot and healthy dermis, the leading-edge kerotinocytes must dissolve the fibrin barrier ahead of them (Martin 1997; Baum and Arpey 2005). The activation of plasmin by plasminogen activator and the production of collagenases (MMPs) by epidermal cells dissolve fibrin and collagen type I, respectively (Yamaguchi and Yoshikawa 2001). An important characteristic of these keratinocytes is that they do not express functional fibrinogen/fibrin receptors such as the integrin αVbβ3 and therefore do not interact with, or become attached to, fibrinogen or its derivatives (Kubo *et al.* 2001). Within two days after injury, epidermal cells at the wound margin begin to proliferate behind the migrating cells (Singer and Clark 1999). As re-epithelialisation continues, basement membrane proteins reappear and epidermal cells revert to their normal phenotype (Singer and Clark 1999). Re-

epithelialisation is usually complete within 24-48 hours (Baum and Arpey 2005). Following the establishment of a monolayer of keratinocytes, epidermal migration stops and a new stratified epidermis attached to the basal lamina re-emerges (Martin 1997; Lorenz and Longaker 2003) within 7-9 days (Li *et al.* 2007). Once the epithelial bridge is complete, enzymes are released to dissolve the attachment at the base of the scab (Diegelmann and Evans 2004), resulting in the dissection of the fibrin eschar from viable tissue (Singer and Clark 1999; Chodorowska and Rogus-Skorupska 2004). Important growth factors in the re-epithelialisation process are EGF, TGF-α, and keratinocyte growth factor (KGF, also known as FGF-7) (Singer and Clark 1999; Chodorowska and Rogus-Skorupska 2004).

## Granulation tissue

Granulation tissue (named for the granular appearance of the blood vessels in the wound) is composed of inflammatory cells, fibroblasts, and newly-formed blood vessels (Wahl 2002) embedded in a loose ECM of collagen, fibronectin and hyaluronic acid (Chodorowska and Rogus-Skorupska 2004). Early in response to the injury, resident dermal fibroblasts near the wound begin to proliferate (Martin 1997). The fibroblast front moves by chemotaxis into the wound led by a wave of phagocytosing macrophages (Thackham *et al.* 2008). Chemotactic agents include PDGF and TGF-β (Martin 1997). From here, fibroblasts become the dominant cell type in the wound area, with fibroplasia and angiogenesis ensuing (see Figure 1.9).

### *Fibroplasia*

Fibroblasts produce an ECM which facilitates further cell migration and provides mechanical support for new capillaries, which in turn supply further nutrients to sustain cell metabolism (Gaffney *et al.* 2002). The main types of ECM components fibroblasts produce are collagens (mainly types I and III), fibronectin, elastin and proteoglycans. Collagens I and III are the major fibrillar collagens of the ECM in both wounded and unwounded skin (Lorenz and Longaker 2003). Type I collagen is the most abundant type of collagen in the normal dermis (approximately 80-90%) and type III collagen (normally 10-20%) is actively secreted by fibroblasts during the early stages of wound healing and may account for up to 30% of the collagen in a healing wound (Fathke *et al.* 2004; Kwon *et al.* 2007). The marked increase in collagen III production after wounding may persist for several weeks (Robins *et al.* 2003) before normalising during the remodelling phase. Although collagen turnover naturally occurs continually in normal skin, collagen expression is suppressed in uninjured skin, supposedly for the benefit of

the healing wound (Ihlberg *et al.* 1993). Procollagens (precursor collagen polypeptides) are synthesised by fibroblasts. A critical step in their production is the hydroxylation of lysine and proline residues; this occurs in the endoplasmic reticulum and is important for the final arrangement of the precursor collagen molecules into mature triple-helix structures (Baum and Arpey 2005). Cross-linking is another post-translational modification which takes both intramolecular and intermolecular forms as triple-helix molecules form fibrils and then fibres (Diegelmann 2001). Like keratinocytes, fibroblasts must also alter their integrin expression profile from collagen binding to fibrin, fibronectin and vitronectin binding receptors in order to crawl into the clot (Martin 1997). The fibronectin matrix also provides a scaffold for collagen fibrils (Li *et al.* 2007).

The various roles played by fibroblasts in the process of wound healing are as important as they are diverse. In a microarray study of the transcriptional response of human fibroblasts to serum, Iyer *et al.* (1999) concluded that fibroblasts can participate in the processes of: hemostasis, clotting, clot dissolution, remodelling, chemotaxis and activation of neutrophils and T-lymphocytes, angiogenesis, migration and proliferation of fibroblasts and their differentiation into myofibroblasts, and migration and proliferation of keratinocytes for re-epithelialisation. Fibroblasts also express receptors for a number of cytokines including PDGF, TGF-β1 and TNF-α which all may play a role in recruitment and activation during wound healing (Darby and Hewitson 2007). While fibroblasts can adequately produce and replace all ECM components of normal skin, a scar forms because their ability to organise those components is impaired (Singer and Clark 1999; Sorrell and Caplan 2004). Interestingly, elastin which is normally present in skin, is not produced in scars, and this may be a cause of the firmness and loss of flexibility seen in scar tissue (Baum and Arpey 2005).

*Wound contraction*

As previously mentioned, fibroblasts can differentiate into myofibroblasts, which as the name suggests, endows them with a smooth muscle-like contractile ability. In fact, these specialised mesenchymal cells exhibit features of both fibroblasts and smooth muscle cells such as the expression of α-smooth muscle actin (Gabbiani 1992; Darby and Hewitson 2007). This ability appears to be important for wound contraction. Wound contraction decreases the size of the wound substantially (e.g. up to 80% in the trunk and perineum of humans), thus reducing the amount of new tissue formation needed (Lorenz and Longaker 2003). Re-epithelialisation is also made easier by underlying contractile connective tissue,

as it brings wound margins closer together (Martin 1997). In mammals whose skin is loosely attached to the underlying tissue layer, for example: rodents, wound contraction results in wound closure with minimal scarring or loss of function, whereas in humans, whose skin is more firmly attached, contraction can be less beneficial, leading to outcomes that range from minor cosmetic scarring in some instances, to loss of joint motion or major body deformation in others (Grinnell 1994). Myofibroblasts also play a significant role in tissue fibrosis, with TGF-β implicated strongly as a mediator (Evans *et al.* 2003). Myofibroblasts are only transient in their presence at the proliferative phase, and as mechanical tension decreases they disappear by apoptosis (Grinnell *et al.* 1999).

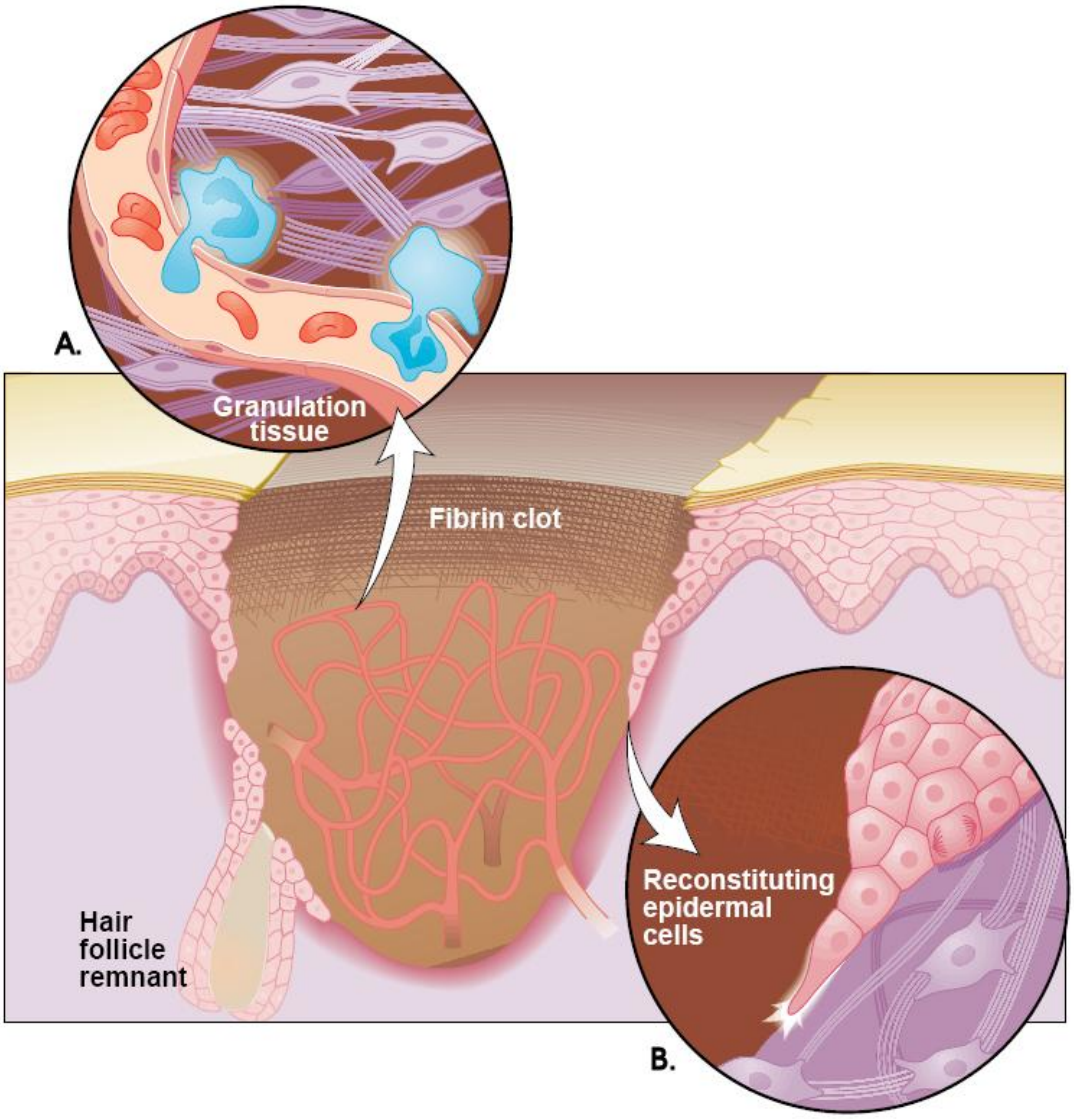



**Figure 1.9: Proliferative phase**
A: The temporary fibrin clot is infiltrated by inflammatory cells and fibroblasts as granulation tissue becomes densely packed with new capillaries. Granulation tissue contracts with the aid of myofibroblasts which tug on surrounding collagen fibres. B: Re-epithelialisation begins at the wound edge and from the remnants of hair follicles to re-establish the epidermal barrier. Leading edge keratinocytes clear a path over the provisional matrix and uninjured dermis (from Martin 1997).

## Angiogenesis

Angiogenesis is defined simply as the formation of blood vessels which sprout from existing blood vessels, while vasculogenesis specifically refers to the mobilisation of endothelial stem cells from bone marrow to form new blood vessels. Both processes occur together to produce new blood vessels in wound tissue (Eming *et al.* 2007a). Angiogenesis is necessary to nourish the newly-formed granulation tissue (Singer and Clark 1999). Unique to wound angiogenesis, as opposed to other physiological and pathological conditions of vessel growth, is the involvement of the innate immune response. As mentioned previously, immediately following injury, different types of leukocytes are attracted to the injury site releasing an assortment of cytokines and growth factors, through which they may trigger, sustain or potentially terminate angiogenesis (Eming *et al.* 2007a).

### *Cytokines and growth factors*

Several reviewers suggest that the most important factors in angiogenesis are VEGF, FGF-2, and TGF-β. These cytokines can be released by epidermal cells, fibroblasts, macrophages, and vascular endothelial cells (Diegelmann and Evans 2004). Other growth factors known to enhance angiogenesis are PDGF, HB-EGF, IGF-1, hepatocyte growth factor (HGF) and FGF-2, along with heparin, leptin and fibronectin (Yamaguchi and Yoshikawa 2001), and also angiogenin, angiotropin and angiopoietin 1 (Singer and Clark 1999). VEGF can be detected at high levels during the proliferative phase, and its transcription is induced by PDGF, endothelial growth factor, TNF-α, TNF-β, interleukin-1β (IL-β), and exposure to hypoxia (Chodorowska and Rogus-Skorupska 2004). With the elevated metabolic activity at the wound site, there is elevated demand for oxygen and nutrients (Diegelmann and Evans 2004). Prolonged hypoxia seems to inhibit wound healing by blocking fibroblast proliferation, collagen production, and angiogenesis, as well as increasing the risk of infection (Thackham *et al.* 2008). However, the development of oxygen tension (Knighton *et al.* 1983), lactate build-up (Zieker *et al.* 2008) and a low pH stimulates the release of factors which promote angiogenesis (Diegelmann and Evans 2004). An interesting signalling pathway involves the role of low oxygen tension, which in turn stimulates the expression of a nuclear transcription factor termed hypoxia-inducible factor-1 (HIF-1) by vascular endothelial cells (Gerber *et al.* 1997). HIF-1 in turn binds as an enhancer to sequences of DNA that regulate the expression of VEGF, stimulating angiogenesis. With new blood vessels entering the wound repair area, the oxygen tension returns to a normal level. Oxygen then binds to HIF-1

and blocks its activity leading to a decrease in synthesis of VEGF (Diegelmann and Evans 2004).

*Endothelial migration*

The development of new blood vessels begins when MMPs are released into the connective tissue to clear the way for new capillaries to sprout. Fragments of the degraded proteins recruit blood monocytes to the site of injury and, following their conversion to macrophages, release angiogenic factors (Singer and Clark 1999). Macrophage-derived angiogenic factors affect endothelial cells mitogenically (FGF-2, TGF-α, IGF-1), or by inducing migration (angiotropin, human angiogenic factor), or are angiogenic by some other mechanism (Sunderkötter *et al.* 1991). Endothelial migration is also stimulated by the aforementioned important angiogenic factors (VEGF, FGF-2, and TGF-β). These factors also stimulate endothelial expression of integrins, especially αVbβ3 (Martin 1997; Baum and Arpey 2005), which bind ECM proteins to facilitate migration. The movement of endothelial cells towards their angiogenic stimulus requires the degradation of parent vessel basement membrane. This is achieved via FGF-2-stimulated conversion of plasminogen to plasmin and procollagenase to collagenase allowing these two proteases to digest basement membranes (Singer and Clark 1999). Migrating endothelial cells then elongate and align with one another to create a solid sprout which increases in length as endothelial cells proliferate (Folkman and Klagsbrun 1987). Two capillary sprouts may join at their tips to form a loop allowing blood flow to begin, after which pericytes position themselves along the base of the loop and new sprouts grow from the apex of the loop to continue the angiogenic process (Folkman and Klagsbrun 1987).

*Extracellular matrix*

Aside from cells and cytokines, the structure and organisation of ECM components is important for angiogenesis. ECM components such as fibronectin, collagen and vitronectin provide structural support and guidance for invading capillaries and migrating cells as well as serving as a reservoir for important growth factors (Baum and Arpey 2005; Li *et al.* 2007). Laminins of the basement membrane have also been shown to be important for angiogenesis (Baum and Arpey 2005; Li *et al.* 2007). The transition of granulation tissue into scar tissue is represented by regression of capillaries no longer needed and differentiation of newly formed blood vessels into mature vascular structures (Eming *et al.* 2007a). Involution of newly-formed capillaries is achieved by endothelial apoptosis (Desmoulire *et al.* 1995; Lokmic *et al.* 2006). Several mechanisms have been identified which might contribute to vascular regression at the wound site: (i)

decreased expression of growth factors necessary for endothelial cell survival; (ii) increased expression of angiogenesis inhibitors; and (iii) the transition from a provisional pro-angiogenic ECM (consisting of vitronectin, fibronectin and fibrin) into a permanent collagenous ECM (Eming *et al.* 2007a).

### 1.3.4 Healing Phase IV - Remodelling

Upon completion of epithelialisation and fibroplasia, cell proliferation and neovascularisation cease, scar tissue forms, and the wound enters the remodelling phase, which continues for several months or even years. Remodelling is characterised by contraction, reduced redness, reduced thickness, and increased strength of the wound area (Baum and Arpey 2005). The central event in remodelling is the reorganisation of the collagen matrix in order to regain the former qualities of uninjured skin as much as possible. This event is characterised by a balance between synthesis of new components of the scar matrix and degradation by proteases (Eming *et al.* 2007a). The balance of newly-formed collagen with the destruction of old collagen determines the final physical quality of the scar (Metcalfe and Ferguson 2007). Degradation is driven by serine proteases and MMPs (also known as collagenases, gelatinases, and stromelysins) under the control of various cytokines (Wahl 2002). Tissue inhibitors of metalloproteinases (TIMPs) are natural inhibitors of MMPs (Birkedal-Hansen 1995), and provide a counter-balance to degradation. Upsetting this balance can result in pathological wound healing states such as chronic ulcers. As remodelling progresses, the soft and gelatinous collagen type III laid down in the granulation phase is gradually replaced by more highly organised type I collagen (Chodorowska and Rogus-Skorupska 2004; Li *et al.* 2007), and there is a reduction in proteoglycan and water content (Baum and Arpey 2005). These events allow collagen fibres to lie closer together, allowing collagen cross-linking and ultimately decreasing scar thickness and increasing wound-bursting strength (Romo III *et al.* 2005). Collagen cross-linking is essential in providing tensile strength and mechanical stability of the collagen fibrils (Oxlund *et al.* 1996). The small leucine-rich proteoglycans (SLRPs) lumican and decorin modulate collagen fibrillogenesis and enhance collagen fibril stability (Neame *et al.* 2000). Wound strength remains inversely proportional to wound thickness over time (Baum and Arpey 2005). By day 21, new collagen production reaches a maximum and from this point on, fibroblasts gradually decrease production and begin to disappear (Baum and Arpey 2005; Li *et al.* 2007). At this point wound strength is only about 20% that of normal skin, reflecting the haphazard arrangement of fibrils in the

dermis. However, the healed skin will reach a maximum strength of around 80% after 6 months as fibrils become more organised in line with directional stress forces on the skin (Baum and Arpey 2005). Regulation of collagen synthesis is controlled by several growth factors, of which TGF-β and FGF are the main ones (Li *et al.* 2007). The reduction in redness over time corresponds to the reduction in capillary density within the wound as it matures (Baum and Arpey 2005). Characteristic of mature scars is the absence of appendages such as hair follicles. Although keratinocyte stem cells easily repopulate the epidermis, stem cells responsible for appendages appear not to repopulate a scar (Baum and Arpey 2005). The formation of a scar may be seen as an unfortunate consequence of mammalian wound healing, but the benefit of scar formation is the prompt reformation of tissue integrity (Lorenz and Longaker 2003).

### 1.3.5 Summary of wound healing events

The phases of wound healing are by no means discrete and overlap considerably, as do the events within each phase. Figure 1.10 below illustrates this overlap and the roles of the various cell types within each phase at the wound site. Table 1.2 summarises the various key cytokines and growth factors present at the wound site including where they come from their target and their effect. It is interesting to note that many factors have autocrine and/or paracrine effects on the cells that secrete them.

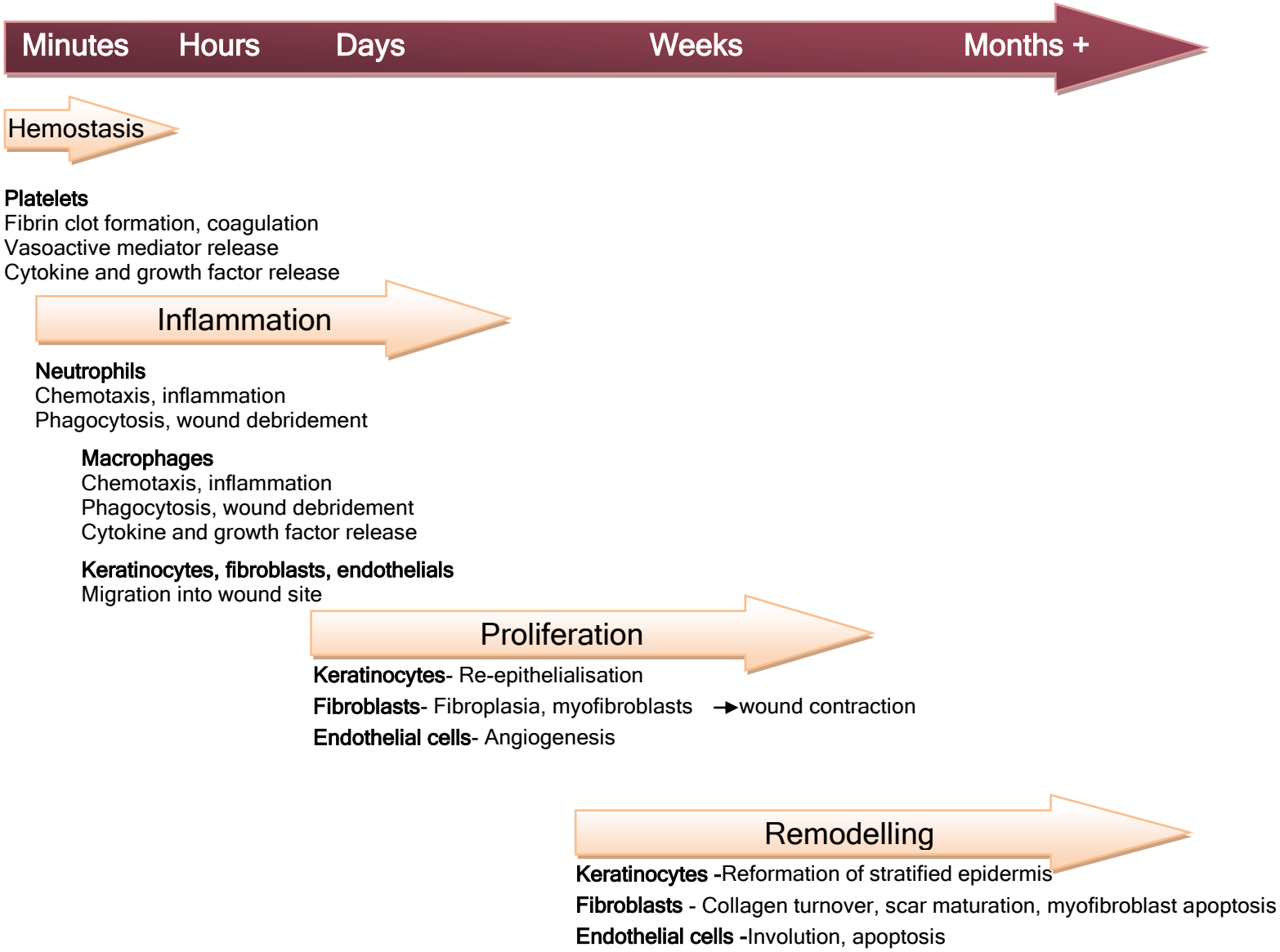



**Figure 1.10: Wound healing phases .**
Generalised diagram of wound healing phases with key players and main events (modified from Li *et al.* 2007).

Table 1.2: Summary of key growth factors present at the wound site

| Growth Factor | Source | Target cells | Biological effect on target |
|---|---|---|---|
| **TGF-β1, TGF-β2** | Macrophages, platelets, fibroblasts, keratinocytes | Inflammatory cells, keratinocytes, fibroblasts | Chemotaxis, proliferation, matrix production (fibrosis) |
| **TGF-β3** | Macrophages | Fibroblasts | Anti-scarring? |
| **TGF-α** | Macrophages, platelets, keratinocytes | Keratinocytes, fibroblasts, endothelial cells | Proliferation |
| **TNF-α** | Neutrophils | Macrophages, keratinocytes, fibroblasts | Activation of growth factor expression |
| **PDGF** | Macrophage, platelets, fibroblasts, endothelial cells, vascular smooth muscle cells | Neutrophils, macrophages, fibroblasts, endothelial cells, vascular smooth muscle cells | Chemotaxis, proliferation, matrix production |
| **FGF-1, 2, 4** | Macrophage, fibroblasts, endothelial cells | Keratinocytes, fibroblasts, endothelial cells, chondrocytes | Angiogenesis, proliferation, chemotaxis |
| **KGF (FGF-7)** | Fibroblasts | Keratinocytes | Proliferation, chemotaxis |
| **EGF** | Platelets, macrophages, keratinocytes | Keratinocytes, fibroblasts, endothelial cells | Proliferation, chemotaxis |
| **HB-EGF** | Macrophages, keratinocytes | Keratinocytes, fibroblasts, smooth muscle cells | Proliferation, chemotaxis (Raab and Klagsbrun 1997) |
| **IGF-1** | Fibroblasts, macrophages, serum | Fibroblasts, endothelial cells | Proliferation, collagen synthesis |
| **IL-1α– and IL-1β** | Macrophages, neutrophils | Macrophages, fibroblasts, keratinocytes | Proliferation, collagenase synthesis, chemotaxis |
| **CTGF** | Fibroblasts, endothelial cells | Fibroblasts | Downstream of TGF-β1 |
| **VEGF** | Macrophages, keratinocytes | Endothelial cells | Angiogenesis |

(from Lorenz and Longaker 2003 unless otherwise indicated).

## 1.4 Aspects of Wound Healing Research

### 1.4.1 Factors affecting wound healing

Stress, poor nutrition, and advancing age are the main factors that affect wound healing negatively. In addition, there are several local and systemic factors which contribute to delayed wound healing or lead to chronic wounds (Table 1.3)

**Table 1.3: Local and Systemic factors affecting wound healing**

| Local factors | Systemic factors |
|---|---|
| Shock of any cause | Advancing age and general immobility |
| Inadequate blood supply | Obesity |
| Increased skin tension | Smoking |
| Poor surgical apposition | Malnutrition |
| Wound dehiscence (rupture) | Deficiency of vitamins and trace elements |
| Poor venous drainage | Systemic malignancy and terminal illness |
| Presence of foreign body and foreign body reactions | Chemotherapy and radiotherapy |
| Continued presence of micro-organisms | Immunosuppressant drugs, corticosteroids, anticoagulants |
| Infection | Inherited neutrophil disorders, such as leucocyte adhesion deficiency |
| Excess local mobility, such as over a joint | Impaired macrophage activity |

(From Grey *et al.* 2006).

### 1.4.2 Aberrant wound healing

As outlined above, the process of wound healing is a highly complex and tightly controlled series of overlapping events facilitated by expression of myriad cytokines and growth factors at specific time points. The importance of these events and their regulation is revealed when the equilibrium of such aspects is upset and abnormal wound healing occurs. This may result in a deficiency of healing which manifests as an ulcer, or excessive healing resulting in fibrotic deformities such as keloids, hypertrophic scar and contracture.

## Deficient healing

*Ulcers (chronic inflammation),*

Ulcers are the most prevalent wound healing disorder in humans (Eming *et al.* 2007b). Normal wound healing is characterised by the progression of the wound through 4 overlapping phases. However, chronic wound progression is not so orderly. Some areas of chronic wounds are found in different phases of the healing process, and keratinocytes at the wound edge have impaired ability to migrate (Li *et al.* 2007). Chronic inflammation is the chief characteristic of non-healing ulcers (Eming *et al.* 2007b), and excessive infiltration of neutrophils is a significant biological marker of this (Diegelmann and Evans 2004). Excessive MMPs and elastase released by neutrophils as well as the presence of excessive reactive oxygen species contribute to further damage of cells and healing tissues (Diegelmann and Evans 2004). Sustained chronic inflammation leads to ECM collapse and formation of necrotic centres (Braiman-Wiksman *et al.* 2007). In chronic wounds there is also prolonged and increased T cell infiltration as well as increased macrophage number, without any evidence of increased autodigestion or phagocytosis (Loots *et al.* 1998).

## Excessive healing

*Fibrosis*

The goal of scar formation is to strengthen the affected area, although healed skin never totally regains normal tensile strength or function. Normally, wounds have stop signals which arrest the repair process when the wound is closed and re-epithelialisation is complete (Lorenz and Longaker 2003). Fibrosis is a pathological condition characterised by excessive matrix deposition and reduced remodelling (Diegelmann and Evans 2004), with major alterations in both the type and quantity of matrix synthesis (Darby and Hewitson 2007). Excessive scar tissue can lead to serious problems by impairing skin function, structure and aesthetic appearance in the area of the scarring (Eming *et al.* 2007b). In addition to disfigurement, severe burns can lead to scarring which restricts joint movement; this is known as contracture. Impaired bone growth can result from scar formation in juveniles after surgery, for example after the repair of cleft palates (Wilgus *et al.* 2003). Various fibrotic disorders can also result from abnormal (excessive) scar formation, and include hypertrophic scars (excessive collagen deposition within the original wound boundary) and keloids (excessive collagen deposition beyond the original wound boundary). Other fibrotic disorders include; tendon adhesions, transmission blockage of nerves, scleroderma,

Crohn's disease, oesophageal strictures, urethral strictures, liver cirrhosis, atherosclerosis and fibrotic non-union in bone (Diegelmann and Evans 2004). TGF-β is often cited as an inducer of fibrosis. A role for TGF-β1 in fibrogenesis has been theorised on the basis that TGF-β1 neutralising antibodies (Border *et al.* 1990) and the natural TGF-β1-binding glycoprotein, decorin (Border *et al.* 1992), abolishes kidney fibrogenesis (Darby and Hewitson 2007).

*Keloids*

Keloids can be thought of as benign skin tumours which are common among people groups with darker pigmented skin (Bock *et al.* 2005; Köse and Waseem 2008) presenting with autosomal dominance (Lorenz and Longaker 2003).Unlike hypertrophic scars, they grow invasively into surrounding healthy tissue beyond the original wound border (Bock *et al.* 2005). Keloids are relatively acellular in central regions, with fibroblasts present along their expanding borders where collagen deposition exceeds degradation (Lorenz and Longaker 2003). Keloids and other fibroproliferative disorders such as hypertrophic scarring (see next paragraph) present abnormalities in cell migration and proliferation, inflammation, synthesis and secretion of ECM proteins and cytokines, and wound remodelling (Singer and Clark 1999). In contrast to hypertrophic scar and normal skin, collagen fibrils of keloids appear thicker, more closely packed and more stretched (Tuan and Nichter 1998; Köse and Waseem 2008). Also in contrast to hypertrophic scars, the myofibroblast is absent from keloids, but energy demands are high, as indicated by the high level of adenosine triphosphate (ATP) expression (Köse and Waseem 2008). Keloid fibroblasts show increased expression of TGF-β1 and its type I receptor TβRI, but decreased expression of TGF-β3 (Bock *et al.* 2005).

*Hypertrophic scar*

It is difficult to distinguish between histological sections of keloids and hypertrophic scar under the microscope (Lorenz and Longaker 2003; Rekha 2004). On the surface, hypertrophic scars present as red, raised, itchy, and inelastic (Scott *et al.* 1996). Burn injuries, traumatic injuries, and surgical procedures can give rise to exuberant scarring that results in permanent loss of function and/or disfigurement (Aarabi *et al.* 2007). Another possible contributor could be delayed or absent induction of apoptosis in myofibroblasts resulting in increased scarring (Darby and Hewitson 2007). Collagen bundles are fine and wavy, well-organised, and run parallel to the epidermis (Köse and Waseem 2008). Wounds on their way to becoming hypertrophic do not demonstrate neovascular involution, but instead become increasingly vascular at this time

(Sheridan and Tompkins 2004). Compared to normal dermis or mature scar, hypertrophic scar tissue contains more water; probably a consequence of higher concentrations of GAGs (Sayani *et al.* 2000).

*Contracture*

Contractures are most likely to occur with severe burns where the severity of the scarring is proportional to the severity of the burn (Mahajan *et al.* 2006). Excessive wound contraction can lead to contracture, which distorts tissue and leads to impaired function. For example, wound contraction across a joint can result in a contracture leading to diminished range of motion and function of the joint (see Figure 1.11). Contractures can develop in the extremities, eyelids, neck, spine, and fingers (Lorenz and Longaker 2003).

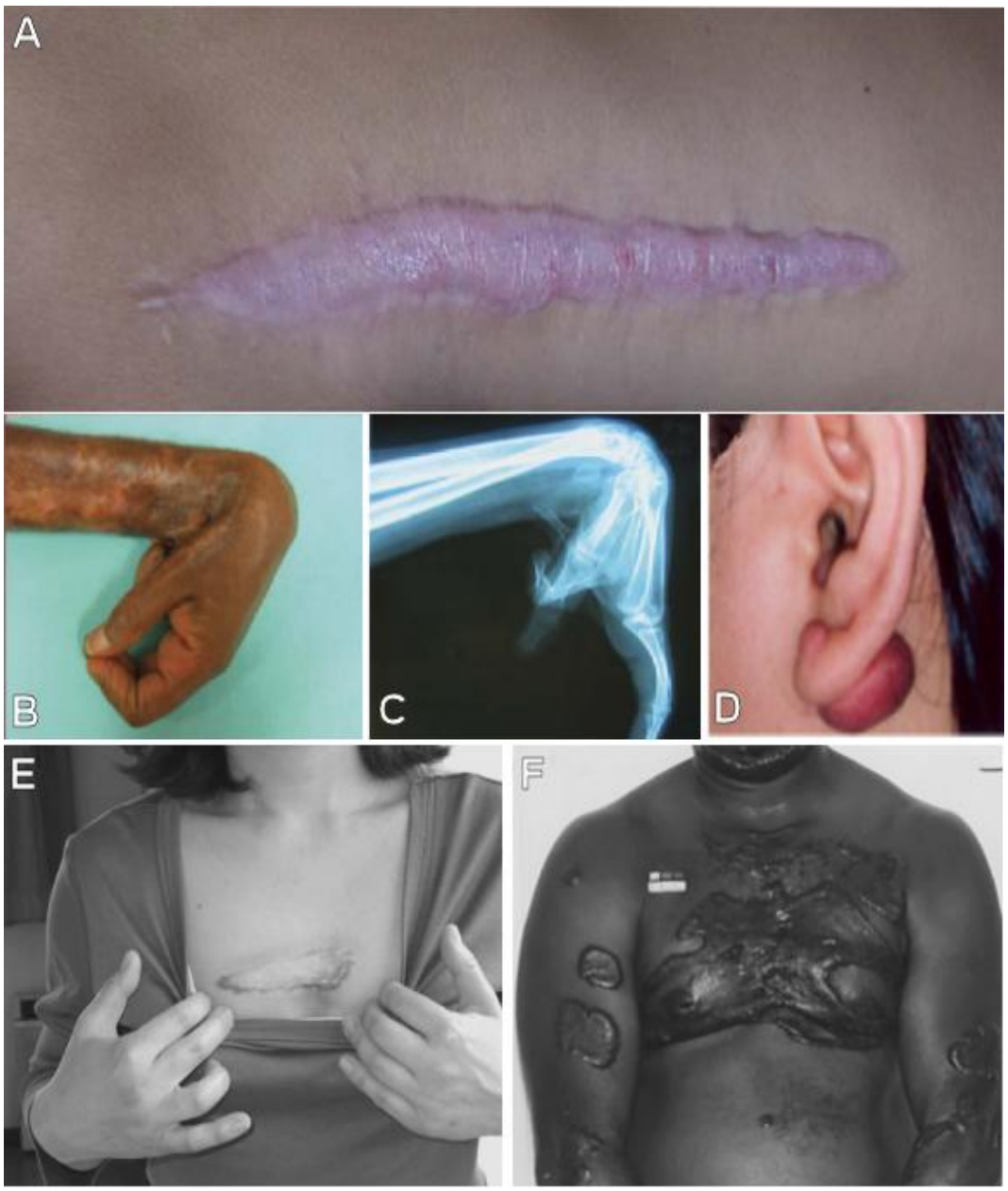



**Figure 1.11: Complications of scarring**
A: Typical hypertrophic scar. B: Contracture as a result of dermal scarring from a burn injury. C: Joint and bone deformation as a result of contracture, viewed under X-Ray (Aarabi *et al.* 2007) D: Keloid as a result of ear piercing (Wu *et al.* 2006). E: Chest keloid on Japanese female. F: Extensive keloids on the trunk, neck, and arms of African American male (Marneros *et al.* 2004).

### 1.4.3 Scarless wound healing

#### Foetal wound healing

It has been observed that mammalian foetal wounds heal without scarring or fibrosis, and that foetal wound healing is more like regeneration than repair. Scar-free foetal wound healing occurs during the first one-third to one-half of gestation (Ferguson and O'Kane 2004) and is believed to heal via different mechanisms from adult healing, resulting in complete restoration of normal skin structure, with normal collagen deposition and regularly spaced hair follicles, capillaries and glands (Metcalfe and Ferguson 2007). One key difference between the healing of foetal and adult skin is the reduced level of inflammation after wounding in foetal wounds (Oberyszyn 2007), with lower numbers of, and less differentiated, inflammatory cells (Ferguson and O'Kane 2004). One theory for this is that adult wounds may be optimised for speed of healing in unfavourable conditions (bacteria, foreign bodies etc) which results in an excessive inflammatory cytokine profile, leading to scarring (Adzick and Lorenz 1994). Additional characteristics of foetal wound healing include rapid re-epithelialisation (Singer and Clark 1999), lack of fibrin clots and platelet degranulation, noticeably elevated levels of molecules involved in skin morphogenesis and growth (Metcalfe and Ferguson 2007), and higher levels of hyaluronic acid (Ferguson and O'Kane 2004). These differences naturally reflect differences in the quality of the growth factor profile. Scar-free embronyic wounds show lower levels of TGF-β1, TGF-β2, and PDGF, and increased levels of TGF-β3 (Metcalfe and Ferguson 2007) in contrast to adult wounds (see Table 1.4 for a summary of the differences between adult and foetal wound healing). Interestingly, experiments conducted in mice, rats and pigs have mimicked foetal scar-free healing in adult tissues by neutralising PDGF, neutralising TGF-β1 and TGF-β2 or adding exogenous TGF-β3 (Ferguson and O'Kane 2004). These studies show that it may be the relative proportions of the TGF-β isoforms, rather than the absolute amount of any one isoform, which determines the wound phenotype (Hantash *et al.* 2008). The effects of TGF-β on wound healing will be reviewed in more detail below.

##### Foetal *extracellular matrix*

Foetal wound healing is seems to be intrinsic to foetal skin and not due to the influence of any unique environmental factors (Lorenz *et al.* 1992; Longaker *et al.* 1994). However, a physiologic solution of cytokines and tissue inhibitors of metalloproteinases produced from amnion-derived multipotent progenitor cells

improves healing in models of acute and chronic adult wounds (Franz *et al.* 2008), showing that the amniotic environment, while not essential, is in all probability still beneficial. Foetal fibroblasts appear to be crucial for scarless repair. Foetal fibroblasts migrate faster, synthesise more total collagen, and a larger proportion of collagen types III and V, than adult fibroblasts (Bullard *et al.* 2003). They also begin synthesising collagen immediately post-injury, whereas in the adult, collagen synthesis begins a few days later (Hantash *et al.* 2008). Foetal skin naturally contains higher levels of collagen III at 30% to 60% of total. The proportion of collagen III progresses toward adult skin levels (10-20%) as development continues, as does the ability to heal without scar (Bullard *et al.* 2003). The ability to turnover ECM during wound healing may also be an important difference in foetal wound healing. Differences in MMP/TIMP ratios have been reported which may allow more rapid ECM turnover and lead to scarless repair (Bullard *et al.* 2003).

**Table 1.4: Qualitative differences between foetal and adult wound healing**

| Foetal factors | Adult factors |
|---|---|
| Amniotic fluid, sterile environment | Dry, contaminated environment |
| Rapid cell proliferation | Slow cell proliferation |
| Rapid closure and epithelialisation | Slow closure and epithelialisation |
| Less differentiated skin | Fully differentiated skin |
| More hyaluronic acid and non-sulfated GAGs | Less hyaluronic acid and more sulfated GAGs |
| Oxygen tension lesser | Oxygen tension greater |
| Little inflammation | Massive inflammation |
| TGF-β3 isoform predominant | TGF-β1 isoform predominant |
| Fibromodulin is predominant TGF-β modulator | Decorin is predominant TGF-β modulator |
| Platelets: less degranulation of active cytokines | Platelets: potent degranulation of active cytokines |
| Faster and organised matrix deposition | Slow and disorganised matrix deposition |
| More fibronectin and tenascin; more fibroblast migration | Slower fibroblast migration |
| High proportion of collagen type III | Low proportion of collagen type III |
| No inflammatory effector cell | Macrophage is inflammatory effector cell |
| Scarless | Scar |

(From Adzick and Lorenz 1994; Bullard *et al.* 2003).

### 1.4.4 Burn wounds

Burn wounds are unique among wound types, and are treated as such in the literature. Therefore, a brief review of the characteristics which make burn wounds unique follows.

#### Depth and degree

Burn injuries can result from thermal, electrical or chemical insult. Superficial burns present with pain, redness, and/or blistering and usually heal within 14 days with minimal scarring (Benson *et al.* 2006). Healing with excessive scarring is a characteristic of deep burn wounds and this can lead to fibrotic disorders such as hypertrophic scarring and contracture. Burn wounds are rated for severity by degree. First degree burns do not go beyond the basal epidermal layer; sunburn is an example of a first degree burn. Second degree or partial thickness burns extend past the epidermis but not totally through the dermis. Second degree burns can be subdivided into superficial and deep second degree burns. The effectiveness of healing of second degree burns is related to the number of adnexal structures left intact, which assist in re-epithelialisation, such as hair follicles and glands (Greenhalgh 1996). Third degree burns of the skin are the most severe as they extend all the way through the dermis. With hair follicles and other adnexa destroyed, these wounds cannot heal by epithelialisation and may require surgical intervention and/or skin grafting (Greenhalgh 1996). Burn depth is the most important factor in determining morbidity and mortality of the patient (Heimbach *et al.* 1992). The depth of injury depends on four factors: temperature of contacting agent, duration of contact, thickness of the skin, and the blood supply (a more vascular tissue will dissipate heat faster and reduce depth of injury) (Greenhalgh 1996).

#### Burn wound zonation

The area of a burn wound has been described in terms of three distinct zones: a central zone of necrosis, surrounded by a zone of stasis, with a further surrounding zone of hyperaemia (increased blood-flow giving red appearance) (Jackson 1953). The tissue in the central necrotic zone is damaged beyond recovery, but the tissue in the outer zone will recover fully (Mahajan *et al.* 2006). The zone of stasis results from significant damage to surrounding tissue occurring after the removal of the thermal source, with cell death occurring through both necrosis and apoptosis (Giles *et al.* 2008). An area of interest in burn wound healing is therefore the zone of stasis where blood flow in the capillaries slows down and can eventually stop, leading to a further progression

of size and depth of injury known as secondary aggravation (Mahajan *et al.* 2006). This zone is viable for the first 24 hours following the trauma, but may progress to necrosis afterwards (Zor *et al.* 2005). Whether the zone of stasis' progression to necrosis can be interrupted or even reversed is the subject of much research with the aim to improve healing and reduce scarring in burn wounds.

### Contrasting elements of burn wounds with other wound types

The zone of stasis is the most obvious difference between burn and other wound types and has a significant impact on healing with regard to blood flow. Damage to blood vessels causes restriction or cessation of blood flow in the immediate area of the wound and modifications to blood flow in surrounding areas (Shakespeare 2001). Another key difference is the extent and duration of the inflammatory phase in burn patients where the wound remains open for prolonged periods (Greenhalgh 1996). For a chronically open burn wound, the inflammatory phase blends with the proliferative phase where continued production of cytokines and growth factors leads to fibroblast proliferation and synthesis of ECM, resulting in increased scarring (Greenhalgh 1996). Burn-injured dermal tissue contains significantly increased amounts of collagen type III for a prolonged time and this may be a marker for the extent of fibrosis (Garcia-Filipe *et al.* 2006).

## 1.4.5 Wound models

Wound healing studies have been successfully conducted on several *in vivo* animal models including rat, mouse, guinea pig, opossum, rabbit, horse, sheep, pig and human. Most wound healing studies aim to contribute to knowledge that may one day benefit human wound healing. Therefore selection criteria for suitable animal models need to balance the cost, availability and ease of handling of animal subjects with the appropriateness of applying such research to human wound healing scenarios. The most appropriate model to use in many studies is the human model (Lindblad 2008), but the obvious practical and ethical considerations often render this option unrealistic. For this reason, animal models can make suitable alternatives. The pig is arguably the most suitable animal to study as a model for human cutaneous wound healing. Pig skin is anatomically and physiologically similar to human skin in several ways including thickness of the epidermis, dermal to epidermal thickness ratio, collagen biochemistry, number and distribution of blood vessels and other cutaneous structures such as hair follicles, and the fact that the skin is well-fixed to the underlying musculature

unlike smaller loose-skinned mammals such as the mouse (Sullivan *et al.* 2001). However, cost, availability and ease of handling, as well as short gestation and short lifespan, dictate that most wound healing studies are conducted on rodents.

### 1.4.6 General effects of cytokine and growth factor manipulation

Most wound healing studies aim to manipulate one or more cytokines or growth factors (or their receptors) involved in wound healing in order to study their effects, or in an attempt to improve wound healing. This can be achieved by methods such as transgenic knockout, gene over-expression or inducible expression, or by topical application or systemic injection of exogenous growth factors.

In most cases the effects of exogenous growth factors on the healing process has been beneficial. To date only PDGF-BB has been approved by the United States Food and Drug Administration (USFDA), though only for use in diabetic foot ulcer (Branski *et al.* 2006; Riedel *et al.* 2006). Transgenic animals are used to give an insight into the potential usefulness of a particular gene for gene therapy. By inserting a desired gene into recipient cells, gene therapy has the potential to make improvements in wound healing where exogenous or systemic application of growth factors has seen limited success (Eming *et al.* 2007b; Hirsch *et al.* 2007). Table 1.5 outlines the typical results of such trials.

Table 1.5: Effect of topically applied growth factors and cytokines

| Growth factor/ cytokine applied | General effect |
|---|---|
| **PDGF-BB** | Increased neovascularisation<br>Increased re-epithelialisation<br>Increased granulation tissue<br>Accelerated wound healing<br>Enhanced ulcer closure |
| **TGF-β2** | Increased collagen content of granulation tissue. |
| **TGF-β3** | Enhanced wound healing<br>Decreased scarring |
| **FGF-2** | Increased neovascularisation<br>Increased re-epithelialisation<br>Accelerated ulcer closure |
| **FGF-1** | Increased neovascularisation<br>Increased matrix deposition |
| **EGF** | Increased re-epithelialisation<br>Accelerated wound healing |
| **TGF-α** | Accelerated healing of chronic wounds |
| **IL-1β** | Accelerated wound healing especially in infected open wounds |
| **CSF-2** | Increased healing in incisional wounds and chronic ulcers |
| **CCL-2 (MCP-1)** | Accelerated healing of chronic wounds |
| **CCL-3 (MIP-1α)** | Accelerated healing of chronic wounds |
| **IFN-γ** | Attenuation of fibrosis in keloids |
| **VEGF-A** | Accelerated healing of ischemic wounds |
| **IGF-1** | Accelerated wound healing |

(Based on reviews by Pierce and Mustoe 1995; Gharaee-Kermani and Phan 2001; Werner and Grose 2003) CSF: Colony Stimulating Factor, MCP: Monocyte Chemotactic Protein, MIP: Macrophage Inflammatory Protein, CCL: Chemokine (C-C motif) Ligand, IFN: Interferon.

## 1.4.7 Specific effects of some key factors

### Myostatin

*Structure, processing and signalling*

Myostatin, also known as Growth and Differentiation Factor-8 (GDF-8), is a member of the transforming growth factor-beta superfamily. The TGF-β

superfamily contains more than 30 structurally-related polypeptide growth factors including TGF-βs (1-3), activins (A, B), inhibins (A, B), bone morphogenetic proteins (BMPs 1-20), growth differentiation factors including myostatin, nodal, leftys (1,2), and Müllerian-inhibiting substance (MIS) (Gordon and Blobe 2008).The superfamily is characterised by a conserved carboxy-terminal feature consisting of seven cysteine residues, six of which form a rigid cystine (the oxidised dimer of cysteine) knot, a structure which almost all family members share (O'Kane and Ferguson 1997) (see Figure 1.12). Like other family members, myostatin is synthesised and secreted as a large inactive pro-peptide with three distinct regions: an inactive precursor protein with a signal sequence required for processing and secretion, the latency-associated peptide (LAP) domain which regulates biological activity of myostatin, and mature myostatin which, as a homodimer, binds to the receptor (Patel and Amthor 2005). Myostatin has a RSRR (arg ser arg arg) proteolytic processing site in the C-terminal half of the protein, which when processed forms the biologically active C-terminal 'mature' protein. Mature myostatin contains nine cysteine residues from which the characteristic cystine knot structure is formed to allow for homodimerisation (Lee 2004).

*Signalling*

TGF-β family members exert their effect by binding to type II receptors, which recruit and activate by phosphorylation type I receptors via serine/threonine kinase activity of the type II receptor (Whitman 1998; Glasgow and Mishra 2008). Following this, the type I receptor phosphorylates receptor-activated Smad proteins (R-Smads) (Faler *et al.* 2006), R-Smad then associates with a co-Smad (Smad4) facilitating translocation into the nucleus (Abdollah *et al.* 1997) (see Figure 1.14). Smad proteins are responsible for transmitting the signals of TGF-β family members from the cell membrane into the nucleus and can be grouped into three subfamilies; receptor-regulated Smads (R-Smads), common Smads (co-Smads), and the inhibitory Smads (I-Smads) (Attisano and Tuen Lee-Hoeflich 2001). Once in the nucleus, Smad complexes activate specific genes through cooperative interactions with other DNA-binding proteins (Padgett *et al.* 1998; Glasgow and Mishra 2008). In the case of myostatin, the mature region binds to Activin type II receptor B (ActRIIB) and, to a lesser degree, ActRIIA. After phosphorylation of the corresponding type I receptor (ActRI), signal transduction continues to the nucleus via Smad proteins.

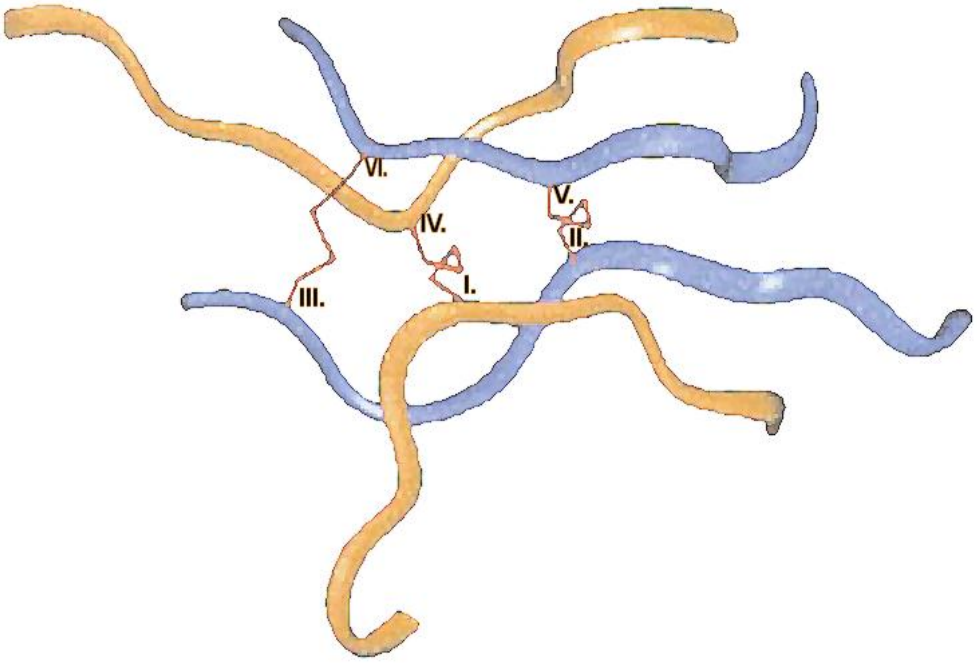



**Figure 1.12: Growth factor cystine knot motif**
All of the structures in the growth factor class of cystine knots have I-IV, II-V, III-VI disulphide linkages with Cys I-IV passing through the ring created by Cys III-VI and Cys II-V linkages (McDonald and Hendrickson 1993; Isaacs 1995; diagram adapted from Hearn and Gomme 2000).

*Function*

Myostatin has been well-characterised as a negative regulator of muscle development (McPherron *et al.* 1997; Lee and McPherron 1999; Lee 2004). A dramatic increase in muscle mass is observed in mice where the myostatin gene has been disrupted (McPherron *et al.* 1997) or in cattle where a naturally-occurring mutation results in absence of myostatin protein or a non-functional myostatin protein product, resulting in the heavily-muscled Belgian Blue and Piedmontese cattle breeds respectively (Kambadur *et al.* 1997; McPherron and Lee 1997). A similar effect has been seen in humans by the identification of a child possessing a myostatin mutation (Schuelke *et al.* 2004) and also in whippet dogs (Shelton and Engvall 2007). In the heavily-muscled Texel sheep breed, a mutation in the 3' untranslated region of the myostatin gene creates a target site for highly expressed interfering micro RNAs in muscle, causing inhibition of translation of myostatin with subsequent muscle hypertrophy (Clop *et al.* 2006). Conversely, clear links have been drawn between over-expression of myostatin and a resulting decrease in skeletal muscle mass. Indeed, in various muscle

wasting disease states such as cancer cachexia, up-regulation of myostatin is observed (Jespersen *et al.* 2006; McFarlane *et al.* 2006).

*Effect on wound healing*

Research on the myostatin gene has tended to focus almost exclusively on its effect on muscle development and myogenesis, where it modulates skeletal muscle satellite cell activation and controls both differentiation and proliferation of skeletal muscle both pre- and post-natally (Walsh and Celeste 2005). However, recent research is uncovering another role of myostatin, in wound healing. It has been shown that myostatin is involved negatively in adult myogenesis and muscle regeneration from injury (Kirk *et al.* 2000). Recent work in this lab has shown that myostatin can impair macrophage chemotaxis, retarding their migration to the site of injury, but is chemotactic for fibroblasts likely contributing to enhanced collagen deposition (Siriett 2007) (see Figure 1.13 for a proposed model of myostatin involvement in wound healing). Enhanced regeneration and reduced fibrosis is seen in myostatin-null mice (McCroskery *et al.* 2005) and in mice treated with a myostatin inhibitor after injury (Nozaki *et al.* 2008). Myostatin is shown to be up-regulated nearly four-fold in gastrocnemius muscle in response to full thickness burn injury to body surface, although *E-coli* endotoxin or induction of polymicrobial sepsis did not significantly alter myostatin mRNA levels in the same study (Lang *et al.* 2001). These studies show that myostatin can be targeted to improve the healing process across the phases of wound healing.

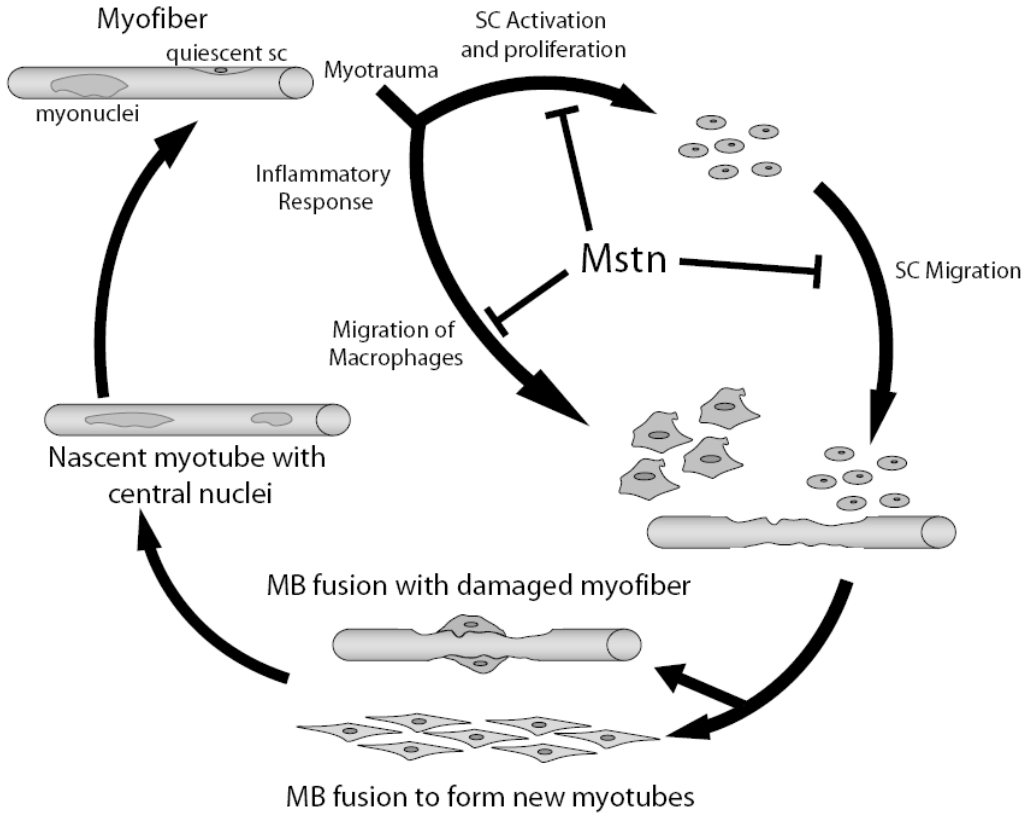



**Figure 1.13: A model for the role of myostatin in skeletal muscle healing**
Following injury to the muscle, satellite cells become activated and migrate to the wound site. myostatin inhibits satellite cell activation and migration, and also macrophage migration (from McCroskery *et al.* 2005). Mstn: myostatin, SC: stem cell, MB: myoblast.

*Therapeutic potential of targeting myostatin*

Due to the potency of myostatin as an inhibitor of regeneration and promotor of fibrosis, it follows that inhibiting myostatin in certain situations would be of therapeutic benefit. Several proteins have been discovered that bind and prevent myostatin from signalling. These include myostatin propeptide, activin receptor, metalloproteases, follistatin and the follistatin-related proteins (Follistatin Related Gene (FLRG), and Growth and Differentiation Factor-Associated Serum Protein-1 (GASP-1)), as well as specific anti-myostatin antibodies which have been developed (Patel and Amthor 2005).

## TGF-β1

TGF-β1 is perhaps the most important factor in the wound healing context and is worth special consideration. It has been discovered to have some effect on almost every aspect of wound healing. This same cytokine can even have different effects depending on cell type and state, for example, cell proliferation may be stimulated or inhibited by TGF-β depending on whether the cell is a fibroblast or a keratinocyte respectively (Massagué and Wotton 2000). Wound TGF-β is released from degranulating platelets and is secreted by all major cell types contributing to wound repair including lymphocytes, macrophages, endothelial cells, epithelial cells and fibroblasts (Liu *et al.* 2004).

*Structure and processing*

TGF-β has a similar structure and processing program to myostatin. The TGF-β1 precursor cleaved of its peptide signal is labelled 'pro-TGF-β' (Lawrence 2001). An endoprotease recognises an RHRR (arg his arg arg) site in pro-TGF-β and cleaves the molecule, but the two parts remain non-covalently associated and are known in dimeric form as 'small latent TGF-β1' (Lawrence 2001). Proteolytic cleavage of LAP activates TGF-β1 and this can be achieved through acidic conditions or by several extracellular proteases (Gordon and Blobe 2008). One free cysteine, which is not involved in the knot structure, forms a disulfide bond with an identical monomeric chain to further stabilise the mature TGF-β dimer (Verrecchia and Mauviel 2002)

*Signalling*

TGF-β binds to and signals through its type II receptor TβRII, which then recruits TβRI to initiate intracellular signalling via Smads, as for myostatin. In the case of TGF-β, a type III receptor on the cell surface usually known as 'TβRIII' or

otherwise known as the membrane-anchored proteoglycan, 'betaglycan', provides high-affinity TGF-β presentation to the type II receptor complex (Derynck and Zhang 2003; Liu *et al.* 2004).

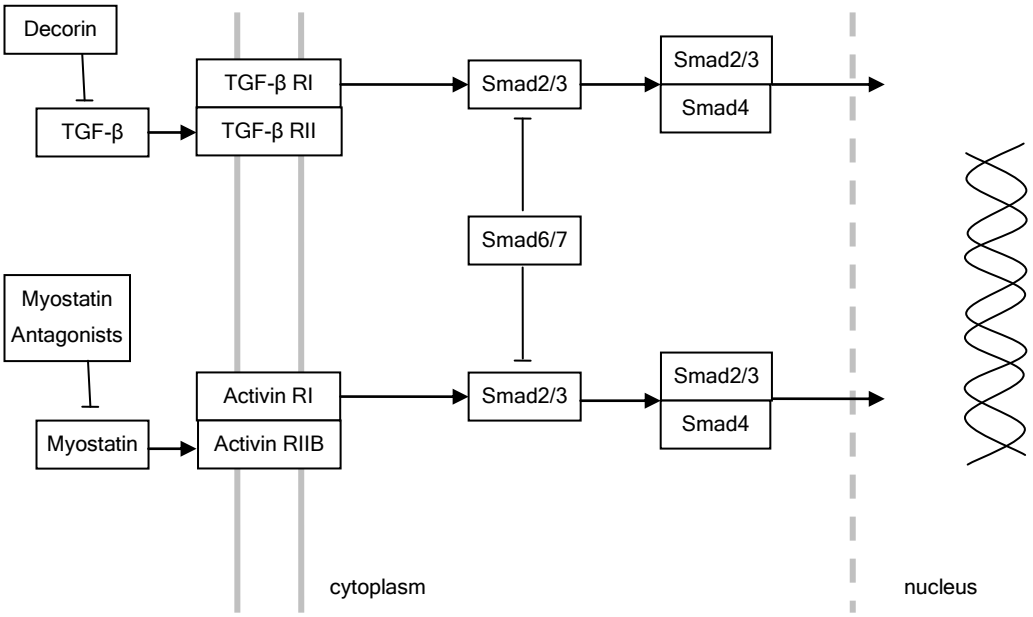



**Figure 1.14: Simplified signalling pathways of two TGF-β superfamily members.**

The question arises of how a relatively simple system can elicit a variety of specific responses. Massagué and Wotton (2000) provide the following useful summary on the sequence of events. An incoming Smad complex is met in the nucleus by a set of partner proteins that are specific to a particular cell type in one of a particular set of conditions. These partners govern the DNA sequences to which the Smad complex will bind the transcriptional co-activators or co-repressors it will recruit, the other transcription factors it will cooperate with, and the duration of these effects. The combination of Smad partners and regulators present in a given cell at the time of TGF-β stimulation accordingly determines the response.

*Effects of TGF-β1 signalling in wound healing*

TGF-β1 has manifold effects in all phases of wound healing by directing the functions of monocytes/macrophages, endothelial cells, fibroblasts and keratinocytes (Faler *et al.* 2006). Table 1.6 outlines these various effects.

Table 1.6: Effect of TGF-β1 on major cell types in wound healing

| Cell Type | Effect of TGF-β1 |
|---|---|
| **Monocyte/ Macrophage** | Production of TGF-β1<br>Chemotaxis and migration into the wound site<br>Production of protease inhibitors<br>Suppress production of proteases<br>Promote macrophage maturation |
| **Fibroblast** | Chemotaxis<br>Production of TGF-β1<br>Proliferation<br>Production of ECM components<br>Production of protease inhibitors<br>Suppress production of proteases<br>Differentiation into myofibroblast (Rolfe *et al.* 2007) |
| **Endothelial cell** | Stimulates and inhibits proliferation<br>Migration for angiogenesis via induction of cell surface integrins in a concentration dependent manner<br>Tube formation and maturation (concentration dependent) |
| **Keratinocyte** | Decreased proliferation *in vitro*<br>Increased migration and maturation *in vitro*<br>Conflicting results *in vivo* |

(Adapted from reviews by Beanes *et al.* 2003; and Faler *et al.* 2006 except where indicated otherwise).

TGF-β1 is principally recognised for its role in scar tissue formation. Evidence of this is apparent by its multi-faceted effect on ECM-producing fibroblasts. Early studies showed the benefit of exogenous TGF-β1 application on the rate of healing and wound-bursting strength. Mustoe *et al.* (1987) showed that a 220% increase in bursting strength was achieved by a single application of exogenous TGF-β1 on day two, an effect that was dose-dependent. Also, decreased expression of TGF-β1 was observed in chronic leg ulcers (Cowin *et al.* 2001), but clinical trials failed to confirm TGF-β1 as a potential therapeutic agent for wound healing (Wang *et al.* 2006). Other studies shed light on why this may have been the case. One group was surprised to observe that mice lacking Smad3 (one of the intracellular signal transducers of TGF-β) actually showed accelerated wound healing characterised by an increased rate of re-epithelialisation, and also displayed impaired inflammatory response with reduced infiltration of monocytes (Ashcroft *et al.* 1999; Ashcroft and Roberts 2000). Delayed healing was observed in TGF-β1 overexpressing mice with full-thickness punch biopsy wounds, but TGF-β1 did not significantly delay re-epithelialisation in the partial-thickness ear wound of animals in the same study (Tredget *et al.* 2005). Another group designed a transgenic animal that over-expressed TGF-β1 in the epidermis

driven by the keratin-14 promoter. They reported significantly inhibited re-epithelialisation of partial thickness $CO_2$-laser-inflicted burns and increased collagen type I expression and hydroxyproline, a marker of total collagen content (Yang *et al.* 2001). To corroborate these findings, Shah and colleagues (1992; 1994; 1995) used neutralising antibodies against TGF-β1 and TGF-β2 which resulted in incisional wounds healing with reduced inflammatory and angiogenic responses and reduced ECM deposition without reduction in tensile strength. Scarring was reduced to the extent that neodermis was similar in architecture to normal skin. In the 1995 study, they showed that neutralising both TGF-β1 and TGF-β2 concurrently gave the best results and that anti-TGF-β1 alone gave only marginally reduced scarring. The above findings, together with the involvement of TGF-β3 in the foetal wound healing paradigm, confirm the comprehensive influence of TGF-β on wound healing. Any pharmacologic improvement in wound healing will likely be due to direct or indirect effects on one or more TGF-β isoforms.

## Small Leucine-Rich Proteoglycans

### *Decorin*

Decorin, named for its decorative appearance on collagen fibrils, is the prototype of the small leucine-rich proteoglycan (SLRP) family (Iozzo 1998; Delehedde *et al.* 2002). SLRPs consist of a central domain of leucine-rich repeats with small cysteine clusters to either side (Hocking *et al.* 1998). Decorin-knockout mice have fragile skin and abnormal collagen fibril morphology showing that decorin is important for collagen fibrillogenesis (Danielson *et al.* 1997). Decorin binds to the fibrillar collagens (types I, II, III, V, and XI) (Beanes *et al.* 2001) and also TGF-β (Burton-Wurster *et al.* 2003), and is important for wound healing because of its ability to regulate ECM assembly and growth factor activity (Beanes *et al.* 2001). While decorin seems to modulate TGF-β, the opposite is also occurs with decorin being down-regulated by TGF-β (Iozzo 1998). During the remodelling phase of wound healing, hyaluronic acid is replaced by sulphated proteoglycans such as decorin, produced by mature scar fibroblasts (Miller and Nanchahal 2005). In the presence of decorin, collagen fibrils form more slowly allowing time for optimal interaction, resulting in structurally ideal fibre diameters (Reed and Iozzo 2002). A specific amount of decorin relative to collagen is probably necessary for optimal fibre formation and any deviation from the optimal ratio appears to have a negative impact on collagen fibre formation and overall dermal structure (Lochner et al. 2007). Decorin also induces the expression of MMP-1 (Huttenlocher *et al.* 1996). The MMPs and their associated TIMPs are thought to be critical for wound

ECM remodelling, and possibly the scarless wound healing phenotype (Beanes et al. 2001).

*Fibromodulin*

Fibromodulin, like decorin, binds to type I and type II collagens (Viola *et al.* 2007; Vélez-delValle *et al.* 2008) and influences the rate of fibrillogenesis and the structure of fibrils (Delehedde *et al.* 2002) as its name suggests. There seems to be no competitive inhibition between fibromodulin and decorin binding to collagen, suggesting that these two members of the gene family bind to different sites on the collagen fibril (Svensson et al. 1999). Fibromodulin seems to be the main modulator of TGF-β in the foetal wound healing context (Bullard *et al.* 2003).

Other SLRPs not discussed here include biglycan, lumican, epiphycan, osteoglycin, chondroadherin, and keratocan.

## Relationships between myostatin, decorin and TGF-β in wound healing

Zhu *et al.* (2007) have recently elucidated the relationship between TGF-β1, decorin, and myostatin. They discovered that myostatin stimulated fibroblast proliferation *in vitro* and induced its differentiation into myofibroblasts. They also found that TGF-β1 stimulated myostatin expression, and conversely, myostatin stimulated TGF-β1 secretion in C2C12 myoblasts. Decorin was found to neutralise the effects of myostatin in both fibroblasts and myoblasts and decorin up-regulated the expression of follistatin, a natural antagonist of myostatin. Their subsequent *in vivo* studies showed improved muscle regeneration with reduced fibrosis in myostatin-null mice and concluded that myostatin has fibrogenic properties. An earlier study (McCroskery *et al.* 2005) concurred with these results and suggested that myostatin negatively regulates decorin mRNA expression. Therefore by removing myostatin, the ensuing up-regulation of decorin neutralises TGF-β and the result is decreased scar tissue formation. Zhu *et al.* (2007) discuss that fibroblasts may be a source of myostatin and that in injured muscle, myostatin activates fibroblasts by stimulating fibroblast proliferation. Myostatin may then attract fibroblasts to an injury site, further inducing them to express myostatin in an autocrine fashion. The fibroblasts then differentiate into myofibroblasts, thereby accelerating the deposition of the ECM. A current study has provided evidence that myostatin directly stimulates muscle fibroblast proliferation and expression of ECM proteins *in vivo* as well as *in vitro*, and that muscle fibroblasts express functional myostatin, which signals through the ActRIIB receptor inducing proliferation via the canonical Smad pathway (Li *et al.* 2008).

## 1.5 Aims, Objectives, and Hypothesis

Up untill the present time, research on the function of myostatin has revealed its role as a powerful negative regulator of muscle development. Recently, work in this lab by McCroskery *et al.* (2005) and Siriett (2007) has been conducted on myostatin's role in adult myogenesis in response to muscular injury, and its negative regulation thereof . These studies indicate that myostatin has a clear role in muscle wound healing, and that removing or antagonising myostatin leads to improved healing and reduced scar tissue formation. The present study will attempt to apply these findings to wound healing in skin, where the wound healing processes and biology are essentially alike, with many of the same molecular pathways operating in both. Myostatin is primarily expressed in skeletal muscle and it is also found, in mature form, in the circulation (Hosoyama *et al.* 2006). Therefore, it is likely that myostatin diffuses into the skin. It has been shown that the same receptor (ActRIIB) that binds myostatin in the muscle is also present in the skin (Hübner and Werner 1996; Lai and Pittelkow 2004; Bamberger *et al.* 2005), indicating the possibility that myostatin may have a function in skin. Consequently, by antagonising myostatin, skin healing could be improved, fibrosis avoided, and progression to a pathologic state prevented.

Presently there is no data available on the effect of myostatin on skin and this represents a gap in the scientific literature. Therefore, the aim of this project is to apply knowledge of myostatin's action in a muscle healing model to a skin healing model, and by doing so, establish whether antagonising myostatin can lead to improved wound healing in skin.

### 1.5.1 Skin burn injury model

Burn wounds differ significantly from other types of wound. The most important difference is the amount of necrotic tissue remaining, and the viability of the surrounding zone of stasis. While the healing of burn wounds follows through the same course of events as other wounds, burn wounds often take longer to heal and lead to increased accumulation of scar tissue. Therefore, the uniqueness of burn wounds necessitates the development of a specific burn wound model.

One of the challenges in establishing a burn injury model is standardisation of the burn wound. Another consideration is whether a partial thickness or a full thickness injury is required. Some of the early models of burn wound healing in the 1970s involved the ignition of ethanol on a standard sized area marked out by

flame resistant plastic (Neely *et al.* 1999). Other types of thermal injury have included scald injury from 100°C water (Ballard-Croft *et al.* 2004), hot wax burn (Bairy *et al.* 1997), flame burn (Sakurai *et al.* 2002), and various metal objects, usually brass, heated in boiling water (Papp *et al.* 2005; Willis *et al.* 2005; Moller-Kristensen *et al.* 2006; Moller-Kristensen *et al.* 2007). All of these methods were not considered suitable in the interests of animal welfare by the Ruakura Animal Ethics Committee, because of the extent of injury, and therefore suffering, caused. Burns inflicted by $CO_2$ lasers possess the advantage of reduced variability by maintaining a consistent depth (Yang *et al.* 2001; Cohen *et al.* 2003; Bilic *et al.* 2005). Other considerations include the use of analgesics for pain relief and whether these interfere with healing (Dorsett-Martin 2004), and the placement of the burn in a position where it cannot be interfered with by the animal itself. The model developed in the present study differs from others in that it uses a small stainless steel bar, heated in a flame before being applied to the skin for five seconds, to cause a severe, though very localised, deep burn injury.

### 1.5.2 *In vivo* trial using skin burn injury model

The major aim of this study is to test the efficacy of antagonising myostatin using the burn injury model described above. The methods for achieving this aim include: total collagen analysis (as an indicator of scar tissue formation), gene expression analysis of collagen type I, collagen type III, TGF-β1, TGF-β3, decorin, and fibromodulin (all involved in wound healing and scar formation), and histological analysis to ascertain the effects of treatment on granulation tissue formation and collagen deposition.

### 1.5.3 Hypothesis and Objectives

It is hypothesised that the administration of a myostatin antagonist will have a similar effect on skin healing as it has been shown to have on muscle healing. In particular, the administration of a myostatin antagonist will result in the expression of genes associated with accelerated healing and deposition of collagen associated with reduced scarring after burn injury compared with the administration of saline as a control. The specific objectives follow from the aims of this study mentioned above. Therefore, the specific hypothesis and objectives are stated as follows:

## Hypothesis:

Antagonising myostatin following burn injury will result in significant and quantifiable improvements in wound healing.

## Objective 1:

To develop and test a murine skin burn injury model suitable for this and future studies of burn wound healing in skin.

## Objective 2:

To determine the effect of a myostatin antagonist on skin healing after severe thermal injury.

# 2 METHODS & MATERIALS

## 2.1 Materials

### 2.1.1 Hydroxyproline assay reagents

Table 2.1

| Reagent | Composition | Amount | Manufacturer |
|---|---|---|---|
| **Standard** | Hydroxyproline standard | 0.1% w/v | Sigma-Aldrich |
| **Acetate Citrate Buffer** | Sodium Acetate Trihydrate<br>Citric Acid<br>Sodium Hydroxide<br>Acetic Acid<br>Adjust to pH 6.5, volume to 100 ml | 12 g<br>4.6 g<br>3.4 g<br>1.2 ml | J.T. Baker<br>BDH Chemicals<br>Merck<br>VWR International |
| **Chloramin T reagent** | Chloramin T<br>50% v/v n-propanol<br>Dissolve then adjust to final vol of 10 ml with acetate citrate buffer | 0.127 g<br>2 ml | Sigma-Aldrich<br>VWR International |
| **Ehrlich's reagent** | p-dimethylaminobenzaldehyde<br>2:1 n-propanol : perchloric acid<br>Dissolve and adjust to 10 ml | 1.5 g<br>8 ml | Fluka<br>VWR International |

### 2.1.2 Gene expression analysis reagents

Table 2.2

| Process | Reagent/Product | Amount | Manufacturer |
|---|---|---|---|
| **RNA extraction** | TRIzol® Reagent<br>Chloroform<br>Isopropanol | 1 ml/ sample | Invitrogen<br>BDH Chemicals<br>BioLab |
| **cDNA synthesis** | SuperScript-II kit | | Invitrogen |
| **PCR** | 10x Choral Load PCR Buffer | | Qiagen |
| | 5x Q solution | | Qiagen |
| | dNTPs | | Invitrogen |
| | Oligonucleotide Primers | | Invitrogen |
| | Taq Polymerase | | Qiagen |
| **RT-PCR** | LightCycler$^{tm}$ Master mix | | Roche |
| | Taq Polymerase | | Roche |
| | Capillaries | 96/box | Roche |
| **DNA purification** | Wizard PCR prep | | Promega |
| **DNA Gel Electrophoresis** | Agarose<br>Low melting point (LMP) Agarose | | Gene Choice<br>Mercury |
| | Ethidium bromide | | Invitrogen |
| | TAE buffer<br>40 mM Tris-acetate<br>2 mM EDTA (pH 8.0) | | <br>J.T. Baker<br>Scientific Supplies |
| | DNA 1kb+ ladder | | Invitrogen |
| | DNA loading dye<br>15% ficoll<br>0.25% bromophenol blue<br>0.25% xylene cyanol<br>1% SDS<br>1 mM EDTA (pH 8.0) | | <br>Sigma<br>BDH Lab Supplies<br>BDH Lab Supplies<br>BioRad<br>Scientific Supplies |

| | | | |
|---|---|---|---|
| **RNA Gel Electrophoresis** | Agarose | | Gene Choice |
| | 2x RNA loading dye | | |
| | 10% MOPS (10 x) | | Sigma |
| | 20% deionised formaldehyde | | Sigma |
| | 50% deionised formamide | | Sigma |
| | 0.02% bromophenol blue | | BDH Lab Supplies |
| | 5% glycerol | | VWR International |
| | 1 mM EDTA (pH 8.0) | | Scientific Supplies |
| | 40 µg/ml ethidium bromide | | Invitrogen |
| | 10 x MOPS | | |
| | 41.8% MOPS | | Sigma |
| | 50 mM NaOAc | | J.T. Baker |
| | 10 mM EDTA | | Scientific Supplies |
| | Formaldehyde | | Sigma |
| **Denaturing Polyacrylamide Gel Electro-phoresis** | Acrylamide/Bisacrylamide | | BioRad |
| | TEMED | | BioRad |
| | APS | | BioRad |
| | Urea | | BDH Chemicals |
| | TBE buffer | | |
| | Tris Base (890 mM) | 27g | J.T. Baker |
| | Boric acid (890 mM) | 13.75g | BDH Chemicals |
| | 0.5 M EDTA, pH 8.0 (20 mM) | 10 ml | Scientific Supplies |
| | Loading Dye | | |
| | 10 bp ladder | | Invitrogen |
| **Silver Staining** | BioRad Silver Staining kit | | BioRad |

### 2.1.3 Histology Reagents

Table 2.3

| Process | Reagent composition | Amount | Manufacturer |
|---|---|---|---|
| **Fixatives** | 10% Buffered Formalin | | |
| | Formalin (40% formaldehyde) | 100 ml | BioLab |
| | Anhydrous disodium phosphate | 6.5 g | VWR International |
| | Sodium acid phosphate | 4.0 g | VWR International |
| | Distilled water | 900 ml | |
| | Bouins Fixative | | |
| | Picric acid (saturated) | 75 ml | BDH Chemicals |
| | Formalin (40% formaldehyde) | 25 ml | BioLab |
| | Glacial acetic acid | 5 ml | BDH Chemicals |
| **Stains** | Celestine Blue | | |
| | 5% ammonium ferric sulfate | 100 ml | BDH Chemicals |
| | Celestine Blue | 0.5 g | BDH Chemicals |
| | Glycerol | 14 ml | VWR International |
| | Gill's (1974) Hematoxylin | | |
| | Hematoxylin | 4 g | Sigma-Aldrich |
| | Sodium iodate | 0.4 g | BDH Chemicals |
| | Aluminium sulfate | 35.2 g | BDH Chemicals |
| | Ethylene glycol | 250 ml | Sigma-Aldrich |
| | Glacial acetic acid | 40 ml | BDH Chemicals |
| | Distilled water | 710 ml | |
| | Scott's tap-water | | |
| | Sodium bicarbonate | 2 g | BioLab |
| | Magnesium sulfate | 20 g | BDH Chemicals |
| | Distilled water | 1000 ml | |
| | Gomori's (1950)Trichrome | | |
| | Chromotrope 2R | 1.2 g | Scientific Ltd, UK |
| | Light green SF or Analine blue | 0.6 g | BDH Chemicals |
| | Dodecatungstophosphoric acid | 1.6 g | Fluka |
| | Glacial acetic acid | 2 ml | BDH Chemicals |
| | Distilled water | 200 ml | |
| | Eosin Yellowish (1%) | | Scientific Ltd, UK |
| **Other reagents** | 1% Glacial acetic acid | | BDH Chemicals |
| | 1% Acid alcohol | | |
| | 70% Ethanol | 99 ml | VWR International |
| | Hydrochloric acid (conc.) | 1 ml | VWR International |

| | | |
|---|---|---|
| | 50%, 70%, 95%, 100%, Ethanol | VWR International |
| | Xylene | BioLab |
| | Toluene | BioLab |
| | DPX mounting solution | VWR International |
| | Poly-lysine adhesive | Sigma |
| | Paraffin wax | BDH Chemicals |

## 2.1.4 Oligonucleotides used for PCR

End-point PCR primers

Table 2.4

| Gene | GenBank Access # | Forward Primer 5' – 3' Reverse Primer 5' - 3' | Product size (bp) | Annealing Temp (°C) | Cycles |
|---|---|---|---|---|---|
| **18S rRNA** | NR_ 003278 | AACGTCTGCCCTATCAACT AACCTCCGACTTTGCTTCT | 699 | 59 | 8 |
| **Collagen Iα1** | BC 050014 | ATGTCGCTATCCAGCTGACC AAGGGTGCTGTAGGTGAAGC | 192 | 59 | 28 |
| **Collagen Iα2** | AK 075707 | ACAACGTAGAAGGGGTGTCC GTAGGTGAACCTGCTGTTGC | 212 | 58 | 35 |
| **Collagen IIIα1** | AK 041115 | ATAAGCCCTGATGGTTCTCG ATAAGCCCTGATGGTTCTCG | 195 | 59 | 28 |
| **Decorin** | NM_ 007833 | TTCCAGGGACTGAAGAGTCTCT GCACCCTGAGGAGTTTGTTGTT | 268 | 59 | 29 |
| **Fibromodulin** | NM_ 021355.3 | TCAATCTCCTTCCAACAGGCAG AGCCATCCGTTTAGCCTCAGAT | 382 | 59 | 31 |
| **GAPDH** | AK 146435 | GTGGCAAAGTGGAGATTGTTGCC GATGATGACCCGTTTGGCTCC | 289 | 62 | 20 |
| **TGF-β1** | BC 013738 | GACTCTCCACCTGCAAGACC AGACAGCCACTCAGGCGTAT | 506 | 58 | 29 |

Real-Time PCR primers

Table 2.5

| Gene | GenBank Access # | Forward Primer 5' – 3' Reverse Primer 5' - 3' | Product size (bp) | Annealing Temp (°C) |
|---|---|---|---|---|
| **Collagen Iα1** | BC050014 | ATGTCGCTATCCAGCTGACC<br>AAGGGTGCTGTAGGTGAAGC | 192 | 60 |
| **Collagen IIIα1** | AK041115 | ATAAGCCCTGATGGTTCTCG<br>ATAAGCCCTGATGGTTCTCG | 195 | 61 |
| **Decorin** | NM_007833 | TTCCAGGGACTGAAGAGTCTCT<br>GCACCCTGAGGAGTTTGTTGTT | 268 | 60 |
| **Fibromodulin** | NM_021355.3 | GCTGTACGTCCGGTTGTCTC<br>GAACTCATTGATCCTGTTGCCC | 175 | 60 |
| **Histone H3.3A** | NM_008210 | GGCTCGTACAAAGCAGACTGCC<br>GCAATTTCTCGCACCAGACG | 225 | 60 |
| **TGF-β1** | BC013738 | GCAACAATTCCTGGCGTTACC<br>GGCTGATCCCGTTGATTTCC | 198 | 60 |
| **TGF-β3** | AK132519.1 | GCGGAGCACAATGAACTGG<br>GCTCATCCGGTCGAAGTATCTG | 193 | 60 |

## 2.2 Methods

### 2.2.1 Burn trial

#### Burn injury

Animal ethics approval was obtained from both the University of Waikato and the Ruakura Animal Ethics Committees (Application number 11315, amendment number 817) prior to any animal manipulations. On day zero, 104 approximately 9 month-old, male C57bl mice were inflicted with a burn injury. Briefly, mice were anaesthetised, shaved, tagged, weighed, and inflicted with a burn injury on each side of the upper back just below the shoulder and parallel to the midline using a red-hot metal rod (1.5 mm wide, 7 mm long) which was applied to the skin for 5 seconds. The skin was lifted between thumb and forefinger and folded over forefinger to prevent the heat from damaging structures underlying the skin. Tattoos were then applied marking around the area 2-3 mm from the edge of the wound. The mice were then placed on a heat pad at 37°C to recover from the anaesthetic.

#### Injection of antagonist or saline

Trial mice were age-matched and divided into two study groups where 48 were saline-treated and 48 were Mstn-ant4-treated. A couple of extra mice were added to each group to compensate for trial mice being withdrawn from the trial because of illness or some other unforeseen reason. Mice were injected subcutaneously with either saline or recombinant Mstn-ant4 antagonist at a concentration of 6 µg/g of body weight in a volume of 0.2 ml (29 gauge needle) on days 1, 3, 5, 7, 10, and 15 after injury.

#### Tissue collection

Six animals from each group were sacrificed on days 2, 4, 7, 14, 21, 28, 35, and 46. Burn wounds were excised along with matching sized areas of uninjured skin from the shaved lower back and either snap-frozen in liquid nitrogen for RNA and collagen analysis or fixed in 10% phosphate-buffered formalin for histology.

A.

B.

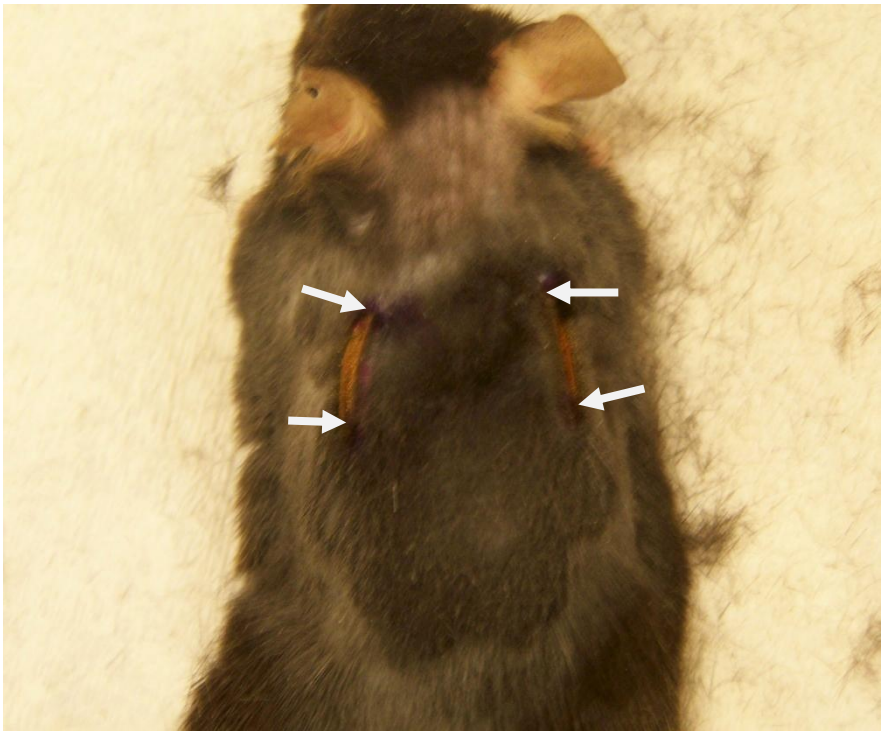


**Figure 2.1: Burn injury**
**A**: Method of causing burn injury. **B**: Position of each burn injury after application of burn causing agent (between arrowheads).

## 2.2.2 Antagonist preparation

Myostatin antagonist preparation was performed by Carole Berry (AgResearch Ltd). Various myostatin antagonists have been developed and tested. They are essentially a truncated form of mature myostatin. The myostatin antagonist used in the present study was named 'Mstn-ant4'. This carboxy-terminal truncation spanning amino acid 266 – 310 was produced using an *E. coli* expression system. Amplified cDNA was purified and subsequently inserted into the cloning vector pET 16-B (Novagen) as described in Sharma *et al.* (1999).

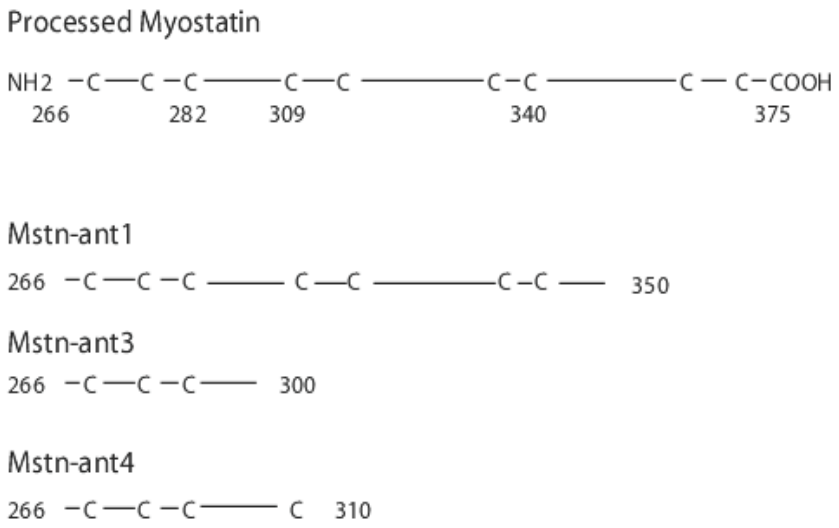



**Figure 2.2: Myostatin antagonists created at AgResearch Ltd.**

## 2.2.3 Hydroxyproline assay

Total collagen was measured by hydroxyproline assay. It was used to assess a difference in treatment with respect to collagen deposition. The amino acid proline occurs at a relatively constant frequency in the synthesis of collagen molecules. Hydroxyproline results from a post-translational modification of proline and this can be oxidised to form a chromophore whose absorbance can be read at 550 nm. In this study, a method for the determination of hydroxyproline was established with modifications from published methods by Stegemann and Stalder (1967), Edwards and O'Brien (1980), and Reddy and Enwemeka (1996).

### Sample hydrolysis

Excised skin samples were retrieved from -80°C, weighed and dried overnight at 55-60°C in an oven. Dried tissue was weighed to determine water content of skin.

Tissue was then cut into small pieces, re-weighed and placed in a 15ml neoprene tube with 6 ml of 6 N HCl. The tube was punched through a tinfoil-covered 1 L beaker of water to hold it in place, which was then placed inside a pressure cooker set at 120°C for 1 h. 1 ml of hydrolysed skin in acid was pipetted into a 1.7 ml eppendorf tube and centrifuged to remove any remaining ash.

### Assay

A standard curve was included for every assay run which included varying concentrations of standard hydroxyproline (Sigma-Aldrich). 10 µl of each sample was added to 40 µl of Milli-Q water in a 1.7 ml eppendorf tube, in triplicate. 450 µl of freshly made Chloramin T reagent was then added, briefly mixed and incubated in a water bath at 27°C for 30 min. Tubes were then placed on ice to stop the reaction. 500 µl of freshly made Ehrlich's reagent was then added, mixed and incubated for a further 20 min at 60°C after which tubes were placed on ice to stop the reaction. 100 µl aliquots were then placed in duplicate in a 96 well plate which was then read at 550 nm on a Versamax plate reader using SOFTmax pro software (Molecular Devices). Hydroxyproline quantities were calculated from the standard curve and then converted to percentage of collagen in sample using a 7.46 conversion factor (Neuman and Logan 1950).

## 2.2.4 RNA extraction

Total RNA was extracted from skin using TRIzol® Reagent (Invitrogen), according to the supplied instructions. 1 ml of TRIzol® Reagent was added to frozen skin which was homogenised using a power homogeniser (Global Science) for approximately 1 min. Following homogenisation, insoluble material from the homogenate was removed by centrifugation at 12,000 × g for 10 min at 4°C. The supernatant was transferred to a new tube. The resulting pellet containing extracellular membranes, polysaccharides, and high molecular weight DNA, was retained for possible later protein extraction.

Phase Separation: Homogenised samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml of chloroform was added and tubes shaken vigorously by hand for 15 s and left to incubate at room temp for 2 to 3 min. Samples were centrifuged at 12,000 × g for 15 min at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase containing the RNA.

RNA Precipitation: The aqueous phase was transferred to a fresh tube, and the organic phase was added to the pellet of insoluble material removed after homogenisation and stored at -80°C for later protein analysis. RNA from the aqueous phase was precipitated by mixing with 0.5 ml of isopropanol. Samples were either incubated at room temperature for 10 min or at 4 or -20°C for 1 – 2 h. Samples were then centrifuged at 12,000 × g for 10 min at 4°C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube.

RNA Wash: The supernatant was removed and discarded. The RNA pellet was washed once with 1 ml of 75% ethanol mixed by vortexing and then centrifuged at 7,500 × g for 5 min at 4°C.

Redissolving the RNA: Ethanol was removed and the RNA pellet briefly dried then resuspended in 20 µl of diethyl pyrocarbonate (DEPC)-treated water by incubating for 10 min at 55 to 60°C.

RNA quantitation: RNA concentration and purity was determined by measuring the absorbance at 260 nm and 280 nm. For the purpose of quantitation, it was assumed that an $A_{260}$ of 1.0 is roughly equivalent to 40 µg/ml of ssRNA. The $A_{260}/A_{280}$ ratio is used to assess RNA purity, where a ratio of 1.8-2.1 indicates highly purified RNA. Absorbance measurements were made on a Nanodrop ND 1000 spectrophotometer using supplied software (NanoDrop Technologies Inc) RNA was stored at -80°C

## 2.2.5 RNA electrophoresis

RNA electrophoresis was carried out on several samples to check for degradation. A 1.2% solution of agarose was made by boiling 0.6 g of agarose in 36 ml of water for 2 min. To the gel solution cooled to 65°C was added 5 ml 10 x MOPS and 9 ml formaldehyde. The gel was poured into a specific RNA electrophoresis gel box and, after setting, was immersed in 1 x MOPS running buffer. 1 µg of total RNA was incubated with an equal volume of 2 x RNA dye (containing ethidium bromide) at 65°C for 5 min, loaded onto the gel, and run at 60 mA until the dye front had crossed half-way. Separated RNA was then visualised by photographing under UV light using a BioRad Gel-doc 2000 and Quantity One 4.4 software (BioRad).

## 2.2.6 Reverse Transcription of RNA to generate cDNA

cDNA was generated from RNA using First-Strand cDNA SuperScript-II Reverse Transcriptase System (Invitrogen). RNA samples were diluted to 0.2 µg/µl. After quantitation, an appropriate amount of suspended RNA was added to a 0.6 ml eppendorf tube and made up to 7 µl with water such that the tube contained 1 µg of total RNA. To this was added: 1µl of 10 mM dNTP mix, 1µl of 0.5 µg/µl Oligo(dT), and 1 µl of 0.2 µM gene-specific primer for 18S rRNA gene. This mixture was then incubated at 65°C for 5 min to denature the RNA then placed on ice for at least 1 min. To each tube was added in the following order: 2 µl 10x RT buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT, 1 µl RNaseOUT$^{tm}$. The mixture was gently mixed and collected by brief centrifugation on a bench-top centrifuge and incubated at 42°C for 2 min. To each tube was added 1 µl SuperScript-II RT, mixed briefly and collected by brief centrifugation. Tubes were then incubated for 50 min at 42°C. Incubation at 70°C for 15 min terminated the reaction, and then samples were chilled on ice. Remaining RNA template was then degraded with 1 µl RNAse H for 20 min at 37°C. cDNA was stored at -20°C.

## 2.2.7 Polymerase Chain Reaction (PCR)

Amplification of target cDNA for PCR standardisation and semi-quantitative PCR was carrier out using a Programmable Thermal Cycler 100 (PTC-100, BioRad). Each 50 µl reaction mixture included: 40.75 µl Milli-Q water, 5 µl 10x Choral Load PCR Buffer (Qiagen) which includes $MgCl_2$ (1.5 mM final concentration), 1 µl 10 mM dNTPs, 1 µl 10 µM forward primer,1 µl 10 µM reverse primer, 0.25 µl Taq DNA Polymerase (Qiagen), and 1 µl of cDNA (or water for control sample). In the case of 18S rRNA PCR, 10 µl 5x Q Solution (Qiagen) was substituted for 10 µl of water. PCR products were electrophoresed for visualisation on a gel as described in 2.2.9.

## 2.2.8 Primer design and PCR standardisation

Primers were designed using a combination of PerlPrimer 1.1.16 (Marshall 2004) and where possible spanned exons to ensure only mRNA was amplified. Both PerlPrimer and Oligocalc (Kibbe 2007) were used to assess chosen primers for melting temperature, secondary structures, and self-complimentarity. A BLAST search of the NCBI database was conducted to ensure primers were unique to the target locus. All oligonucleotides were 19-22 nucleotides long. Cycling conditions were determined by trial-and-error beginning with predicted melting temperatures from Oligocalc. PCR products were electrophoresed and visualised

to determine if a single clear band was present. If visualised gel was not satisfactory, annealing temperature was increased by 1°C each time until satisfactory results were obtained.

### 2.2.9 DNA electrophoresis

A 1% solution of agarose was made by boiling 0.5 g of agarose for 2 min in 50 ml 1 x TAE. The gel was cooled to 65°C and cast by pouring into an appropriate gel box. 1 µl of ethidium bromide was mixed into the gel and allowed to set. The gel was covered with 1 x TAE running buffer. PCR reactions that used Choral Load buffer did not require mixing with DNA loading dye. 10 µl of PCR product was loaded into each well with one well reserved for a '1 kb plus' molecular weight marker (Invitrogen). Electrophoresis was carried out at 80 V for approximately 1 h. DNA was then visualised by photographing using a BioRad Gel-doc 2000 and Quantity One 4.4 software (BioRad).

### 2.2.10 Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using a Roche LightCycler$^{tm}$. Reaction capillaries were placed into position in a lead block to maintain cool conditions. A master mix for *n* reactions was created which included *n* x 4.5 µl Milli-Q water, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, and 2 µl LightCycler$^{tm}$ master mix (Roche) which includes 14 µl Taq polymerase (Roche). 7.5 µl of this mixture was added to each capillary. 3 µl of 1:10 diluted cDNA template was added to the master mix in each capillary by pipetting up and down. Capillaries were loaded into the LightCycler$^{tm}$ Carousel. The reaction mixtures were collected at the bottom of each capillary using the supplied Roche centrifuge with preset parameters. The carousel was then loaded into the LightCycler$^{tm}$ thermal cycler. The cycler was set to run for 45 cycles then perform a melt curve. For each gene analysed, a standard curve was constructed first by creating a series of dilutions of a sample then running a standard RT-PCR on the LightCycler$^{tm}$. Cycle thresholds for each sample were calculated using the standard curve for each gene. These were used to calculate concentrations relative to a sample used for calibration which was designated a concentration of 1 (or 0.1 for a 1:10 dilution).These concentrations were then used to calculate gene expression relative to the house-keeping gene; in the case of all RT-PCR reactions this was Histone H3.3A. Amplified products were run on a 1% agarose gel to check for correct sizing.

### 2.2.11 DNA purification

Occasionally, for genes expressed at a low level, a series of sample dilutions did not result in enough points to create a standard curve. In such cases a band from the electrophoresed amplified gene product was excised and purified and then RT-PCR repeated with a larger amount of DNA. A Wizard PCR prep (Promega) system was used to purify electrophoresed DNA. PCR products were separated on a low melt gel using low melting point (LMP) agarose (Invitrogen). The DNA band of interest was visualised under UV light and excised using a razor blade before being placed into a 1.7 ml eppendorf tube. The tube was incubated at 65-70°C until the gel melted. 1 ml of purification resin was added and mixed gently for 20 s. The resin/DNA slurry was then passed through a Wizard mini-column using a syringe. 2 ml of 80% isopropanol was then washed through. The syringe was removed and the mini-column was centrifuged at 10,000 x g for 2 min. The mini-column was then transferred to a new tube and DNA was eluted from the column by adding 50 µl Milli-Q water, incubating for 1 min and centrifuging at 10,000 x g for 20 s. The purified DNA was quantitated on the NanoDrop 1000 and diluted appropriately for RT-PCR standard curve construction after calculating copy number.

### 2.2.12 Copy number calculation

It was necessary to calculate template copy number so an appropriate dilution could be made to calculate a standard curve for a gene using RT-PCR. After quantitation this formula was used to calculate copy number:

pmol dsDNA = (µg DNA x 1515)/Nbp where Nbp is the no. of base pairs

This formula could be used since the DNA solution contained an amplified PCR product of known length. This method ensured that for the lowest concentration of diluted sample there was at least more than one template copy in the reaction mixture.

### 2.2.13 Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

Denaturing PAGE was used to assess for degradation of oligonucleotide primers. A method was established by modification of a published protocol (Ellington and Pollard 1998). 12 ml of 20% acrylamide (BioRad) was made up for a 10 cm x 10 cm x 1.5 mm gel. 8 µl of tetramethylethylenediamine (TEMED, BioRad) and 60 µl

ammonium persulfate (APS, BioRad) was added before pouring. Running buffer was TBE. Wells were washed by squirting TBE with a Pasteur pipette and 5-50 ng of ssDNA was loaded per well with loading dye. A 10 bp ladder was loaded in lane 1.

### 2.2.14 Silver staining of polyacrylamide gels

Silver Stain Plus from BioRad was used to visualise polyacrylamide gels. After electrophoresis, gels were placed in fixative enhancer solution for 20 min with gentle agitation containing 50% methanol, 10% acetic acid, 10% Fixative Enhancer Concentrate, and 30% Milli-Q Water. Gels were then washed in two changes of water with gentle agitation for 10 min each. A staining solution containing 35% Milli-Q water, 10% Silver Complex Solution, 10% Reduction Moderator Solution, 10% Image Development Reagent and 50% Development Accelerator Solution was used to develop staining until the desired level of staining was achieved. Gels were placed in 5% acetic acid for 15 min to stop the reaction.

### 2.2.15 Histology

#### Fixation

Individual skin samples were placed inside histology cassettes and fixed immediately after excision. The fixative used was 10% buffered formalin for at least 48 h duration to ensure proper penetration. After this time samples were transferred to, and stored in, 70% ethanol until processing.

#### Processing

Samples were processed in a Leica Tissue processor (Global Science) overnight, which involved dehydration and paraffin wax infusion. The following day, skin samples were mounted in wax blocks in preparation for sectioning. Sections were cut at a thickness of 7-8 µm, placed in a 60°C water bath and mounted on glass slides pre-treated with poly-lysine adhesive solution and dried at 37°C overnight.

#### Hematoxylin & Eosin (H & E) Staining

Sections were deparaffinised and rehydrated by immersion in 100% xylene (2 x 5 min), then 100% ethanol (2 min), then 95% ethanol (2 min), then 70% ethanol (2 min), then 50% ethanol (2 min). Slides were then washed briefly in distilled water. Sections were then stained with Gill's Hematoxylin (5 min). Slides were rinsed

with tap-water until clear. Sections were blued with Scott's tap-water (2 min), and then rinsed with tap-water (2 min). Sections were stained with 1% Eosin Y (2 min), rinsed in tap-water until clear (plus 2 min). Sections were dehydrated through 50% ethanol (3 dips), then 70% ethanol (3 dips), then 95% ethanol (2 min), then 100% ethanol (2 min x 2). Slides were cleared in xylene (5 min x 2). Once slides had dried, DPX mounting solution was applied before placing on a cover-slip and allowing to dry overnight.

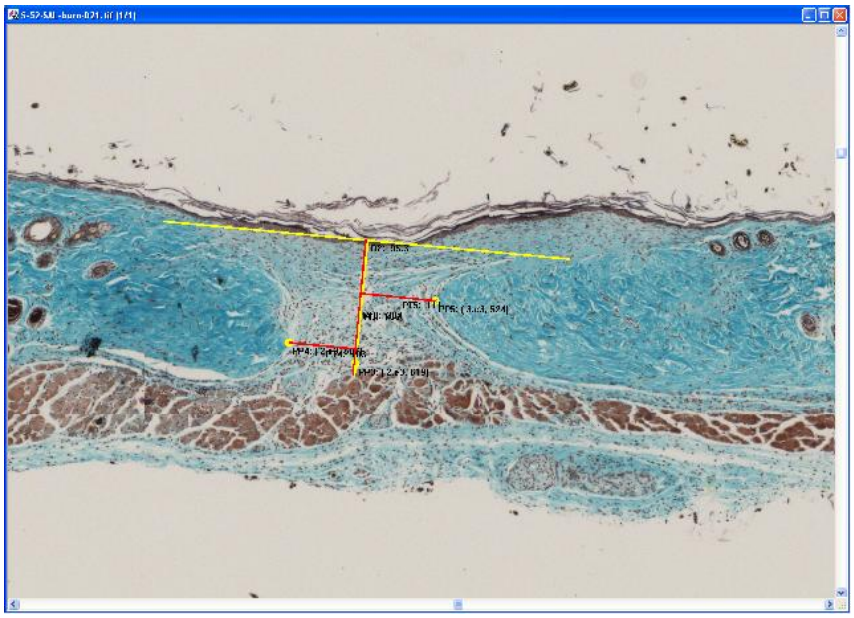
## Trichrome Staining

A method for trichrome staining was adapted from Gomori (1950). Slides were de-waxed and rehydrated as for H & E staining and re-fixed in Bouin's fixative at room temperature overnight or for 1 h at 60°C. Slides were removed from Bouin's fixative and washed in running water until the yellow colour disappeared. Slides were rinsed in distilled water and stained in Celestine Blue (10 min), rinsed in distilled water and then stained with Gill's Hematoxylin (3 min). Slides were then washed in running water (2 min), differentiated in 1% acid alcohol to remove over-staining (2-3-dips), rinsed thoroughly in water, and then blued in Scott's tap-water (2 min), before being rinsed again in distilled water. Slides were then stained in Gomori's trichrome stain for 15 min, after which they were checked for staining progress and stained for a further 5 min if necessary. Slides were then rinsed briefly and differentiate in 1% acetic acid (glacial) for 2 min before dehydrating, clearing and mounting as for H & E staining.

## Microscopy

Stained sections were viewed on a Leica AF6000 microscope (Leica Microsystems Ltd.) with an attached Leica DFC300FX digital camera. Images were captured using the Leica Application Suite Version 2.5.0.R1 software (Leica Microsystems Ltd.). Sections were analysed using Image-Pro Plus (Media Cybernetics) software and measured for wound area, wound depth, wound length, and epidermal thickness. Wound contraction was assessed histologically in a similar manner to Fukai *et al.* (2005). The shortest distance between the dermal wound margins, which corresponded to the span of the granulation tissue was measured (van Rossum *et al.* 2007), and presented in most cases with an hour-glass type morphology. Granulation tissue thickness was assessed by measuring the vertical span of the granulation tissue in three regions of the wound which were then averaged to determine approximate thickness after Joseph *et al.* (1997).

A.



B.

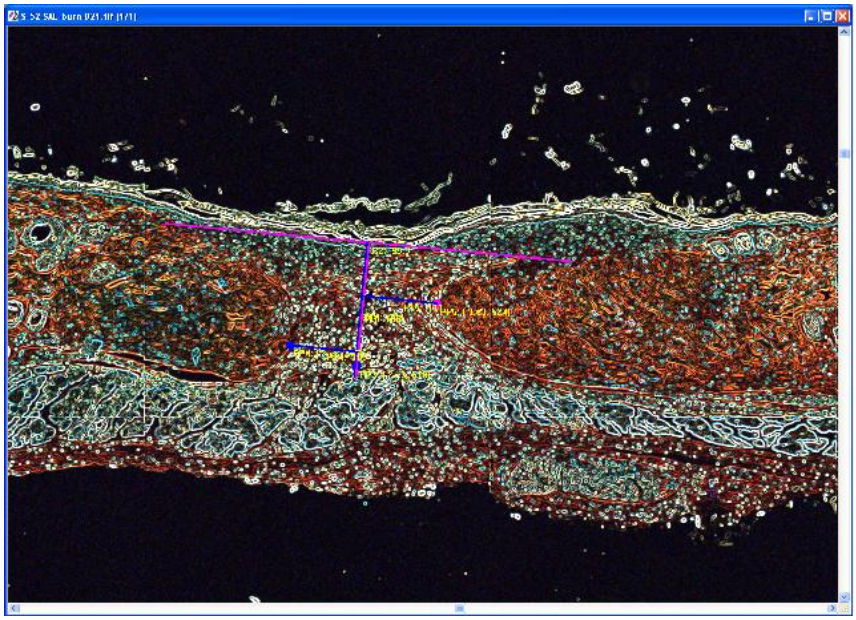


**Figure 2.3: Histological analysis using Image-Pro Plus**
**A**: Granulation tissue width was determined by bisecting the wound area by drawing a line perpendicular to the general plane of the epidermis, then measuring the distance to the nearest wound edge perpendicular to that line. **B**: Sometimes a 'Sorbel' filter was useful for more clearly distinguishing the wound edge. These measurements were used to indicate the degree of wound contraction.

### 2.2.16 Statistics

Data were analysed using analysis of variance (ANOVA) to compare the treatments and the burn effect; the ANOVA also accounted for the repeat observations (burn & control) being on the same animal. GenStat software was used for the ANOVA analysis.

For the gene expression analysis, the data were transformed to approximate normality with constant variance by using the $\log_e$ (natural log) transformation, and error was computed as the standard error of the difference between means (SED) which was pooled across all days. Tests of significance (t-tests) were derived after performing ANOVA on rank transformed data with pooled standard deviation. Statistically significant differences were also detected using Student's t-tests of individual time points.

For all data, a probability (p) value of less than or equal to 0.05 was considered significant, where (*) represents a p-value of less than or equal to 0.05, (**) represents a p-value of less than or equal to 0.01, and (***) represents a p-value of less than or equal to 0.001.

# 3 RESULTS

Myostatin is well established as a powerful negative regulator of embryonic and post-natal muscle development (Kambadur *et al.* 1997; McPherron *et al.* 1997). Myostatin's inhibitory role in myogenic wound healing has also recently been described (McCroskery *et al.* 2005; Siriett 2007; Nozaki *et al.* 2008). Various methods of inhibition or antagonism targeting myostatin have been investigated as a means of improving muscle development (Tsuchida 2004; Bishop *et al.* 2005; Patel and Amthor 2005) and healing (Nozaki *et al.* 2008). Myostatin expression has not been detected in skin, but its receptor ActRIIB is present in both keratinocytes and dermal fibroblasts (Hübner and Werner 1996). Therefore, it remains a possibility that myostatin might have some effect on wound healing in skin, and that antagonising myostatin may result in quantifiable improvements in wound healing.

## 3.1 Effect of myostatin antagonist on weight recovery after injury

Since blocking myostatin has been shown to alleviate muscle wasting conditions such as sarcopenia (Siriett *et al.* 2007), cachexia (Liu *et al.* 2008) and muscular dystrophy (Bogdanovich *et al.* 2002), it may also help to regain losses in normal body weight which occur following traumatic injury (Jeschke *et al.* 2008). In the present study, body weights of all mice were measured on day zero of the burn trial. Body weights were re-measured at each collection time point when 6 saline-treated mice and 6 Mstn-ant4-treated mice were sacrificed for tissue collection. The following formula was used to calculate the percent change in body weight where $PW_n$ is the percentage of day 0 body weight on day n, $W_i$ is the initial body weight on day 0, and $W_n$ is the body weight on day n.

$$PW_n = \frac{W_i - W_n}{W_i} \times 100$$

The burn trauma (and likely anaesthetic effects also) did appear to cause a significant (p=0.04) loss of body weight of up to 10% of initial weight during the first four days after injury. Figure 3.1 shows that the Mstn-ant4-treated group had recovered their lost body weight by day 21, whereas the body weight of saline-treated mice was significantly lower (p=0.02), and did not fully recover until day 28.

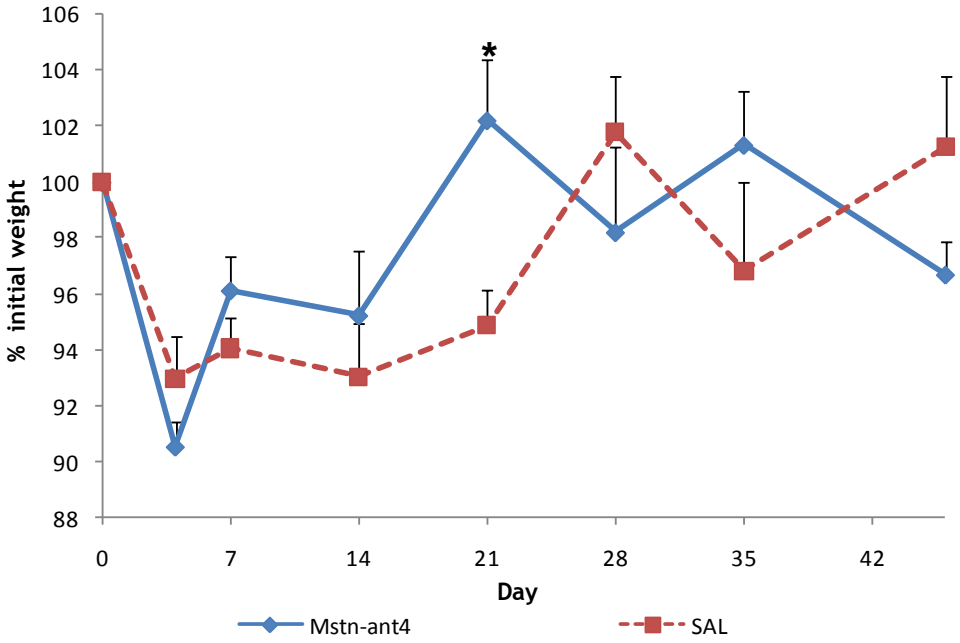



**Figure 3.1: Body weight data for duration of trial**
Body weight data is expressed as a percentage of body weight at day 0. Error bars represent standard error of the mean (SEM). For each point, n=6 (data for day 2 was not collected).

## 3.2 Effect of myostatin antagonist on total collagen

Collagen was estimated using the hydroxyproline assay. In the present study, collagen was expressed as a ratio of the amount of collagen in control skin compared to burned skin for each treatment. There did not appear to be significant differences between treatments for the amount of collagen in skin samples except at day 28 ($p=0.007$) (Figure 3.2 B). This increase in ratio seen on day 28 was due to similar values obtained from burn and control samples for Mstn-ant4 (Figure 3.2 A), giving a ratio of close to 1:1. This would seem to indicate that the wound had fully healed. However, this is unlikely to be the case as subsequent time points re-establish the differences between burn and control samples seen at earlier time points. There is an unexpected significant difference in hydroxyproline in control skin at day 21. This could be a response to withdrawal of treatment on day 15, leading to an increase in collagen levels in Mstn-ant4-treated mice.

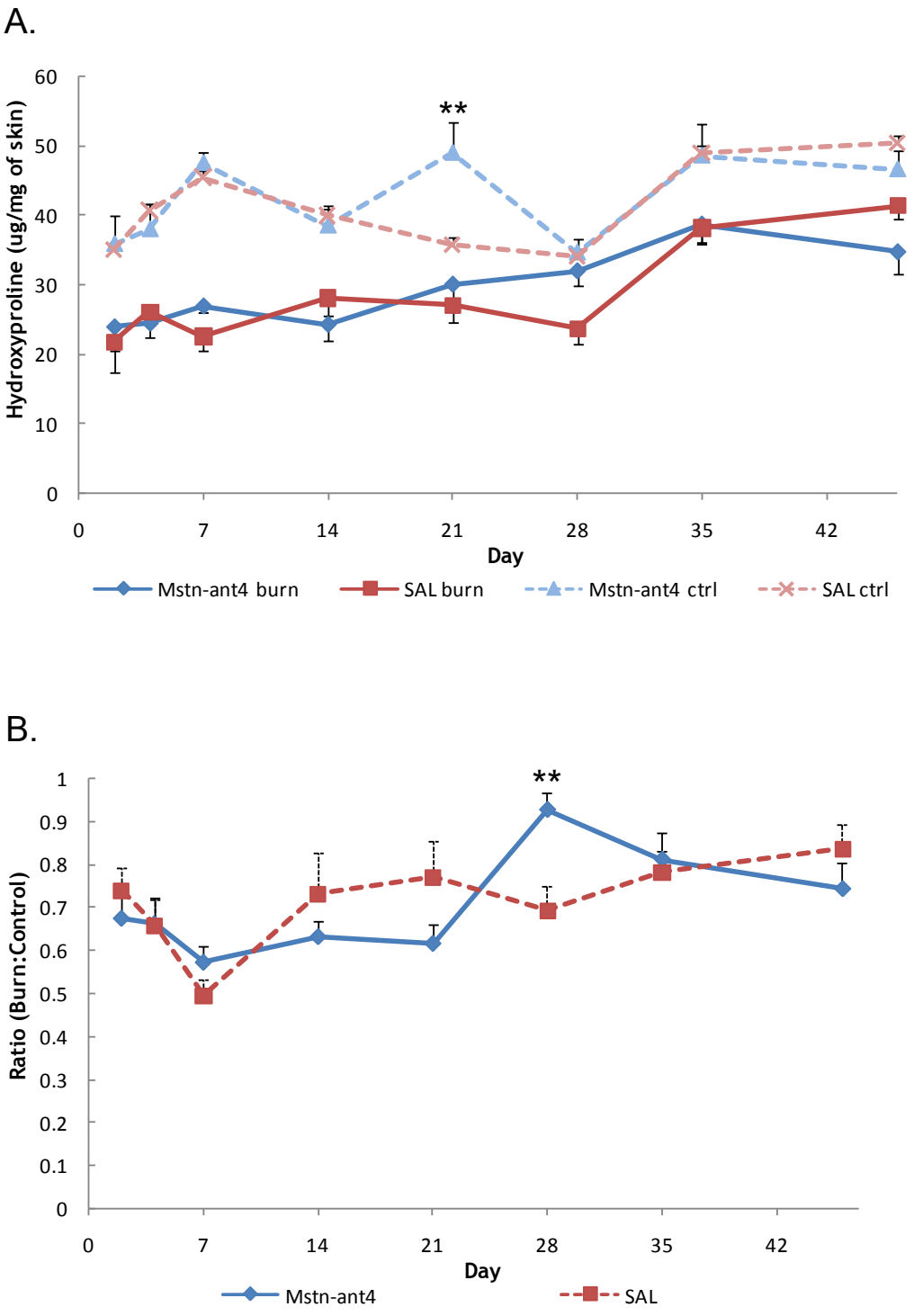


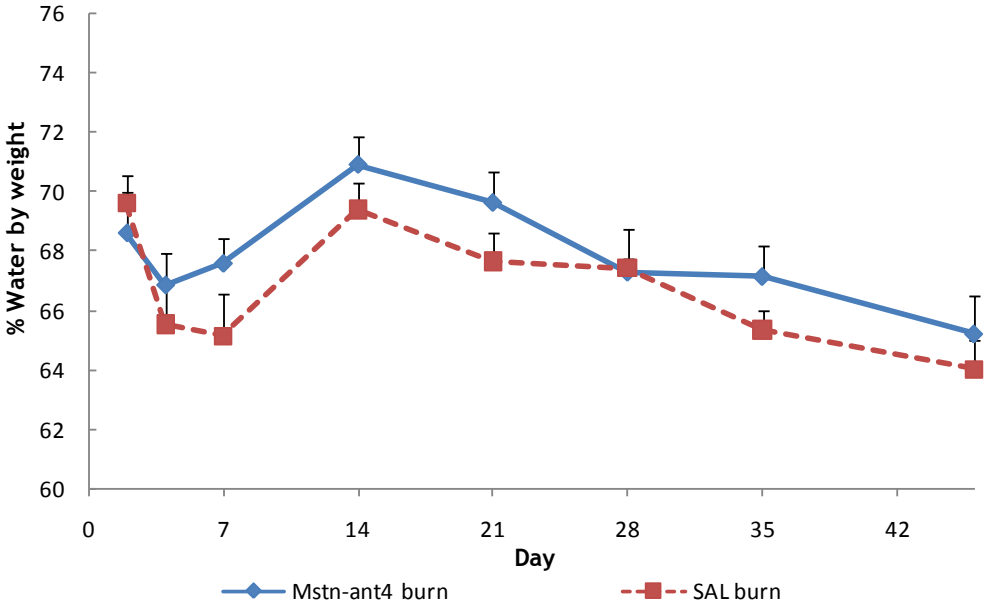

**Figure 3.2: Hydroxyproline content and percentage of collagen as a ratio of burned to uninjured skin**

**A**: Actual hydroxyproline measurements for each treatment/effect. For each time point, n=6. Error bars represent SEM. **B**: Hydroxyproline values were converted to percentage of collagen. These values were then expressed as a ratio of burned skin to uninjured skin and averaged to compare the different treatments. Errors are expressed as SEM. Each time point represents the mean of 6 ratios, except Mstn-ant4-treated samples of day 7 where n=5 due to loss of sample.

## 3.3 Effect of myostatin antagonist on skin hydration

Well hydrated skin is an indication of its healthy status. Increased hydration of keratinocytes positively influences their cytokine profiles and rate of re-epithelialisation (Dumas *et al.* 2007; Tandara *et al.* 2007). Water content of excised skin samples was able to be compared between treatments by dehydrating the samples and measuring the weight change. For uninjured skin the data showed that water content was significantly higher at day 7 ($p=0.01$), remained higher at day 14 ($p=0.1$), and was again significantly higher at day 21 ($p=0.02$) for Mstn-ant4-treated skin as compared to saline-treated skin. A similar trend was seen in burned skin samples, but was not significant (Figure 3.3). Water content is calculated as the difference between wet and dry weights of skin samples and expressed as a percentage of wet weight.

Another observation is that skin hydration decreased significantly between days 2 and 4 for both saline-treated burned and uninjured skin. The same effect was not observed in Mstn-ant4-treated burned or uninjured skin. This indicates that Mstn-ant4 treatment can also prevent dehydration in surrounding tissue as a result of burn injury.



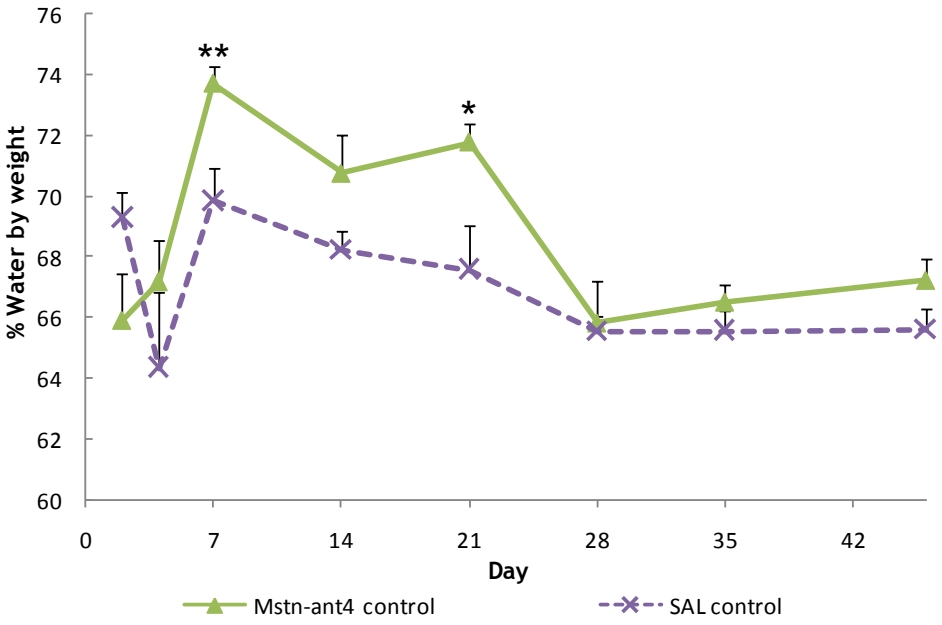




**Figure 3.3: Skin hydration**
**A**: Water content of Mstn-ant4-treated burned skin compared with saline-treated burned skin. **B**: Water content of Mstn-ant4-treated uninjured skin compared with saline-treated uninjured skin. Error bars for both plots represent SEM.

## 3.4 Gene expression analysis

In accordance with the main aim of this thesis, gene expression analysis was conducted for changes in the expression of genes considered to be indicative of improved wound healing in skin. The genes chosen for expression analysis were: collagen types I and III as indicators of scar tissue formation; TGF-β1 as an indicator of fibrosis; decorin which binds and inhibits TGF-β1; TGF-β3 as implicated in scarless foetal wound healing; and fibromodulin as also implicated in foetal scarless wound healing. The house-keeping gene for all RT-PCR samples was Histone H3.3A. Semi-quantitative PCR was also performed to analyse expression for some genes but proved to be inferior in sensitivity to RT-PCR and therefore results are not included. To make efficient use of time and resources, the most important time period to study the effect of administering Mstn-ant4 on gene expression by RT-PCR was determined to be days 4 to 21. No significant changes in gene expression were observed after day 21.

Mouse number 37 proved to be a consistent outlier according to Grubbs' test for outliers (Grubbs 1969) in many gene expression datasets, even when transformed. After careful consideration, it was removed from all gene expression analyses. Mouse 37 was a saline treated animal from day 7; expression values from both burn and uninjured control skin for this animal were therefore not included in the analysis.

Tests of significance (t-tests) were calculated after ANOVA was performed, and at individual time points on $\log_e$ transformed data. For consistency, only ANOVA derived t-tests are included with figures, and where individual time point (ITP) t-tests are reported in the text, this is explicitly stated. Where fold differences are reported, these have been calculated after back-transforming mean log values.

### 3.4.1 Collagen expression

Levels of collagen gene expression are indicative of the amount of ECM and hence scar tissue being produced by fibroblasts. Depending on degree, a decrease in collagen expression may be related to a decrease in scarring or at least a decreased risk of fibrosis.

In this study, collagen type I expression rose sharply during the first week, did not change significantly during the second week, and subsequently fell to around day 4 levels by the end of the third week for both treatment groups (Figure 3.4 A). These observations are consistent with other studies (Scharffetter *et al.* 1989). Collagen type III levels followed a similar pattern (Figure 3.4 B). There were no significant differences in collagen expression of any type on either day between saline-treated and Mstn-ant4-treated burn-injured mice. However, a trend toward a higher ratio of collagen I to collagen III (COL1:COL3) for saline-treated burn wounds developed from day 7, and by day 21, this difference was significant ($p=0.038$, ANOVA) (Figure 3.5). An ITP t-test performed on day 7 values also demonstrated a significance ($p=0.04$) between treatments with a higher ratio seen in saline treatment (not shown on figure). The implication of this result will be discussed in chapter four. Collagen expression levels were only assessed in samples of burned skin.

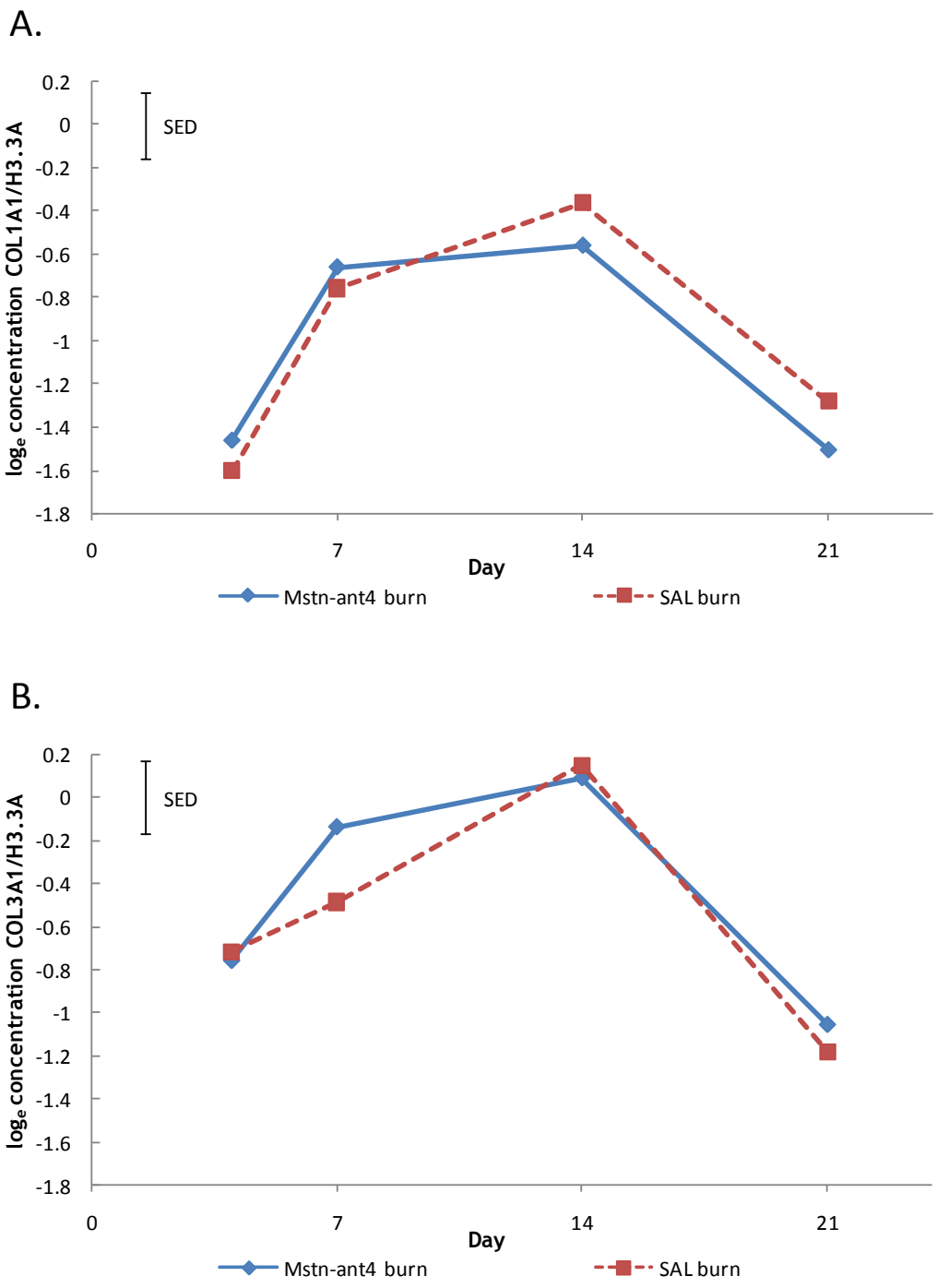



**Figure 3.4: Collagen Type I (A) and Collagen type III (B) expression**
**A**: Collagen type I expression is displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A. **B**: Collagen type III expression is displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A. Error bar for both plots represents pooled standard error of the difference between means (SED). For each point, n=6 except SAL day 7 where n=5.

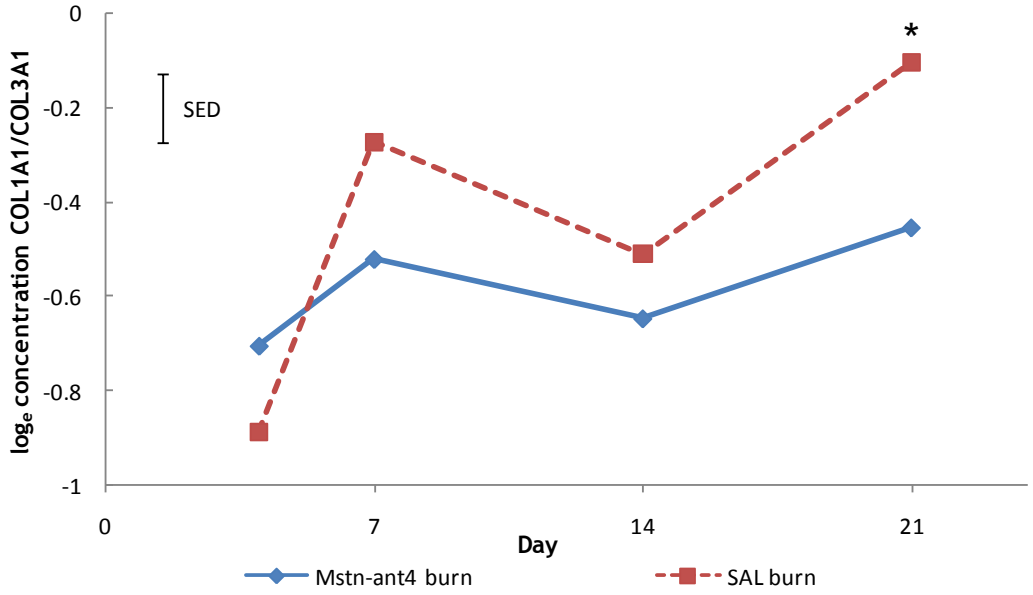



**Figure 3.5: Collagen I relative to Collagen III expression**
Collagen type I expression as a ratio to collagen III. $Log_e$ values are used for ratio calculation. Error bar represents pooled standard error of the difference between means (SED). For all points, n=6 except SAL day 7 where n=5.

## 3.4.2 TGF-β1 expression

TGF-β1 was analysed because of the known correlation between its up-regulation and fibrosis. Typically, expression levels peak at around day 8 (Wang *et al.* 1998), depending on depth and degree of injury.

In this study, TGF-β1 expression peaked at day 7 in burned skin. During the following two weeks TGF-β1 levels declined gradually (Figure 3.6). Both saline-treated and Mstn-ant4-treated burns showed remarkable similarity in these trends. The gradual decline in TGF-β1 from day 7 was also true for control skin of both treatments. However, on day 4 there was a marked difference in TGF-β1 expression between treatments ($p<0.001$, ANOVA) where TGF-β1 in Mstn-ant4-treated control skin was up-regulated by 4.1-fold (Figure 3.6 B). This result suggests that the myostatin antagonist is having an effect on TGF-β1 expression in uninjured skin, even though the effect is not seen in burned skin.

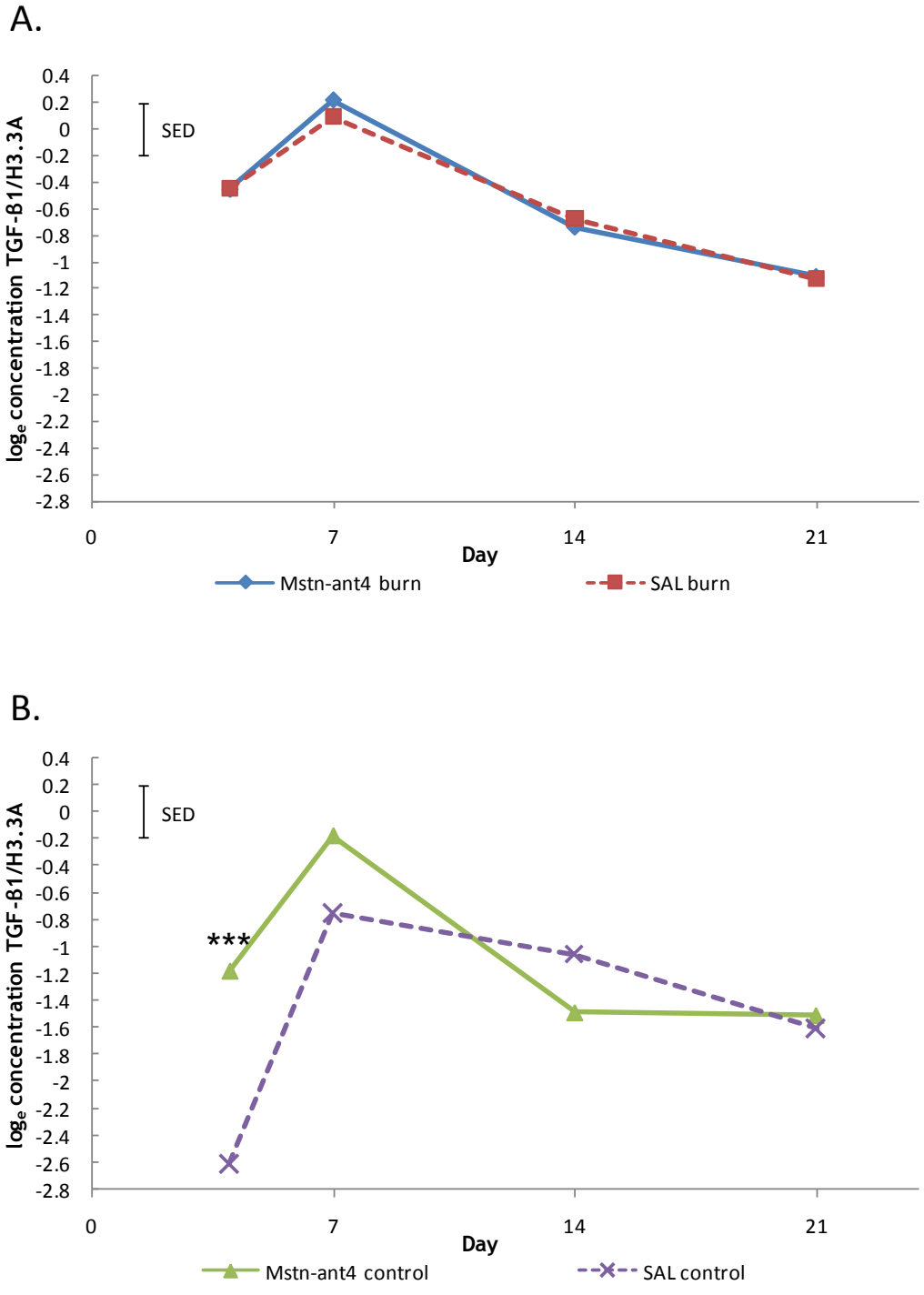



**Figure 3.6: TGF-β1 expression**

**A**: TGF-β1 expression in burned skin for each treatment displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A **B**: TGF-β1 expression in control skin for each treatment displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A. Error bar for both plots represents pooled standard error of the difference between means (SED). For all points, n=6 except SAL day 7 where n=5.

### 3.4.3 Decorin expression

Decorin was analysed because of its ability to bind and sequester TGF-β1. Decorin is also an important organiser of collagen, which is essential during wound healing. Decorin expression changed little during the first week, but there was a gradual though significant decline between day 7 and day 21 in burned skin of both treatment groups (Figure 3.7). There was no significant difference between treatments on any day for burned skin, although on day 7 an ITP t-test gave a p-value of 0.056 bordering on significance. In uninjured skin decorin expression levels increased sharply during the first week up to day 7 for both treatment groups. Mstn-ant4-treated skin exhibited significantly higher levels of decorin on day 7 (2.1-fold, $p=0.02$, ANOVA), before declining to base levels by day 21. An ITP t-test gave a p-value of 0.007 for Mstn-ant4 treatment on day 7 (not shown on figure).

### 3.4.4 TGF-β3 expression

Up-regulation of TGF-β3 is associated with scarless healing in early foetal mammals. Paradoxically, its effect markedly contrasts with that of its closest relative, TGF-β1, in wound healing. However, in the present study, its expression profile was similar to that of TGF-β1. For burn-injured skin, regardless of treatment, levels do not significantly change up until day 14, after which they drop significantly (Figure 3.8 A). For uninjured skin, Mstn-ant4-treatment showed a similar pattern of expression as it did for burn-injured skin. Saline-treated uninjured skin however showed significantly less TGF-β3 expression at day 4, before showing a similar pattern as Mstn-ant4 treated skin for the remaining days. In this case, Mstn-ant4 treatment resulted in a 6.1-fold higher TGF-β3 expression level than saline-treated control skin ($p<0.001$, ANOVA) was seen on day 4. By day 7 the difference had decreased to 2-fold due to an increase in TGF-β3 expression in saline-treated skin, however this difference was only significant by ITP t-test ($p=0.01$, not shown on figure).

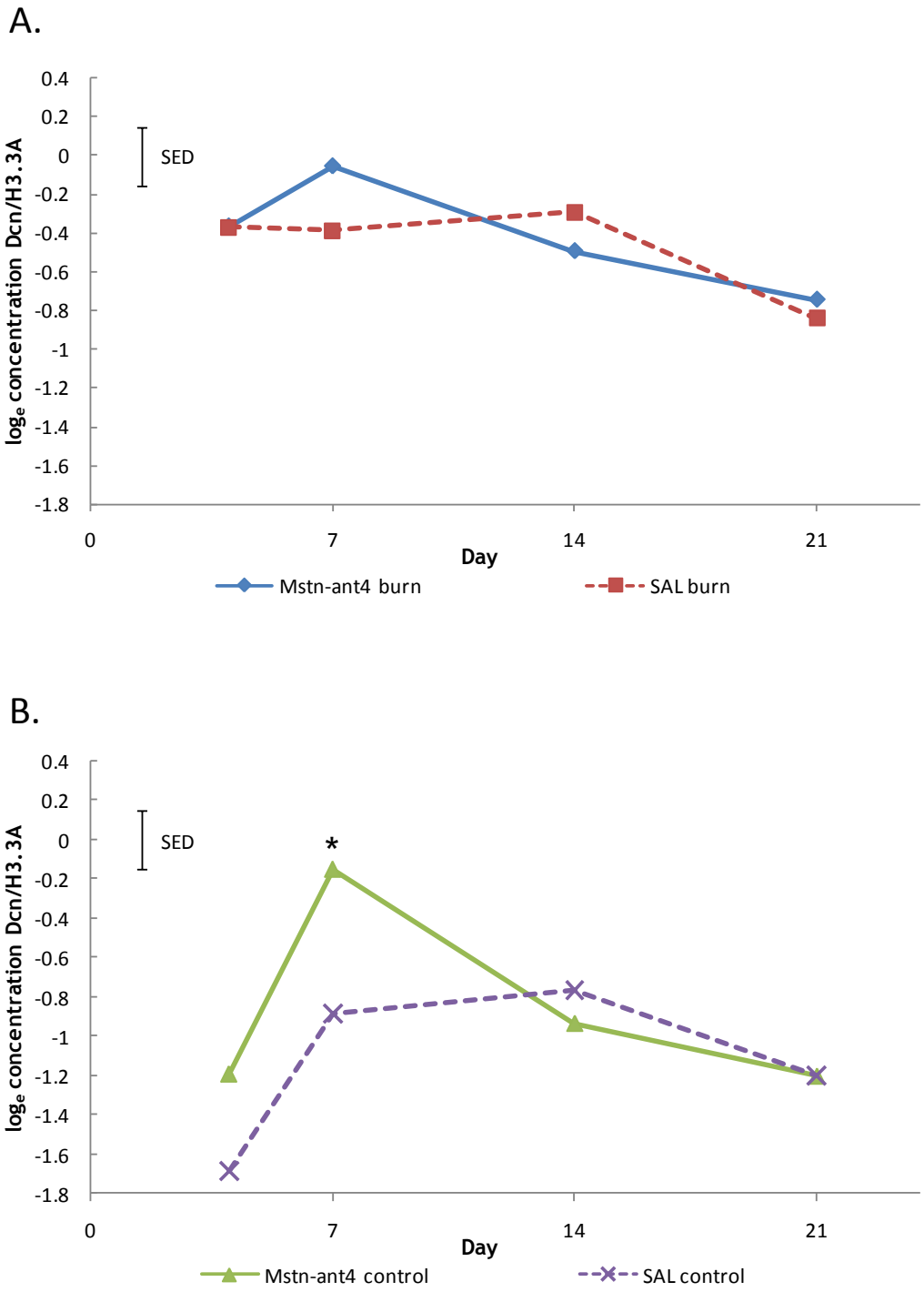



**Figure 3.7: Decorin expression**

**A**: Decorin (Dcn) expression in burned skin for each treatment displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A. **B**: Decorin expression in control skin for each treatment displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A. Error bar for both plots represents pooled standard error of the difference between means (SED). For all points, n=6 except SAL day 7 where n=5.

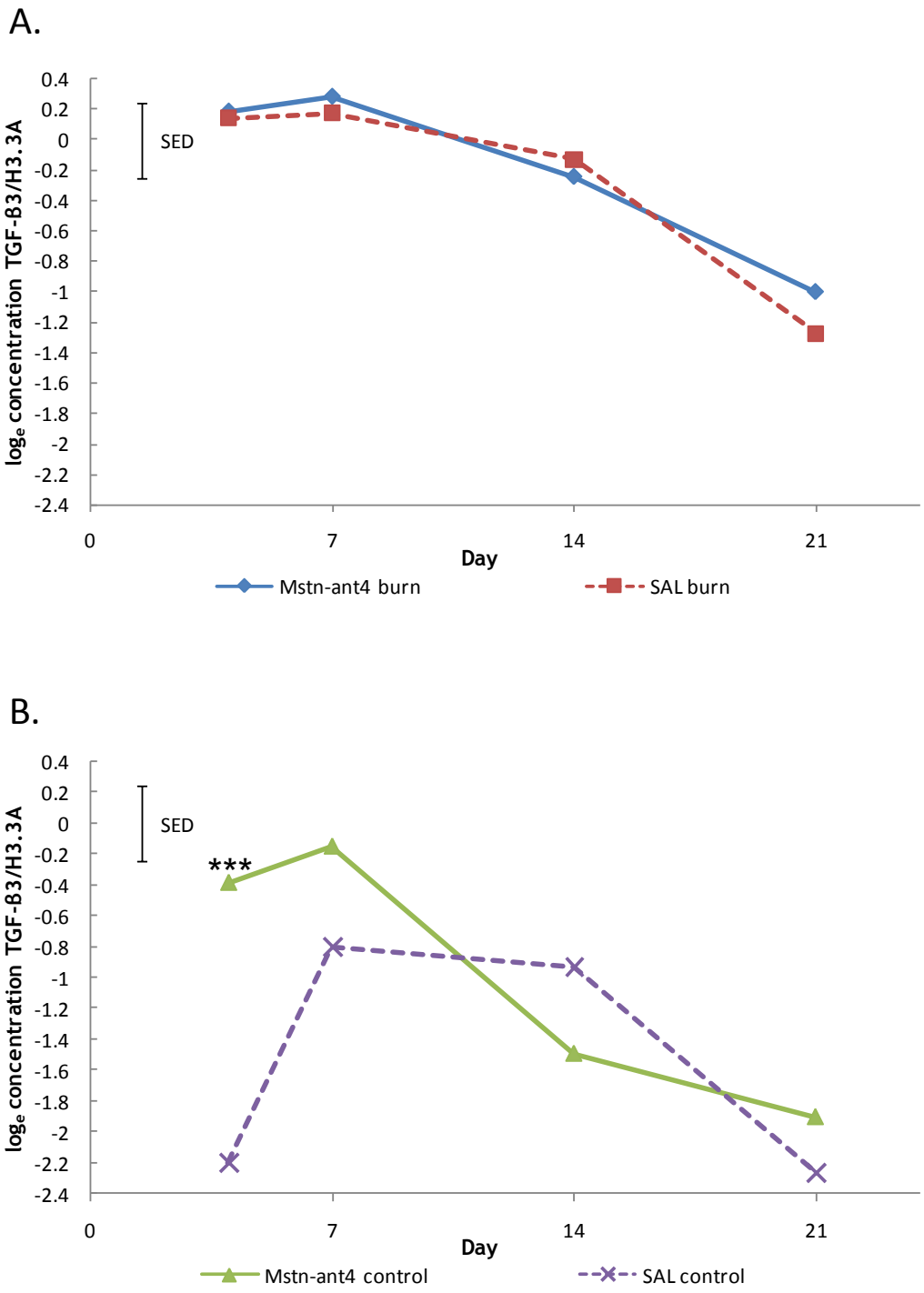



**Figure 3.8: TGF-β3 expression**

**A**: TGF-β3 expression in burned skin for each treatment displayed as log concentration values relative to house-keeping gene Histone H3.3A. **B**: TGF-β3 expression in control skin for each treatment displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A. Error bar for both plots represents pooled standard error of the difference between means (SED). For all points, n=6 except SAL day 7 where n=5.

### 3.4.5 Fibromodulin expression

Fibromodulin is up-regulated strongly in foetal scarless wound healing studies and as a modulator of TGF-β, is considered to be involved in the anti-scarring phenotype of foetal wounds. It also plays a similar role to decorin in the organisation of collagen fibres. For fibromodulin expression levels burned skin in the present study, there appeared to be an early difference, where Mstn-ant4-treated wounds showed a higher expression of fibromodulin in the early stages (Figure 3.9 A). However, this difference was not significant, except on day 7 by ITP t-test ($p=0.01$, not shown on figure). A similar trend is seen in uninjured skin where, Mstn-ant4 treatment results in a 3.8-fold ($p=0.01$, ANOVA) increase in fibromodulin expression. By day 7, the difference is 2.6-fold and borderline significant ($p=0.066$) (Figure 3.9 B).

### 3.4.6 Gene expression ratios

Two gene expression ratios were considered important in the context of this study. The ratio of TGF-β isoforms 1 and 3 are considered to be a more important factor in scarless wound healing than absolute amounts of any individual form (Hantash *et al.* 2008). Regarding TGF-β isoform ratios in the present study, there is a general increase in the ratio of TGF-β1:TGF-β3 at the 4 time points studied, possibly owing to the more rapid decline of TGF-β3 compared to TGF-β1 over that time period for all treatments/manipulations (Figure 3.10 A & B).

The ratio of decorin to TGF-β1 expression can be indicative of the levels of decorin available to neutralise TGF-β1. There is no significant difference in the ratio of decorin to TGF-β1 over the time points studied between any combinations of treatment/manipulation, except on day 4 for saline-treated uninjured skin. The ratio for this time point shows 4-fold more decorin than TGF-β1 expression (Figure 3.10 C & D). This could be explained by the significant lag in TGF-β1 expression for uninjured skin observed on this day (see Figure 3.6 B).

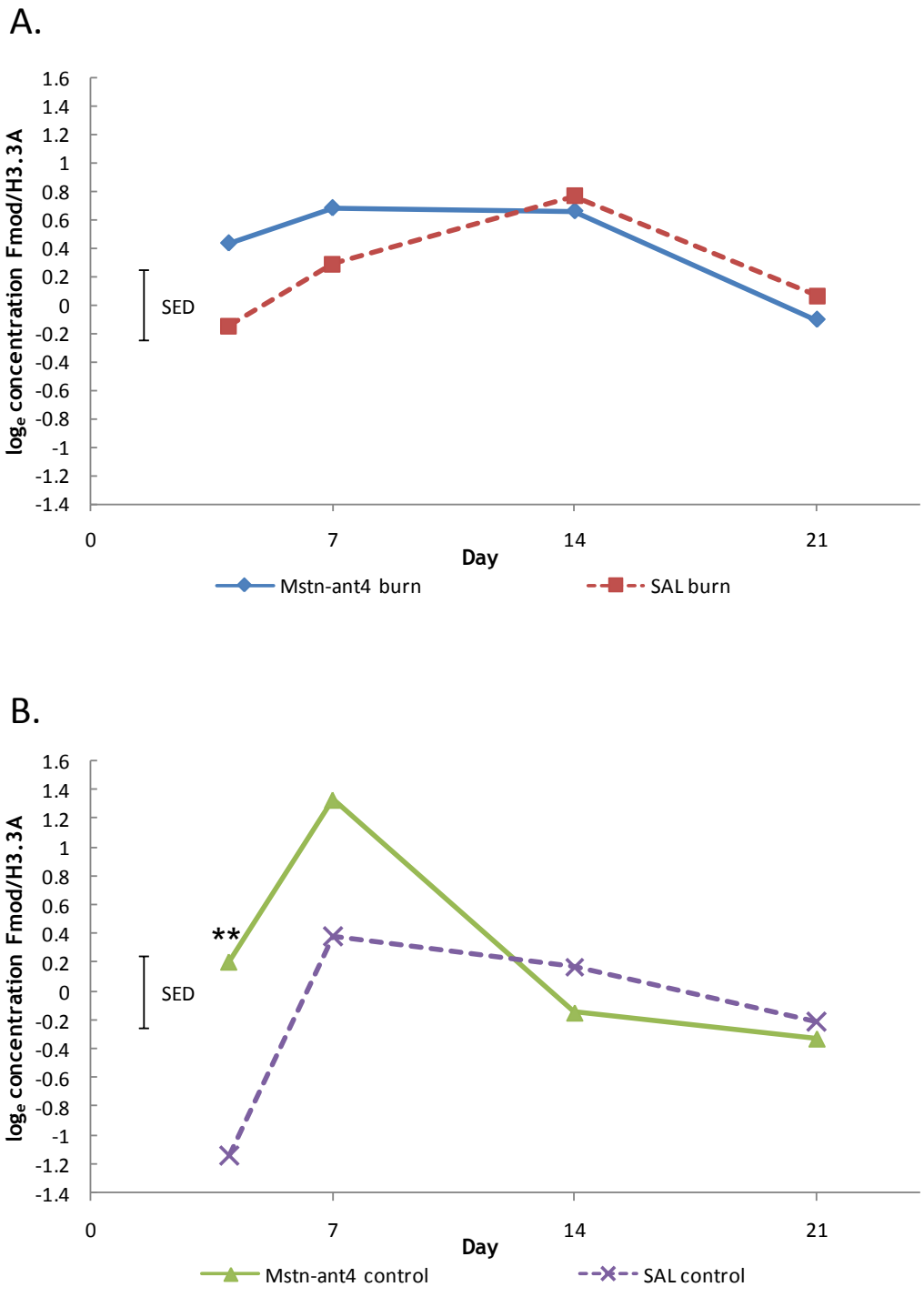



**Figure 3.9: Fibromodulin expression**

**A**: Fibromodulin (Fmod) expression in burned skin for each treatment displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A. **B**: Fibromodulin expression in control skin for each treatment displayed as $\log_e$ concentration values relative to house-keeping gene Histone H3.3A. Error bar for both plots represents pooled standard error of the difference between means (SED). For all points, n=6 except SAL day 7 where n=5.

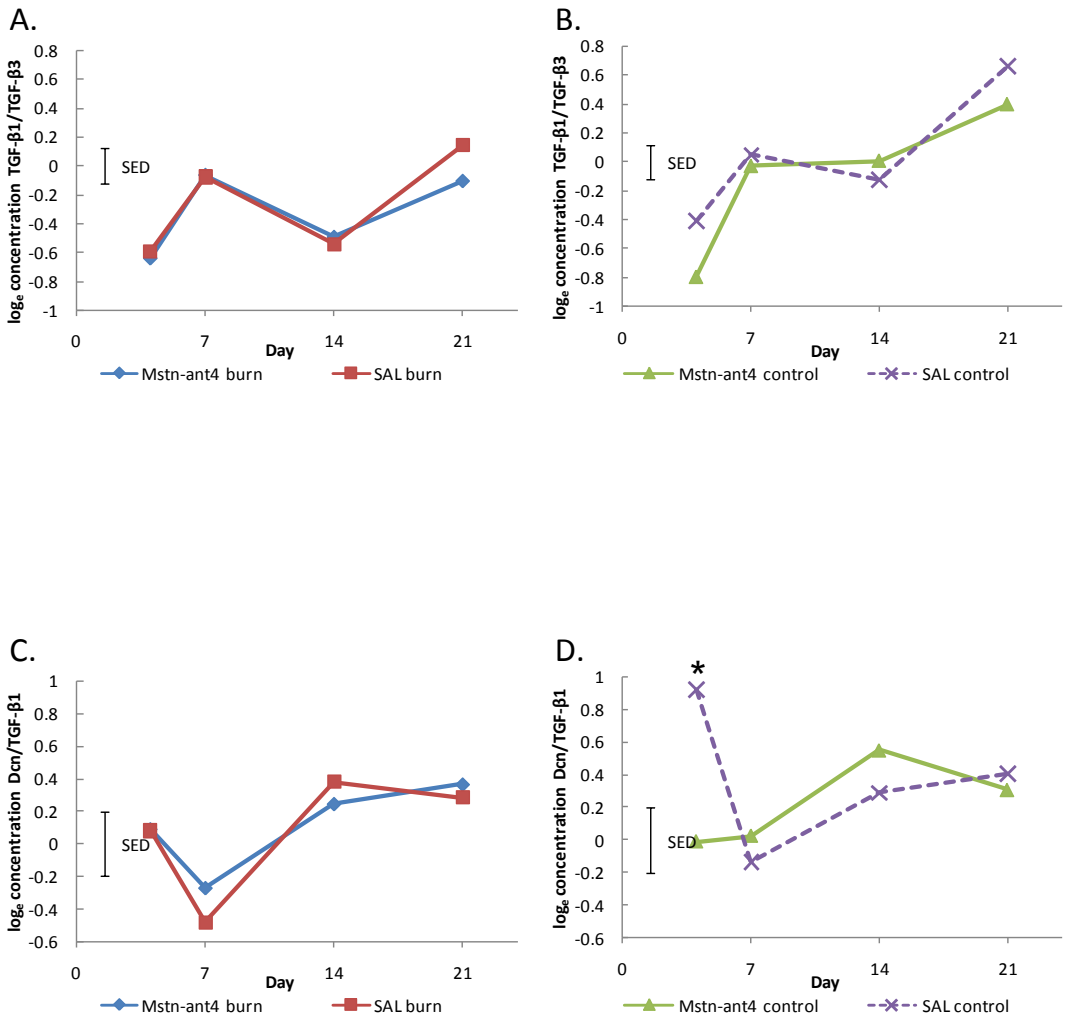



**Figure 3.10: Gene expression ratios**
**A** & **B**: TGF-β1 expression as a ratio to TGF-β3. $Log_e$ values are used for ratio calculation. Error bar represents pooled standard error of the difference between means (SED). For all points, n=6 except SAL day 7 where n=5. **C** & **D**: Decorin expression as a ratio to TGF-β1. $Log_e$ values are used for ratio calculation. Error bar represents pooled standard error of the difference between means (SED). For all points, n=6 except SAL day 7 where n=5.

## 3.5 Histology

The devastating effect of the burn injury can be seen clearly by trichrome staining of sections. This devastation, it would appear, can cause various patterns of regeneration. This variation made it difficult to make consistent measurements, particularly with respect to wound area. Re-epithelisation occurred sometime between days 7 and 14 for both treatment groups and therefore could not be assessed, as no histological data was available between these time points. Therefore, histological analyses were restricted to days 14 to 28, as all wounds had fully healed by day 35. The measurements made on sections from days 14 to 28 included measurements of wound depth or granulation tissue thickness, wound width or granulation tissue width, and epidermal thickness over the wound area.

Wound depth or granulation tissue thickness was significantly less in Mstn-ant4-treated wounds ($p=0.031$, Figure 3.11 A) on day 14 but this significance disappeared on subsequent days. There was also significance in wound or granulation tissue width measurements for day 14 ($p=0.003$) where Mstn-ant4-treated wounds were wider (Figure 3.11 B), but again results were not significant on subsequent days. Wound width measurements were made as an indication of wound contraction as in Fukai *et al.* (2005). This revealed that Mstn-ant4-treated wounds had contracted to a lesser degree than saline-treated wounds. It should be noted that values could not be obtained for all skin samples due to the quality of some tissue sections. The wounds of three animals were deemed to be fully healed by day 28 (one Mstn-ant4-treated and two saline-treated) and were not included in the analysis for day 28. One specimen was removed from the study at day 14 due to an unusual healing pattern; this was possibly the result of an infection which presented with granulation tissue development outside the original wound area. Figure 3.12 shows representative skin sections from the time points studied for each treatment.

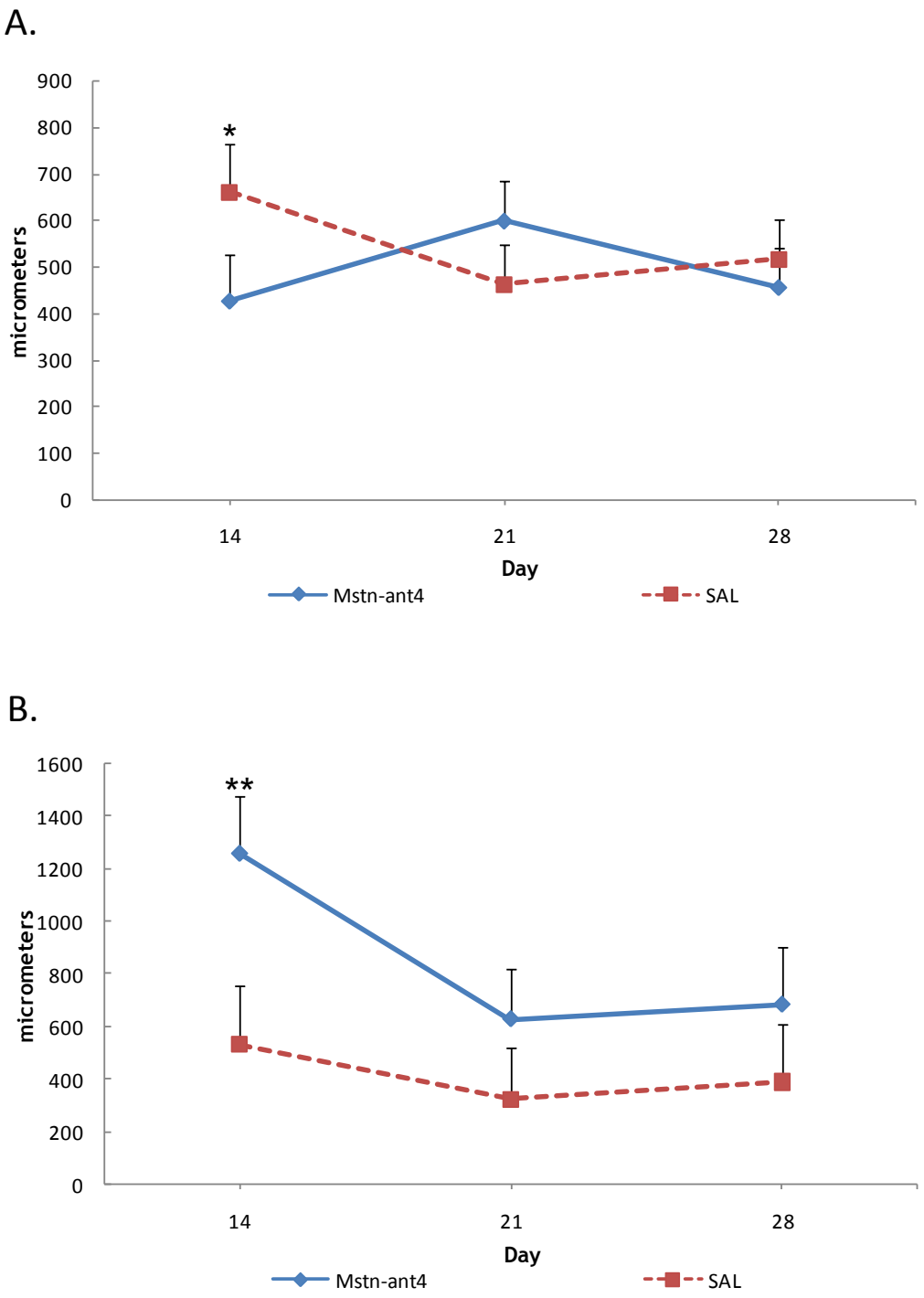
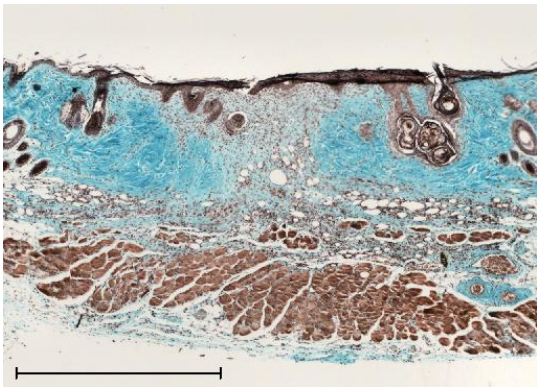
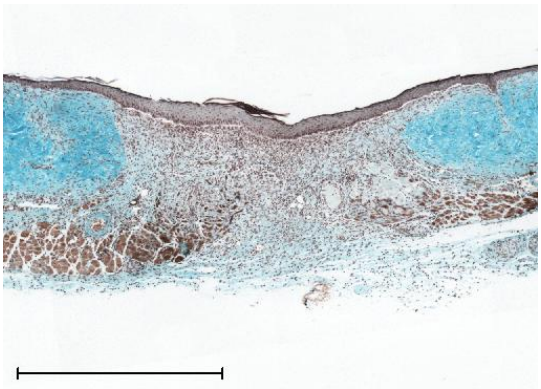


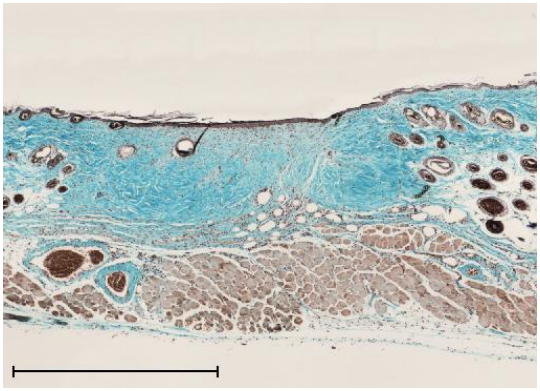

**Figure 3.11: Histology – granulation tissue thickness and width**
**A**: Granulation tissue thickness from days 14 – 28 measured in micrometers using Image-Pro Plus. **B**: Granulation tissue width from days 14 – 28 measured in micrometers using Image-Pro Plus. Errors for both plots represent individual SEDs. For both plots, day 14: Mstn-ant4 n=5, SAL n=4; day 21: Mstn-ant4 n=5, SAL n=4; day 28 Mstn-ant4 n=4, SAL n=3.



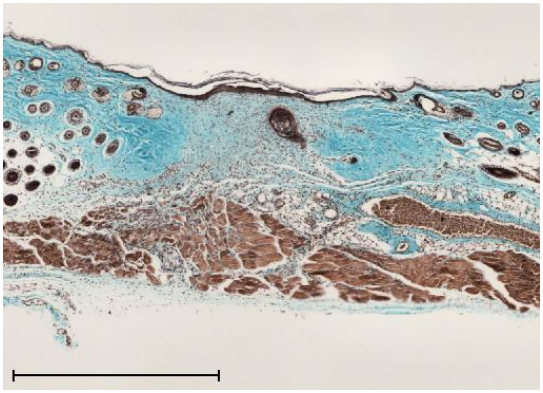
Day 14 - Saline



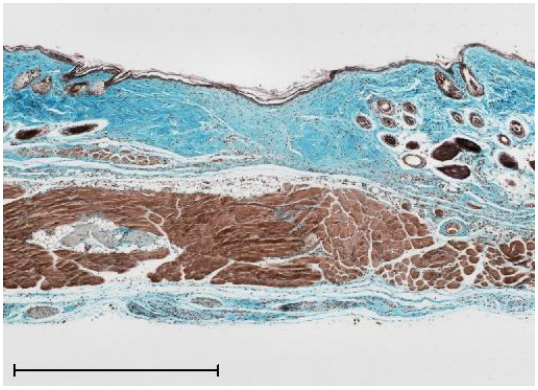
Day 14 - Mstn-ant4



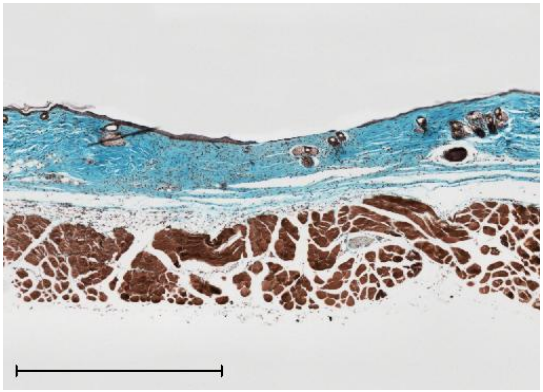
Day 21 - Saline



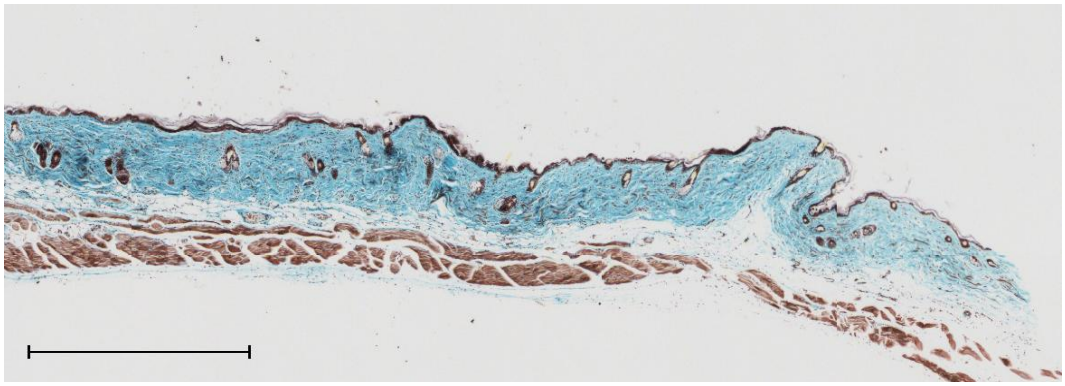
Day 21 - Mstn-ant4



Day 28 - Saline



Day 28 - Mstn-ant4



Uninjured skin

**Figure 3.12: Representative images of trichrome stained sections; days 14-28.** Sections stained using modified Gomori's Trichrome showing collagen stained green, muscle and keratin (epidermis) red and nuclei dark red/black. Differences in wound contraction more pronounced on day 14. Scale bar = 1000 µm

# 4 DISCUSSION

Solid evidence for the involvement of myostatin in the biology of skin is non-existent. A PubMed search which combines the search terms 'myostatin' and 'skin' retrieved two results, both of which refer to fish (Radaelli *et al.* 2003; Patruno *et al.* 2008). Fish usually have two myostatin genes which are expressed in many tissues, but as yet are functionally poorly defined (Rodgers *et al.* 2007). In 1997 the function of GDF-8 was discovered and appropriately named myostatin (McPherron *et al.* 1997). Like myostatin, many genes are given names according to their initially discovered function. Often this has meant that almost all successive research has been limited to the gene's function as dictated by the name. For some genes, other, sometimes more important functions, are subsequently discovered, regularly leading to confusion, and occasionally a name change is required. Therefore myostatin could have additional functions other than those already documented and reviewed by Bass *et al.* (1999), Sharma *et al.* (2001), Lee (2004), Patel and Amthor (2005), and McFarlane *et al.* (2008). The therapeutic potential of blocking myostatin to alleviate muscle wasting disorders has also been specifically reviewed (Tsuchida 2004; Bishop *et al.* 2005; Patel and Amthor 2005; McFarlane *et al.* 2008). Consequently, the main hypothesis of this study; that antagonism of myostatin can improve burn-wound healing in skin, falls outside the scope of research on myostatin to date. However, the formulation of this hypothesis is founded on several converging lines of evidence which are worthy of the brief discussion that follows.

Although myostatin's main organ of expression is skeletal muscle, one of the unsolved issues with myostatin is the extent and locality of its distribution in active form. Inactive (latent) myostatin is present in serum (Patel and Amthor 2005) and recent evidence shows that the active form of myostatin is secreted into the circulation (Hosoyama *et al.* 2006). This is underlined by observations that circulating myostatin is increased in aged men and women suffering sarcopenia (Yarasheski *et al.* 2002) and in HIV-infected men with muscle wasting (Gonzalez-Cadavid *et al.* 1998). This, together with the fact that cachexia can be induced by systemically administering myostatin (Zimmers *et al.* 2002), reveals a hormone-like endocrine behaviour for circulating myostatin.

The role that myostatin plays in tissues other than muscle hasn't yet been uncovered (Bishop *et al.* 2005). It is well known that myostatin signals through ActRIIB receptor together with ActRIB/TβRI (ALK4/ALK5), and it is likewise

established that all known activin receptors are expressed in both the mesenchymal and epithelial compartments of normal and wounded skin (Wankell *et al.* 2001). Therefore, with the presence of active form myostatin in the circulation, and the availability of receptors in skin, there is substantial potential for an important function for myostatin in skin.

Myostatin is now also recognised as a factor in wound healing. This has been reviewed in chapter 1 so a brief summary will suffice here. Myostatin negatively regulates adult myogenesis and muscle regeneration from injury (Kirk *et al.* 2000), and can impair macrophage and myoblast chemotaxis (McCroskery *et al.* 2005), but is chemotactic for fibroblasts *in vitro* (Kambadur *et al.* 2006a). Enhanced regeneration and reduced fibrosis are seen in myostatin-null mice (McCroskery *et al.* 2005) and in mice treated with a myostatin antagonist after muscle injury (Kambadur *et al.* 2006c; Siriett 2007; Nozaki *et al.* 2008). In the absence of myostatin, a resulting increase in decorin leads to reduced scar tissue formation after muscle injury, suggesting that myostatin negatively regulates decorin expression (McCroskery *et al.* 2005). Decorin is known to neutralise TGF-β, and in a similar way decorin binds and neutralises myostatin sequestering it into the ECM (Miura *et al.* 2005), as well as directly affecting its activity (Kishioka *et al.* 2008). Myostatin directly stimulates muscle fibroblast proliferation and expression of extracellular matrix proteins (Li *et al.* 2008). In addition, muscle fibroblasts express functional myostatin, which signals through the ActRIIB receptor inducing fibroblast proliferation via the canonical Smad pathway (Li *et al.* 2008).

Apart from these studies, work completed in this lab has specifically established the efficacy of Mstn-ant4. This particular myostatin antagonist has been shown to positively affect myoblast proliferation, as well as chemotaxis of macrophages, myoblasts, muscle fibroblasts, and dermal fibroblasts. It also affects myostatin signalling by competitive inhibition (Kambadur *et al.* 2006a; Kambadur *et al.* 2006b; Kambadur *et al.* 2006c).

The findings mentioned above provide a strong rationale for research focussing on the role of myostatin in skin wound healing. A further reason for the research was to specifically test whether a myostatin antagonist could improve skin wound healing for the purpose of satisfying wound healing and myostatin antagonist patents. The reason for this was to establish the commercial value of myostatin antagonist Mstn-ant4 developed at AgResearch for Orico Ltd. This research was conducted by analysing the effect of antagonising myostatin via systemic

administration of a Mstn-ant4 as a means to improve skin healing from burn wounds by reducing scar tissue formation.

Currently, there are no prescription therapeutics available for the prevention or treatment of dermal scarring, and existing treatments are unreliable and unpredictable (Ferguson and O'Kane 2004; Hantash *et al.* 2008). Wound healing is an intricate and highly orchestrated process, involving complex interplay between cell influx into the wound bed, environmental factors in the surrounding skin, and various cytokine mediators (Aarabi *et al.* 2007). This is likely a good reason why there has been limited success in the treatment of scarring. Therefore the task of identifying possible anti-scarring treatments can be extremely challenging.

Strategies to reduce scarring have focused on inhibiting collagen deposition, either directly by impeding synthesis, or indirectly by blocking TGF-β pathways (Tandara and Mustoe 2008). A discussion of the myostatin antagonist effect on collagen deposition and TGF-β expression will be followed by a discussion of its effects on other wound healing genes of interest such as SLRPs, and finally some ideas on a possible mechanism of action for the antagonist.

## 4.1 Effect of myostatin antagonist administration

### Collagen

Collagen type I predominates in normal human skin and exceeds collagen type III by a ratio of at least 4:1. During wound healing, this ratio decreases to 2:1 because of an early increase in the deposition of collagen type III (Fathke *et al.* 2004). These ratios are usually obtained by measuring the two different collagen protein types in a sample. In this study, total collagen was quantified using the hydroxyproline assay. Although this method does not distinguish between collagen types I and III, it is employed by most wound healing/scarring studies together with collagen mRNA expression data. Collagen expression measurements by RT-PCR represent amplified mRNA of collagen types I and III, and therefore correspond to gene expression levels of respective collagens. When viewing gene expression and protein data together, it should be acknowledged that gene expression doesn't necessarily correspond to protein translation and that amplified mRNA may not reflect actual mRNA because of assumptions about PCR efficiency (Wadenback *et al.* 2005). However, many

studies validate the use of collagen expression data to discuss the relative proportions of the collagen types, as is the case with this study.

Type III collagen is seen as important for rapid tissue regeneration because of its ability to form intermolecular disulfide bridges which are assumed to be favourable to tissue regeneration (Masuda et al. 2002). Type III collagen is therefore considered a marker of early wound repair processes (Davidson 2001). As healing proceeds, the proportion of type I collagen increases with a corresponding decrease in the proportion of type III collagen, proteoglycans, and water (Baum and Arpey 2005). This occurs during the remodelling phase where type III collagen is replaced by type I, (which becomes divalently and trivalently cross-linked (Barnard *et al.* 1987)), restoring the normal dermal collagen profile (Kwon et al. 2007). Remodelling can take up to a year or more (Li et al. 2007), but it is assumed that the COL1:COL3 ratio normalises quite rapidly during the remodelling phase (Robins et al. 2003). In contrast, burn-injured dermal tissue contains significantly increased amounts of collagen III (Ulrich *et al.* 2002), and this may cause delays in normalisation of the collagen ratio. Garcia-Filipe and colleagues (2006) suggest that collagen III may be a marker for the extent of fibrosis by citing that the presence of a relative increase in collagen III is often associated with fibrosis and excessive scarring in many diseases. Garcia-Filipe *et al.* (2006) coined the term “fibrotic index” where as a ratio, collagen III production is expressed relative to collagen I production. In the present study, there appears to be no significant difference in either collagen I or collagen III expression respectively between Mstn-ant4-treated and saline-treated groups. However when collagen I and III are viewed proportionally to each other as a ratio (fibrotic index), an interesting trend emerges. While initially there is no difference in COL1:COL3 between saline and Mstn-ant4-treated mice, by day 21 the ratio is significantly lower in Mstn-ant4-treated burns than saline-treated burns. This indicates that collagen III expression remains higher relative to collagen I expression for longer in Mstn-ant4-treated healing burn wounds. Therefore, according to Garcia-Filipe’s *et al.* (2006) fibrotic index, Mstn-ant4-treated burn wounds may be at higher risk of progressing to a fibrotic state. However, this assumption is controversial, because of the role of collagen III in scarless foetal wound healing.

Studies in foetal wound healing suggest that a difference in collagen-type deposition contributes to scarless healing. Collagen I is the main type in both foetal and adult skin, but foetal skin contains a higher proportion of type III than adult skin. In human foetal skin, collagen type III comprises 30% to 60% of the

total, whilst in adult skin it comprises 10% to 20% (Bullard *et al.* 2003; Baum and Arpey 2005). During development, the COL1:COL3 ratio in foetal skin increases approaching that of adults, correlating with the transition from scarless repair to scar formation after wounding (Bullard *et al.* 2003). In early adult wound healing, type III collagen is increased to about 30% (Baum and Arpey 2005), but in contrast, foetal wounds comprise significantly more type III than type I collagen (Beanes et al. 2003). The strength of type I collagen fibres relies on the formation of covalent cross-links. A predominance of non-cross-linked collagen type III in early foetal wounds might allow for a more flexible matrix, contributing to scarless repair in this manner; but it remains unknown whether scar formation is caused by a higher proportion of type I cross-linked collagen or whether a higher proportion of type I cross-linked collagen is caused by scar formation (Bullard *et al.* 2003). When collagen I:III ratios are viewed in this light, it seems that a higher proportion of type III collagen may contribute to reduced scarring, and this supports the proposition that Mstn-ant4 treatment may result in reduced scarring.

In view of the total collagen estimation data, there appeared to be a non-significant trend from day 14 to 21 where total collagen amounts were lower in Mstn-ant4-treated burn skin than saline-treated skin. However a reversal of this trend was seen on day 28, where the opposite effect was seen at a significant level. It might be tempting to suggest that the antagonist was causing the potentially fibrotic effect seen on day 28, but since administration of the antagonist had ceased by day 15, it is more likely that the result from day 28 reflects a rebound compensatory effect which resulted in extra collagen being laid down to balance the previous deficit caused by the antagonist. By day 46, there was no significant difference in collagen amount in burned skin of either treatment. This observation suggests that a longer treatment regimen of the Mstn-ant4 antagonist should be implemented.

Histologically, new collagen deposition resulting in scar tissue could be identified by a deeper degree of staining of the affected area as well as a general absence of cutaneous structures, at later stages of healing. In the earlier stages of healing, the injured area was dominated by granulation tissue, which stained with a reduced intensity to the surrounding intact ECM. Granulation tissue was gradually replaced by collagen fibres up to day 28, after which the staining intensity of the affected area was comparable to normal dermis. The area of granulation/scar tissue is a combination between collagen deposition and wound contraction. Therefore, a small scar area is likely due to increased wound contraction rather than decreased collagen deposition, which is why hydroxyproline and collagen

expression data are important. There was large within-group variation in many of the histological measurements, which is a reflection of the magnitude of tissue damage caused by the burn injury, resulting in variation in regeneration patterns from animal to animal.

## TGF-β

TGF-β1 affects all phases of healing and is clearly pro-fibrotic when added exogenously to foetal and adult wounds (Bullard *et al.* 2003). Three isoforms of TGF-β have been identified in mammals. The most abundant isoform, TGF-β1, is known to cause an increase in collagen gene expression and protein deposition (Beanes *et al.* 2003). TGF-β1 also reduces matrix degradation through inhibition of MMPs via increased expression of TIMPs (Bullard *et al.* 2003). Furthermore, TGF-β1 is chemotactic for fibroblasts and macrophages and enhances angiogenesis (Sporn and Roberts 1992). In this study, expression levels of TGF-β1 between saline and Mstn-ant4-treated burns were indistinguishable over the time points studied, a result which leaves little to be discussed except that it fits well with the observation that there were no significant differences in collagen expression either, given TGF-β1's influence on collagen expression. However, there was a significant increase in TGF-β1 response in saline-treated uninjured skin compared to Mstn-ant4-treated skin on day 4. Whether this result has an effect on wound healing, will be discussed later.

In contrast to TGF-β1, TGF-β3 has been suggested as an anti-scarring factor (see Table 4.1). Neutralisation of TGF-β1 and TGF-β2 has a similar effect to exogenous addition of TGF-β3 to cutaneous rat wounds in reducing scarring (Shah *et al.* 1995). Another of TGF-β3's functions might include the inhibition of inflammation (Rundle *et al.* 2005). Even though TGF-β1 is also present in foetal wounds, albeit transiently, scarless repair still occurs (Rolfe *et al.* 2007). This suggests that the relative proportion of isoforms is more important than the absolute amounts of any one form (Dang *et al.* 2001; Colwell *et al.* 2003). One of the reasons for a difference in expression profiles of TGF-β isoforms is that TGF-β3 is produced mainly by keratinocytes and fibroblasts, whereas TGF-β1 and TGF-β2 are initially sourced from degranulating platelets, and later from monocytes, macrophages, and fibroblasts (Ferguson and O'Kane 2004; Miller and Nanchahal 2005). Interestingly, the clinical potential of TGF-β3 as an anti-scarring agent has been recognised and it has recently entered phase II clinical trials in humans for scar prevention and/or reduction (Hantash et al. 2008).

**Table 4.1: Summary of the evidence for the central role of TGF-β3 in scarring**

- present at high levels in developing skin and in embryonic wounds that heal with no scar
- present at low levels in adult skin and wounds that scar
- induced late in adult wound healing when levels of TGF-β1 start to fall
- neutralisation of TGF-β3 in adult wounds makes the scar worse
- addition of TGF-β3 to adult wounds reduces or eliminates scarring
- genetic deletion of TGF-β3 causes scarring following embryonic wounding (litter mate +/+ embryos heal with no scar)

(from Ferguson and O'Kane 2004)

In the present study, like TGF-β1, the expression of TGF-β3 showed little variance between treatments in burned skin. However, also like TGF-β1, the significant (p=0.001) increase (6.1-fold) in Mstn-ant4 treated skin was seen in uninjured skin on day 4. Because TGF-β3 is expressed at a higher level than TGF-β1 (6.1 vs 4.1-fold), the ratio of TGF-β3:TGF-β1 is important when considering whether TGF-β3 is going to have any anti-scarring effect. In this study, the relative proportion of TGF-β3 is higher than TGF-β1 at day 4 in uninjured skin of Mstn-ant4-treated mice, although this difference (p=0.1) is not considered significant.

## Decorin

Decorin is the predominant proteoglycan in skin (Sayani *et al.* 2000) and is therefore worthy of study in any wound healing context. Indeed, decorin has been implicated as an anti-fibrotic factor in wound healing and therefore, its up-regulation in this study would be indicative of an anti-fibrotic activity for the myostatin antagonist. It is well-established that decorin binds TGF-β via its core protein (Yamaguchi *et al.* 1990) and this binding neutralises all three TGF-β isoforms (Hildebrand *et al.* 1994). It likely acts by sequestering TGF-β, while itself is bound to the ECM (Markmann *et al.* 2000). This appears to be a legitimate function of decorin as part of a negative feedback loop that regulates TGF-β activity, and, since the binding of decorin to TGF-β is reversible, any TGF-β bound to it in tissue forms a reservoir of the growth factor (Ruoslahti and Yamaguchi 1991). The TGF-β-binding property of decorin has been used to prevent fibrosis in several studies (Border *et al.* 1992; Giri *et al.* 1997; Fukushima *et al.* 2001; Shimizu 2001; Sato *et al.* 2003; Li *et al.* 2004; Grisanti *et al.* 2005; Huijun *et al.* 2005). Interestingly, decorin also binds myostatin and suppresses its activity in myogenic cells (Miura *et al.* 2005; Kishioka *et al.* 2008), and myostatin-

null mice show increased expression of decorin mRNA, reduced fibrosis and improved healing (McCroskery *et al.* 2005). In addition, decorin has been shown to up-regulate the expression of follistatin, a natural antagonist of myostatin (Zhu et al. 2007). Consequently, it is possible for any anti-fibrotic effect of antagonising myostatin to be potentiated by an up-regulation of decorin.

In this study the greatest effect of the antagonist on decorin expression was seen on day 7 where it induced a significant 2.1-fold increase ($p=0.007$, ITP t-test) in uninjured skin and a 1.4-fold increase in burn skin, which bordered on significance ($p=0.056$). The effect observed in uninjured skin would seem to correlate with the increase in water content of uninjured control skin between days 7 and 21.

Even though studies of foetal wound healing reveal that decorin is down-regulated during the gestational age when scarless healing occurs (Beanes *et al.* 2001), decorin's importance in adult wound healing for preventing fibrosis should not be underestimated (Yamaguchi *et al.* 1990; Border *et al.* 1992; Costacurta *et al.* 2002; Jahanyar *et al.* 2007; Zhu *et al.* 2007). Furthermore, hypertrophic scars exhibit a marked lack of decorin (Scott *et al.* 1996; Sayani *et al.* 2000), and therefore restoring decorin levels could be one possible application of the antagonist treatment, as decorin treatment inhibits cell proliferation and down-regulates TGF-β1 production in hypertrophic scar fibroblasts (Zhang *et al.* 2007). In addition, decorin's appearance in burn wounds is delayed (Sayani *et al.* 2000), so the early induction of decorin in Mstn-ant4-treated skin compared with saline-treated skin could be another important function of Mstn-ant4 in burn wounds.

One striking observation with all gene expression data is that where significant increases in gene expression occured in the early stages of wound healing in antagonist treated skin, they had largely disappeared by day 14. During the trial, mice were injected with either saline or Mstn-ant4 on days 1, 3, 5, 7, 10, and 15. It is therefore noteworthy that during the first week after wounding, injections were more frequent than after day 10. Therefore, the decrease in frequency of injection coincides with a loss of the effect that is seen by antagonist administration on earlier time points when injections were more frequent. Consequently, if the frequency of treatment given in the first several days was maintained for several weeks, then the significant differences observed in the early stages of healing may also be maintained for longer.

## Fibromodulin

Post-translational modifications of fibromodulin clearly distinguish it from decorin (Hocking *et al.* 1998). Both decorin and fibromodulin bind to active TGF-β 1, 2 and 3 via their core proteins, however, the chondroitin/dermatan sulfate chains of decorin appear to hinder this binding, making TGF-β1 binding to fibromodulin more effective than to decorin within the tissue (Hildebrand *et al.* 1994; Burton-Wurster *et al.* 2003). In addition, fibromodulin can bind (though slightly) TGF-β1 precursor whereas decorin cannot (Hildebrand *et al.* 1994). In contrast to decorin, fibromodulin decreases with increasing gestational age in foetal skin. A study in rats by Soo et al. (2000) clearly outlines contrasting aspects between decorin and fibromodulin. They observed that decorin was up-regulated during adult wound healing whereas fibromodulin expression was initially down-regulated. They also observed that fibromodulin is significantly up-regulated in gestation day-16 foetal wounds, but not in gestation day-19 wounds, which is generally observed to be the transition time point between scarless and scar-forming foetal wound healing in rats. They also note that scarless foetal wound healing occurs despite the presence of TGF-β and that the observed down-regulation of fibromodulin in adult wound healing allows increased activity and auto-induction of TGF-β, which may lead to scar and/or fibrosis. These observations strongly implicate fibromodulin as an anti-fibrotic agent. There are a few findings that may shed light on fibromodulin's anti-fibrotic properties.

TGF-β1 is rapidly induced in mouse embryo (E11.5) wounds, but is also rapidly cleared within 18 hours (Martin *et al.* 1993). In association with elevated fibromodulin levels, this may decrease overall TGF-β bioavailability (Soo *et al.* 2000; Stoff *et al.* 2007), leading to decreased ECM deposition and therefore decreased scarring. Stoff *et al.* (2007) reported that over-expression of fibromodulin induced a decrease in expression of TGF-β1 and TGF-β2 precursor proteins, and an increase in expression of TGF-β3 precursor protein and TGF-β type II receptor in cell culture. As a result, they showed that fibromodulin over-expression improves wound healing *in vivo*. Moreover, while fibromodulin can bind activated TGF-β and suppress TGF-β activity, with fibromodulin exhibiting the greatest affinity for TGF-β1, as well as the latent TGF-β1 precursor, fibromodulin appeared to be a less effective binder of TGF-β3 than decorin (Hildebrand et al. 1994). This is significant because of TGF-β3's association with scarless foetal wound healing. It is also significant because, assuming most of the TGF-β1 mRNA was translated to protein, TGF-β1 protein could be sequestered by fibromodulin in the ECM, thereby neutralising its fibrotic ability.

In the present study, there were observed increases in fibromodulin expression in both burned and uninjured Mstn-ant4 treated skin at day 4, which remained significantly increased by day 7 ($p=0.01$, individual t-test). Again, like decorin and TGF-β, differences in fibromodulin expression had disappeared by day 14 suggesting that while the antagonist is available in the circulation, it can induce favourable gene expression patterns for improved wound healing.

## Skin hydration

The observation that the skin of antagonist-treated mice contained a significantly higher percentage of water, as determined by weight, up to one week after the treatment regime ended is strong evidence for the positive effect of the antagonist on skin hydration levels, and once again suggests that a longer injection period would allow this effect to persist. It seems intuitive that proper hydration is essential for proper wound healing, but very little research has been conducted to support it. Adequate systemic hydration is important for maintaining blood volume and pressure and therefore sufficient nutrient and oxygen delivery. But at a local level, it may be that increased water content is the secondary result of the presence of other essential skin constituents for wound healing. The fluid content of skin is determined by proteoglycans, and their polysaccharide side chains called GAGs. As mentioned in the section 1.1.2, GAGs are strongly hydrophilic. Their strong negative charge attracts osmotically active Na+, causing large amounts of water to be drawn into their structure (Chakravarti *et al.* 1998; Schultz *et al.* 2005). Hyaluronate is the most abundant GAG in skin and is important in wound healing processes, such as inflammation, for its role in stimulating inflammatory cytokines and cellular infiltration into the provisional wound matrix, as well as stimulatory effects of angiogenesis and re-epithelialisation (Chen and Abatangelo 1999). Foetal skin is known to have a high proportion of hyaluronate in place of collagen and fibroblasts are also known to have increased density of hyaluronate cell surface receptors, which may be linked to foetal skin's scarless healing ability (Manuskiatti and Maibach 1996). Although hyaluronate content was not a subject of this study, it could be an interesting future direction. On the other hand, the proteoglycan decorin was a subject of this study and it too has water retentive ability. As was shown, decorin was significantly up-regulated in antagonist-treated skin on day 7. Subsequent protein analysis may have confirmed the presence of increased amounts of actual decorin protein, and this may have correlated with the elevated water content observed in Mstn-ant4-treated skin, which persisted until day 21, while differences in decorin expression levels seemed to be gone by day 14.

Fibromodulin, which is similar to decorin, was also significantly up-regulated on the seventh day following injury in Mstn-ant4-treated skin. The observed water retention is likely a downstream effect of both decorin and fibromodulin expression. In further support of a role for fibromodulin in tissue hydration, it has been shown to determine stroma matrix structure and fluid balance in experimental carcinoma (Oldberg *et al.* 2007).

Epidermal hydration is determined by the intracellular water content of keratinocytes. Aquaporins are proteins that facilitate water transport across cell membranes (Dumas *et al.* 2007) and are believed to be important in wound healing, by facilitating cell migration, and enhancing keratinocyte proliferation and differentiation (Boury-Jamot *et al.* 2009). Tandara and colleagues (2007) have shown that hydrated keratinocytes reduce collagen synthesis by fibroblasts via paracrine mechanisms in co-culture. If this effect occurs *in vivo*, the result would be decreased collagen deposition and therefore reduced scarring (Tandara and Mustoe 2008). Because hydration modifies the levels of cytokines keratinocytes secrete, it has been shown by the same lead author in another study that the reduced collagen content of the keratinocyte-fibroblast co-culture was likely due to a large increase in MMP activity (Tandara *et al.* 2004). Keratinocyte hydration also seems to be important for other aspects of wound healing. Re-epithelialisation is aided by hydration via keratinocyte differentiation with faster skin barrier reformation and recovery (Dumas *et al.* 2007). It is likely that in the present study, increased skin hydration in Mstn-ant4-treated mice was due to increased secretion of proteoglycans and their associated GAG chains. However, one could speculate that a well-hydrated dermis could also influence the hydration of the epidermis, and produce the favourable effects on fibroblasts seen when well hydrated keratinocytes are present.

It has been observed that major changes in the properties of skin occur as a result of aging, and this in turn has a major effect on wound healing. Among those changes are: decreased keratinocyte maturation, decreased fibroblast proliferation, flattening of dermis and dermal/epidermal junction, reduced sebaceous and sweat gland activity, and decreased vascularity (Worley 2006). A decrease in dermal hydration results either directly or indirectly from these changes. It has also been established that an age-related change in proteoglycans occurs. Carrino and others (2000) report that as a function of age, there is a decrease in the proportion of large chondroitin sulfate proteoglycans (versican) and an increase in the proportion of small dermatan sulfate

proteoglycans (decorin). Interestingly, they elaborate on decorin by documenting that foetal decorin is slightly larger than adult decorin and that a proteoglycan which is smaller than decorin, but has the same terminal amino acid sequence, is abundant in mature skin. They speculate that this decorin analogue may affect normal decorin's ability to modulate collagen fibrillogenesis, which may in turn affect skin elasticity (Carrino *et al.* 2000). In view of this, there could be some beneficial effect for the myostatin antagonist on aging skin. Antagonist-treated skin has significantly higher hydration levels, and this is likely due to up-regulation of proteoglycans such as decorin and fibromodulin. This could restore the balance of normal decorin in aged skin and by improving hydration in the skin of the aged may also elicit noticeable improvements in wound healing. In addition, since it has been shown that antagonising myostatin reduces age-related sarcopenia (Siriett *et al.* 2007), there could be a dual benefit in administering the myostatin antagonist to the aged. Furthermore, poorly hydrated skin seems to be considered by those in the industry as adversely affecting cosmetic appearance, making Mstn-ant4 a potential candidate for cosmetic applications.

## 4.2 Consideration of cumulative evidence

Before proceeding, the body of evidence should be considered in the context of some possible limitations of the study. One of the assumptions, and perhaps a limitation of this study, was the standardisation of the burn injury. There was a certain art in applying and maintaining constant pressure of the burn-causing implement by hand and this invites some human error and variability. With a burn injury, especially in the early stages, the majority of the area will consist of dead, necrotic tissue. Also, the regeneration of the wounded area will result in an induction of a massive gene expression program, on top of which any response to the Mstn-ant4 treatment will need to be detected, which may prove difficult, considering that even genes used as internal controls may vary under these circumstances (McKinnell *et al.* 2005). This may help explain why burned skin showed fewer and smaller significant differences in gene expression between treatments, whereas when there were differences, the significance was greater in uninjured skin at corresponding early stages of healing. For these reasons, it may be appropriate, and even insightful, to consider the genetic expression response to Mstn-ant4 treatment of uninjured tissue, which has not suffered the influence of a burn injury, as homeostatic skin would not be required to alter constitutive gene expression, in contrast to the enormous physiologic demand to which burn injured skin is prone. Therefore, if it was observed that certain genes important for wound

healing responded to Mstn-ant4 treatment in uninjured skin near the burn site, this may be informative of what is occurring at the burn site but which may be somewhat masked by the effect of the burn wound. Therefore, a future approach might be to excise the dead necrotic centre of the burn wound and analyse the tissue immediately surrounding the burn. Histological examination of the burn injury could still be used to ascertain morphological effects of treatment. In the present study, gene expression data from both injured and uninjured skin, together with histological analysis, is used to state the cumulative case for the improvement of wound healing in skin by the administration of a myostatin antagonist.

Of considerable interest are the aspects of the evidence which relate to scarless healing in foetal wound healing studies. Antagonist-treated wounds had a higher proportion of type III collagen expression and a larger induction of fibromodulin and TGF-β3 expression at early time points. This could have been responsible for the significant reduction in wound contraction ($p=0.03$) and reduced granulation tissue thickening ($p=0.003$) seen at day 14. However, unlike foetal wounds, there was an increase in TGF-β1 in Mstn-ant4-treated uninjured skin, although this is inevitable in postnatal wound healing because TGF-β1 is initially released by degranulating platelets, which is lacking in foetal wounds (Ferguson and O'Kane 2004). Also unlike foetal wounds decorin expression was significantly up-regulated in Mstn-ant4 treated skin at day 7. While the TGF-β-binding anti-fibrotic effects of decorin are noteworthy and well documented, there is no evidence to suggest that decorin preferentially binds TGF-β1 over TGF-β3. So the anti-scarring potential of TGF-β3 may be reduced by an increased presence of decorin. Having said this, the up-regulation of decorin expression in Mstn-ant4-treated skin is still important because of its ability to bind and inhibit both TGF-β1 and myostatin directly. Therefore, Mstn-ant4 may antagonise myostatin directly, and via decorin up-regulation, may cause further indirect antagonism of myostatin, as well as indirect antagonism of TGF-β1. Myostatin may also be further antagonised by decorin's ability to cause up-regulation of follistatin.

The positive effect of the myostatin antagonist on body weight recovery, and skin hydration were unexpected, though significant findings of this study, because of their importance for wound healing. Another significant observation was that in all gene expression data and histology measurements where a significant effect of Mstn-ant4-treated skin was observed, this subsequently disappeared following a change in the frequency of, or complete withdrawal of treatment. This is evidence

that Mstn-ant4 was causing the observed effects. A summary of the evidence for Mstn-ant4 treatment to improve wound healing in skin is given in Table 4.2.

**Table 4.2: Summary of effects seen in Mstn-ant4 treated animals which are favourable to improved wound healing**

- Increased decorin expression
- Increased fibromodulin expression
- Increased TGF-β3 expression
- Decreased COL1:COL3 expression ratio
- Increased skin hydration
- Decreased time until body weight recovery
- Decreased granulation tissue contraction
- Decreased granulation tissue thickness

## Possible mechanisms

There are two main possibilities for a general mechanism. The first and most obvious mechanism to consider is that myostatin, and therefore the antagonist, has some direct effect on wound healing in skin. As in muscle, it may have some effect on macrophage and fibroblast chemotaxis and/or proliferation. The other possibility is that myostatin has some indirect effect on wound healing in skin. Since lack of myostatin results in an increase in decorin (McCroskery *et al.* 2005), this could improve wound healing via an effect on TGF-β. Alternatively, the antagonist could be affecting other wound healing related genes indirectly. These possibilities will be discussed further below.

If in this study Mstn-ant4 is antagonising myostatin directly, then it may occur in two possible ways. One possible mechanism is that Mstn-ant4 binds to, and occupies, the ActRIIB receptor in competition with myostatin. The other is that Mstn-ant4 may bind to myostatin, preventing its binding to the receptor. The tertiary structure of the myostatin antagonist Mstn-ant4 is likely to be quite different from mature myostatin, because Mstn-ant4 lacks four of the six cysteines required to form the typical growth factor cystine knot (see Figure 4.1 A). It is not clear whether the cystine knot would be required for receptor binding or for binding to myostatin, although the BMP antagonist Noggin contains a cystine knot topology similar to that of BMPs (Groppe *et al.* 2002). Berry *et al.* (2002) have shown that in Piedmontese myostatin, substitution of cysteine with tyrosine results in modification of the cystine knot structure such that the resulting altered

structure of mature myostatin acts in a dominant negative fashion inhibiting normal myostatin function *in vitro*. A myostatin splice variant (MSV), whose mature form also has fewer cysteines than myostatin, antagonises myostatin in a similar manner (McMahon pers. comm.). Moreover, evidence suggests that MSV can bind receptors and elicit its own unique signal in competition with myostatin (McMahon pers. comm.). Recent work in this lab has attempted to shed light on a possible mechanism of action for Mstn-ant4. Binding assays were conducted which included binding radio-labelled Mstn-ant4 to ActRIIB, and radio-labelled Mstn-ant4 to recombinant myostatin. A strong concentration-dependent effect was seen from the assay with the Mstn-ant4 bound to ActRIIB and it seems that Mstn-ant4 also binds myostatin albeit to a lesser degree of affinity than to ActRIIB (Hennebry and Plummer unpublished observations).

Regarding the possible mechanism for the antagonist binding to and sequestering myostatin, Huang (2002) and colleagues examined the effect of a synthetic TGF-β peptide antagonist which had an amino acid sequence identical to residues 41-65 of mature TGF-β1 (see Figure 4.1 B). Its effect on wound healing was studied in a pig model using a standardised burn injury. They successfully showed accelerated wound healing and reduced scarring. Unfortunately, they did not discuss a possible mechanism of action for the peptide antagonist. The primary structure of the peptide antagonist contains two cysteine residues and it may be more likely to bind TGF-β1 rather than bind to TβRII receptor, because its sequence doesn't contain the putative receptor binding residues R94 and R25 of mature TGF-β1 (Shimanuki *et al.* 2007). Both active myostatin and active TGF-β1 occur naturally as dimers. This dimeric association becomes stable by hydrophobic interactions and is usually further stabilised by an inter-subunit disulfide bridge (Lin *et al.* 2006). TGF-β superfamily members activin, inhibin, and a few BMPs have been shown to form heterodimers, with evidence suggesting many heterodimers have more potent activity than corresponding homodimers (Lin *et al.* 2006). It seems possible that Mstn-ant4 could form a heterodimer with myostatin, and either inhibit myostatin from binding to the receptor, or increase myostatin's receptor binding capacity, and compete with normal myostatin. The primary structure of the Mstn-ant4 antagonist consists of the first 44 amino acids identical to mature myostatin including four cysteine residues at positions 7, 16, 17, and 44 (Figure 4.1 A). Therefore, Mstn-ant4 could bind to mature myostatin in the same way that the TGF-β1 antagonist binds to TGF-β1 shown by Huang *et al.* (2002). There is a small overlap in sequence similarity between the TGF-β antagonist used in Huang *et al.* (2002) and the Mstn-ant4 antagonist used in this study. The

possibility that Mstn-ant4 may also dimerise with TGF-β1 is a possible mechanism of action worth considering.

A comparison of myostatin and TGF-β may be useful in discussing the possibility that Mstn-ant4 could be directly affecting TGF-β. Budasz-Swiderska *et al.* (2005) and Kollias and McDermott (2008) provided constructive reviews of the similarities between myostatin and TGF-β which will be summarised below. While their observations concern the effects of myostatin and TGF-β on myogenic cells, there may be several of these aspects that apply to other types of cells, namely fibroblasts and keratinocytes, which share receptor-signalling properties with myoblasts. It is well noted firstly that TGF-β1 is much more pleiotropic than myostatin, and TGF-β1 activity is very specific to the cellular and intracellular context of the cell. The multi-faceted nature of TGF-β1 seems to be necessary for its *in vivo* function, since it regulates many tissues and processes (Kollias and McDermott 2008) by stimulating growth of the majority of the mesenchymal cells and inhibiting growth of epithelial, lymphoreticular, hematopoetic and endothelial cells. Myostatin is only regarded as an inhibitor of myogenic cell proliferation and differentiation (Budasz-Swiderska *et al.* 2005), and interestingly, TGF-β1 also has these effects on myogenic cells (De Angelis *et al.* 1998). The close structural and processing parallels between Myostatin and TGF-β have been alluded to in Chapter 1 and are further detailed in Figure 4.1. A sequence analysis reveals approximately 51% homology at the nucleotide level (34% at amino acid level, see Figure 4.1 C) between the functional domains of each polypeptide. The receptors and transcription factors used by myostatin and TGF-β1 in signal transduction pathways belong to the same families. Type II receptors for myostatin and TGF-β are ActRIIB and TβRII respectively. ActRIB (also known as ALK 4) is usually cited as the myostatin type I receptor, but both myostatin and TGF-β can signal through TβRI (also known as ALK 5) (Rebbapragada *et al.* 2003; Budasz-Swiderska *et al.* 2005; Kollias and McDermott 2008). The same intracellular Smad proteins are involved in transmitting the signal to the nucleus. Finally Budasz-Swiderska *et al.* (2005) observed that the inhibition of myoblast proliferation and terminal differentiation by myostatin and TGF-β1 occurs through similar molecular mechanisms. In addition to the observations made by the above authors, Zhu *et al.* (2007) suggest that myostatin can also bind the TβRII receptor by observing that because TβRII-soluble receptor antagonises, at least in part, the effect of myostatin on muscle cells. This therefore allows for another possible mechanism of action, where the myostatin antagonist may be able to bind TβRII receptor, and inhibit TGF-β1 signalling, leading to improved wound healing.

If the antagonist is affecting TGF-β signalling, there should be some evidence of this with expression of collagen or even autocrine-directed expression of TGF-β itself. But both TGF-β1 and TGF-β3 are up-regulated by the antagonist at early time points in uninjured skin and there are only minor differences in collagen deposition between treatments, although this could be a limitation of the treatment regimen. Based on findings from this study and others (McCroskery *et al.* 2005; Zhu *et al.* 2007), it seems more likely that antagonism of myostatin causes decorin to be up-regulated, which in turn, sequesters TGF-β1, and this may be reflected in the histological observation that antagonist-treated wounds exhibited wound contraction to a lesser degree than saline-treated wounds. This effect may have been a result of decreased differentiation of fibroblasts into myofibroblasts. However blocking myostatin may also have had a similar effect (Zhu *et al.* 2007), although data from Artaza *et al.* (2008) suggests this is controversial. It should be noted also that decorin can neutralise the stimulatory effects of myostatin in fibroblasts (Zhu *et al.* 2007). Differences in granulation tissue width were significant in the wound area at day 14. This could result in decreased collagen deposition in response to neutralisation of TGF-β1. At day 14, Mstn-ant4 treatment also resulted in less dermal thickening. The development of wound contraction force has been found to be proportional to the thickness of granulation tissue (Snowden and Cliff 1985), and the same correlation between decreased wound contraction and reduced granulation tissue thickness observed here, is also reported in other studies (Joseph *et al.* 1996; Joseph *et al.* 1997). Decreased wound contraction may cause wounds to heal over a longer period of time, but the ultimate result would be reduced scarring. The significance of the granulation tissue width and thickness differences had disappeared by day 21. Once again, the loss of significant differences from day 21 onwards could be a result of the response of the healing wound to withdrawal of treatment almost a week earlier, on day 15.

It should be considered that antagonising myostatin may give rise to downstream effects on the expression of other genes. The powerful myogenic effect observed by blocking myostatin may even be in part due to the effect of inhibiting other genes that affect myogenesis such as TGF-β. In support of this idea, Se-Jin Lee (2007) recently showed that muscle mass was likely regulated by genes other than, but related to, myostatin, by over-expressing follistatin in myostatin-null mice to achieve a quadrupling of muscle mass.Another possibility is that myostatin antagonism may affect intracellular Smad signalling which both myostatin and TGF-β share. Wound healing studies which focus on Smads have

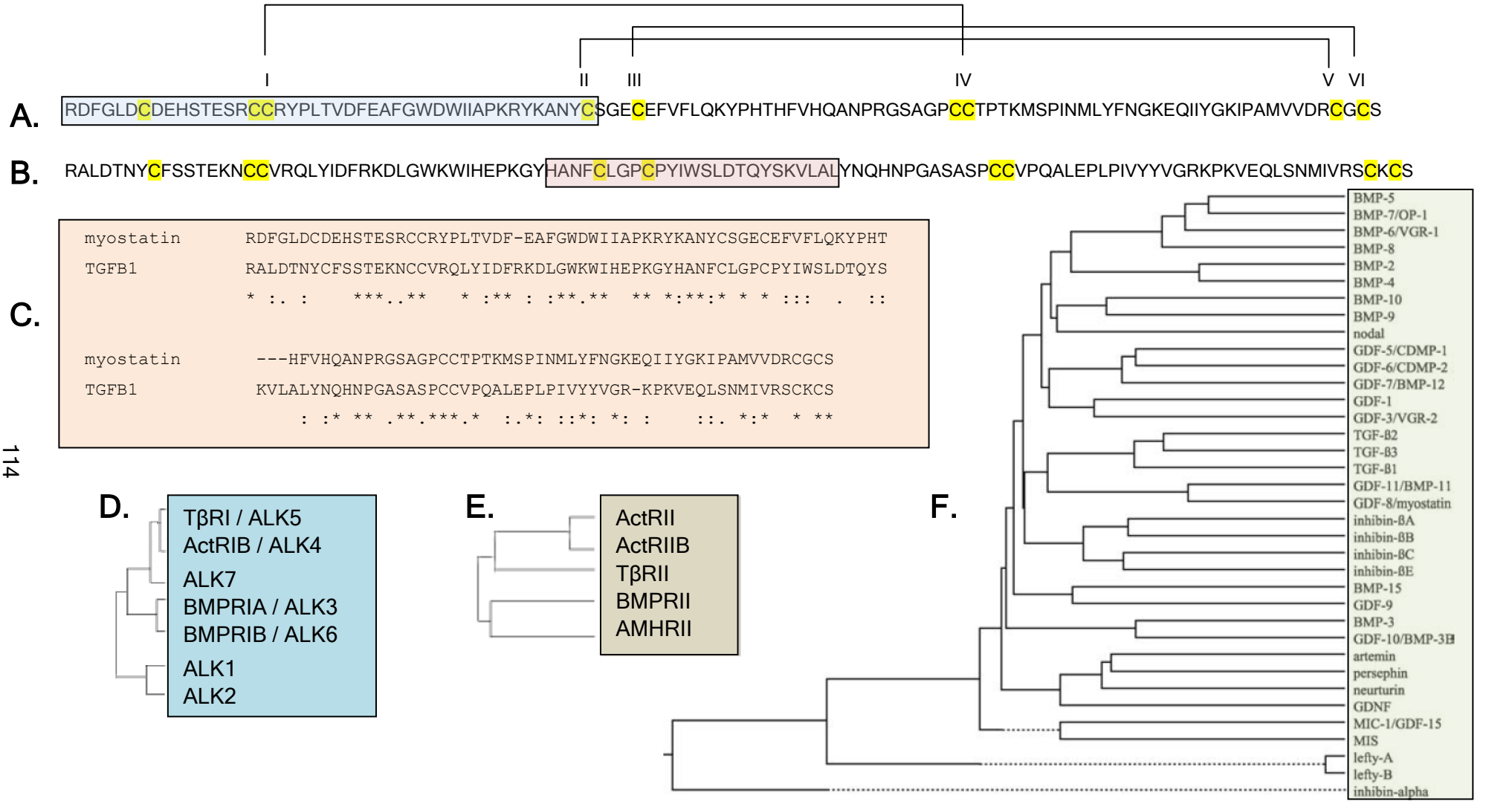



**Figure 4.1 Similarities between TGF-β superfamily members**

**A**: Mature myostatin with antagonist sequence indicated in boxed section. Cysteines highlighted yellow, cysteine bonds indicated which give rise to cystine knot. **B**: Mature TGF-β1 with peptide antagonist from Huang *et al.* (2002) indicated in box. **C**: Amino acid alignment between mature portions of myostatin and TGF-β1. **D** & **E**: similarities between type I and type II TGF-β superfamily member receptors, respectively (adapted from Massagué 2000). **F**: Sequence similarities between TGF-β superfamily members (Lee 2004), notice that myostatin is very closely related to TGF-β.

shown they make a considerable contribution to wound healing (Ashcroft *et al.* 1999; Ashcroft and Roberts 2000; Chong *et al.* 2007). This includes inhibitory Smads, such as Smad-7, which regulate both TGF-β and myostatin signalling (Zhu *et al.* 2004; Forbes *et al.* 2006).

A consideration of other genes that could be suppressed by the antagonist rendering the ActRIIB receptor unavailable is worthy of discussion as far as they relate to wound healing. Activin binds to activin receptors and this factor is purported to have a role in wound healing. Activin stimulates fibroblasts to produce fibronectin and collagen *in vitro*, and mice over-expressing activin in the epidermis exhibit accelerated wound healing by enhanced granulation tissue and ECM deposition (Munz *et al.* 1999). In their study, Munz *et al.* (1999) showed that there was no significant difference in either collagen type I or type III expression in the over-expressing mice suggesting a different mechanism of activin-mediated ECM deposition to that of TGF-β, although like TGF-β, activin may still be a stimulator of fibrosis, as high levels of the cytokine are found in fibrotic (Munz *et al.* 1999) and chronically inflamed tissues (McLean *et al.* 2008). Therefore, there could be therapeutic potential in blocking activin signalling during wound healing, especially in burn wounds, which have a higher potential to become fibrotic. This could therefore be a beneficial mechanism of action for the antagonist. The therapeutic potential of activin antagonists have been recognised (Harrison *et al.* 2005) and McLean *et al.* (2008) speculated that administration of follistatin, a natural activin (and myostatin) antagonist could be of therapeutic relevance here. However, a study conducted by Wankell and colleagues (2001) showed that over-expression of follistatin in transgenic mice impairs wound healing. It should be noted though that while wounds took longer to heal and granulation tissue formation was significantly reduced, the resulting scar area was smaller than in controls. Therefore, from these complementary studies it can be inferred that over-expression of activin in the epidermis accelerates wound healing but increases scar formation, while inhibition of activin activity in the skin delays wound healing but improves the quality of the healed wound (Sulyok *et al.* 2004). Although completely blocking activin may not be the best approach to improve healing outcome, antagonising activin to some degree could have beneficial effects by preventing excessive matrix deposition. The myostatin antagonist could fulfil this role. If the antagonist has the same type II receptor binding characteristics as myostatin, which is high affinity for ActRIIB but lower affinity for ActRIIA (Lee and McPherron 2001), then this action would obstruct activin signalling through ActRIIB.

Bone Morphogenic Proteins (BMPs) are the largest subfamily of the TGF-β superfamily and play an important role in development and homeostasis of postnatal tissues. They are also implicated in some pathological states as well as wound healing (Botchkarev 2003). BMP-2 can signal through ActRIIB, although not with high affinity (Weber *et al.* 2007), and BMP-6 also makes use of ActRIIB to elicit its signal (Ebisawa *et al.* 1999). Research suggests that BMPs, like TGF-β and activin have a role to play in the fibrogenic process. After both acute wounding and in chronic wounds, BMP-6 is up-regulated in keratinocytes and fibroblasts in skin, and delayed re-epithelialisation and scar formation is observed in BMP-6 over-expressing transgenic mice, suggesting that BMP-6 represses keratinocyte proliferation (Blessing *et al.* 1996; Kaiser *et al.* 1998). BMP-2 seems to induce scar formation in much the same way as TGF-β (Stelnicki *et al.* 1998; Botchkarev 2003). Therefore, by blocking activin receptor binding sites, Mstn-ant4 may be able to antagonise BMPs 2 and 6 causing improvements in wound healing.

In summary, the myostatin antagonist used in this study could be having its effect on wound healing in three possible ways. Firstly, Mstn-ant4 could be inhibiting myostatin directly, either by binding myostatin itself or competing with myostatin for its receptor, to prevent myostatin signalling. Secondly, Mstn-ant4 could be antagonising TGF-β1 by causing an up-regulation of decorin or by interfering with TGF-β1 signalling directly. Thirdly, by binding to and occupying ActRIIB cell surface receptors, other factors which use these receptors may be blocked from signalling, leading to improvements would healing.

## 4.3 Concluding remarks

Because of the complexity of the wound healing process and the interaction and synergism of several growth factors and cytokines, manipulating the wound environment to promote regeneration is a challenging prospect. At present, most therapies involve application of a single factor in the hopes that this will result in perfect healing, but mono-therapy is unlikely to be effective (Aarabi *et al.* 2007). Because foetal wounds heal with true tissue regeneration they represent the ideal model for wound repair. The fact that myostatin antagonist treatment in skin wound healing can produce up-regulation of several genes implicated in scarless foetal wound healing is indicative of its potential as a therapeutic agent.

## 4.4 Future directions

The completion of this study has answered several pending questions and has provided a basis for future studies to be conducted. In particular, the main purpose of the study was two-fold: to develop an *in vivo* wound healing model with respect to burn wound healing in skin, and to assess the performance of the antagonist Mstn-ant4 in skin wound healing using the aforementioned *in vivo* model. Previously in this lab, myostatin antagonists Mstn-ant1 to 3 have been used in various acute and chronic muscle wasting conditions such as notexin and incision injury (acute), sarcopenia, and muscular dystrophy (chronic) models, with impressive results (Siriett 2007; Siriett *et al.* 2007). In acute trials, myostatin antagonist injections in the first few days after injury produced significant reductions in scar tissue formation after muscles were allowed to fully heal. The treatment regime for the present study was adapted from an acute muscle healing model. Results from this study now suggest that skin wound healing from a severe burn may benefit from a chronic wound healing treatment regime. In such a treatment schedule, the antagonist would be injected three times per week for the duration of the trial, instead of gradually reducing injection frequency from day 7 of the trial to end treatment by day 15. Since remodelling in skin continues for months to years, a greater benefit from the antagonist may be seen if treatment continued for longer than 15 days.

Having now tested Mstn-ant4 *in vivo*, several *in vitro* methods could be employed to further study its effect on skin wound healing. *In vitro* assays involving proliferation and differentiation of keratinocytes, and dermal fibroblast differentiation into myofibroblasts could be employed. The purpose of these assays would be to study the effects of both endogenous and exogenous myostatin on these processes and whether they can be reversed by administration of Mstn-ant4.

For the initial findings of this study to be established, some further gene expression studies and possibly some further trials would be worthwhile. In particular it would be useful to conduct further gene expression studies of the impact of myostatin antagonism on – MMPs, TIMPs, ActRIIB receptor expression, Smad-2/3 expression and activation, Smad-7 as a negative feedback inhibitor of myostatin and TGF-β, as well as hyaluronan and GAG levels in skin. A closer look at inflammatory processes and the factors involved thereof would provide further evidence of whether Mstn-ant4 can mimic the beneficial characteristics of foetal wound healing, given that foetal wounds heal with a lack of inflammation.

A skin wound healing trial could also be conducted with myostatin-null mice, which would provide a good model for the role of myostatin in skin healing. In fact there is anecdotal evidence that myostatin null mice heal with noticeably reduced scarring from incisional chest wounds resulting from heart surgery (McMahon and Matthews pers. com.).

While the evidence presented here for a positive effect of a myostatin antagonist on burn wound healing in skin is credible, it does warrant further study. If anything, its role in wound healing, or at least skin hydration, could be a positive side effect for those being administered a myostatin antagonist for other medical conditions.

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