Histological and immunohistochemical studies of excisional wounds in the rat with special reference to the involvement of the hair follicles in the wound healing process

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Histological And Immunohistochemical Studies Of Excisional Wounds In The Rat With Special Reference To The Involvement Of The Hair Follicles In The Wound Healing Process.

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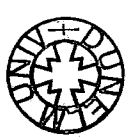


Abstract.

It is well known amongst surgeons that scalp skin heals better than non-hairy skin and is used for repeated split thickness skin graft harvesting in patients with severe burns.In biology literature it is well established that the skin of hairy animals heals relatively quickly. The contribution of the epithelial components of the hair follicle to skin wound healing has been studied to some extent however little or no attention has been given to the possible contribution of the hair follicle dermal components especially in view of the morphological similarities between dermal sheath cells and wound fibroblasts and myofibroblasts.Moreover little is known about any possible differences in the wound healing process between anagen and telogen hairy skin.The PVG rat model was used to look for any differences in wound healing between anagen and telogen skin and to study the contribution of the dermal components of the hair follicle to skin wound healing.The reparative process of the basement membrane of the hair follicle and the wound epithelium were also studied.Excisional punch biopsy wounds were analysed macroscopically and microscopically.The latter involved Weigerts' Haematoxylin stainning of paraffin sections and alpha smooth muscle actin (α SMA), collagen IV and laminin indirect immunoflourescence staining.Light microscopy,UV light microscopy and confocal microscopy were used.Anagen skin was found to have a statistically significant smaller wound diameter than telogen skin at 7 and 8 days post wounding.Epithelial basement membrane was observed to start regenerating in the central part of the wound epithelium as well as in the periphery.The dermal sheath of the hair follicles in the wound merged with the wound fibroblast network. α SMA staining showed concentric spiral patterns of marking around anagen follicles in the wound highly suggestive of dermal sheath cell migration into the wound.The implications of these findings with regards to improvements in living skin equivalents and to further the scope for the use of the scalp as a split thickness skin donor site are discussed.

Preface.

To all who may read this work,

Wound healing is one of the main areas in surgical research that is progressively being better understood.Hair follicles are well known for their helpful role in wound healing by contributing epithelial cells that help to re-epithelialize skin wounds.Hair follicles also contain a dermal component which so far has not been implicated in wound healing.I conducted this work in order to investigate the possible role of the hair follicle dermal sheath cells in the process of hairy skin wound healing.During this work I also made observations on hair follicle repair and regeneration after wounding as well as on the behaviour of basement membrane proteins during the wound healing process.

The data presented in this thesis has not been submitted for any other degree and is my original work which I performed at the Department of Biological Sciences,Durham University,under the supervision of Dr C.A.B. Jahoda Ph.D.

I dedicate this work to my wife,Lisa,for her support and understanding and to my son,Luke.

I am greatly indebted to Dr Jahoda for his great help and advice both during the practical part of the experiments and during the writing of this thesis.I would also like to thank Mr M. Robinson for his practical help during my early days in the laboratory.

Tuesday 16th of November 1999

Raymond Debono MD FRCSEd FRCSGlas.

Abbreviations.

a FGF	Acidic fibroblast growth factor.
α SMA	Alpha smooth muscle actin.
b FGF	Basic fibroblast growth factor.
BM	Basement membrane.
BrdU	Bromodeoxyuridine.
DS	Dermal sheath.
EGF	Epidermal growth factor.
HB-EGF	Heparin binding epidermal growth factor.
IGF	Insulin-like growth factor.
IL-1	Interleukin-1.
IL-8	Interleukin-8.
KGF	Keratinocyte growth factor.
ORS	Outer root sheath.
PBS	Phosphate buffered saline.
PGDF	Platelet-derived growth factor.
TGF α	Transforming growth factor α.
TGF β	Transforming growth factor β.
TNF α	Tumour necrosis factor α.
VEGF	Vascular endothelial growth factor.

Contents.

Section 1.

Introduction.

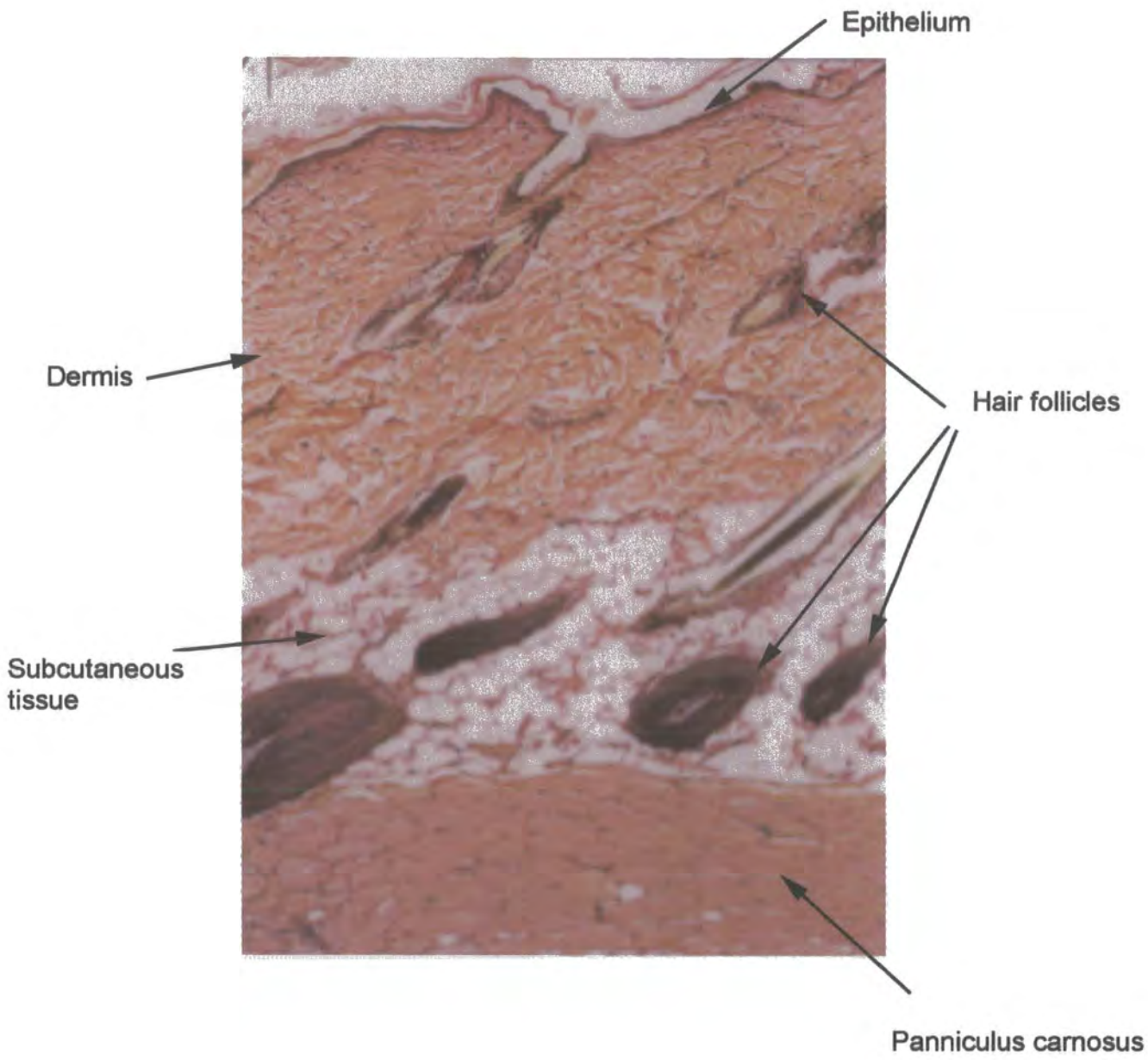
1:1 The Anatomy Of Mammalian Skin.

Skin is the largest organ of the mammalian body. Mammalian skin is composed of two layers:the epidermis and the dermis. The epidermis is derived from the ectoderm whilst the dermis is of mesodermal origin. Underneath the dermis lies the subcutaneous tissue composed of a reticular connective tissue and adipose cells.The junction between the epidermis and the dermis is marked by the basement membrane. The epidermis provides the first level of protection from the external environment and has excellent powers of regeneration and repair.The epidermis, in conjuction with the dermis, also generates the skin appendages namely: hair follicles, sebaceous glands sweat glands and nails. These appendages in turn provide a source of epithelial cells during epithelial wound healing (Ordman and Gillman,1966).The dermis is a mechanically stronger layer and possess elastic properties.The dermis is composed of a rich network of collagen fibres embedding cells,including fibroblasts and macrophages that are important in the healing of deep wounds and for defence against infection.The dermis also incorporates the blood supply to the skin,through which all nutrients are distributed to the various skin components.

The anatomy of skin differs amongst mammalian species even though the general architecture of the dermis and epidermis is essentially similar.The differences are in particular due to the variations in density of the hair follicles and other skin appendages as well as to variations in the relative thickness of the dermis and epidermis and the thickness of the underlying subcutaneous fat (Moserova and Houskova,1989).Furthermore there are also differences in skin thickness amongst different parts of the body of the same animal,for example in the human the dermis is thicker in the back than in the eyelids.Small laboratory animals like the rat,mouse,guinea pig and the rabbit,have a high density of hair follicles, a relatively thin dermis and a limited amount of subcutaneous fat (Figure 1). Pig skin,however, has a thicker dermis,a thicker layer of subcutaneous fat and fewer hair follicles than rodent skin (Montagna and Billingham,1964) and therefore resembles human skin (Figure 2) more closely. Furthermore in small laboratory mammals the density and the depth of the hair follicles as well as the vascularity of the dermis varies with the stage of the hair follicle cycle of that part of the skin (Zawacki and Jones,1967 and Haddow et al,1945). Specifically skin with

Figure 1. **Low Power Histology Of PVG Rat Skin**

(Weigert's Haematoxylin Staining)



Note:the relatively thin epithelium,the high hair follicle density in the dermis and the subcutaneous tissue,and the relatively thin layer of subcutaeous fat.Also note the presence of the panniculus carnosus.

Figure 2. Diagramatic Representation Of Human Skin.

Note:the relatively thick epidermis,the low density of hair follicles in the dermis and subcutaneous tissues,and the relatively thick layer of subcutaneous fat.Also note the absence of the panniculus carnosus.

(Adapted from:Gray's Anatomy,36th edition,Williams and Warwick, Churchill Livingstone.)

growing hair follicles tends to have a thicker and more vascular dermis and a higher density of hair follicles than skin with inactive hair follicles.

1:2 Wound Healing.

History Of Wound Healing.

Since the time of Hippocrates surgeons were aware of the need to clean contaminated wounds in order to help wound healing. Hippocrates advised the washing of wounds in warm sea water and the application of olive leaves (Donnelly,1998). In the eighteenth century French surgeons also became aware that wound healing can only start when any necrotic tissue and foreign material has been removed from the wound:the origin of surgical debridement. They were also aware of the need to apply soothing dressings and bandages to favour the healing of open wounds (Donnelly, 1998). Pasteur (1822-1895) developed the germ theory of disease and later Lister (1827-1912) explained the delay in wound healing as a result of wound infection and used carbolic acid spray to sterilise the air around surgical wounds,the surgeons hands and the instruments with success. Further advances in the types of wound dressings were made in 1880 when the 'Gamgee' dressing (Gamgee 1880) was first used and during World War I by Lumiere when he developed the paraffin impregnated gauze dressing ('tulle gras'), (Donnelly,1998).

During the mid-nineteenth century, in parallel with the antisepsis theory,a great interest in the pathological processes of inflammation developed amongst medical investigators.A strong controversy developed regarding the precise origin of the granulation tissue that developed to obliterate the skin defect produced by the injury. Schwann (1847) believed that wound fibroblasts cells actually split up into connective tissue fibres leading to the granulation tissue architecture formation.However this was strongly opposed by Virchow (1852) who believed the fibroblast cells actually secreted the connective tissue fibres or their precursors (Ashley,1969).The whole controversy was only clarified in 1940 when Stearns showed that collagen fibres were not present until fibroblasts had arrived in the wound (Stearns,1940). He also observed the migration of fibroblasts from the periphery in to centre of the wound. Abercombie et al (1956) further advanced the knowledge about fibroblasts and implicated that the tensile force of wound contraction was derived from the connective tissue cells. They described the fibroblast as a "cell of irregular, branching shape found distributed throughout vertebrate connective tissue". Billingham and Russell (1956) further supported this work and identified the granulation tissue as the "organ of

contracture". Watts et al (1958) showed that the force of wound contraction was coming from cells located at the periphery of the wound rather than in the centre of the granulation tissue.
Ordman and Gillman (1966) made a landmark contribution when they performed the first comprehensive and detailed studies of healing cutaneous wounds and put forward detailed histological descriptions of epithelial and dermal repair. A further important contribution was made by Gabbiani et al (1972) when they demonstrated that strips of granulation tissue contracted in vitro and noted cells with smooth muscle cell features especially actin filaments. The word 'myofibroblast' was coined to describe this specialised group of cells.
From this work to date a great deal of work has been done on the molecular and cellular functions of wound healing myofibroblasts,and wound healing in general as will be described in other sections of this introduction.However since the work of Ordman and Gillman (1966) very little direct attention has been given to the involvement/contribution of the specific cell layers of the skin appendages to the skin wound healing process.

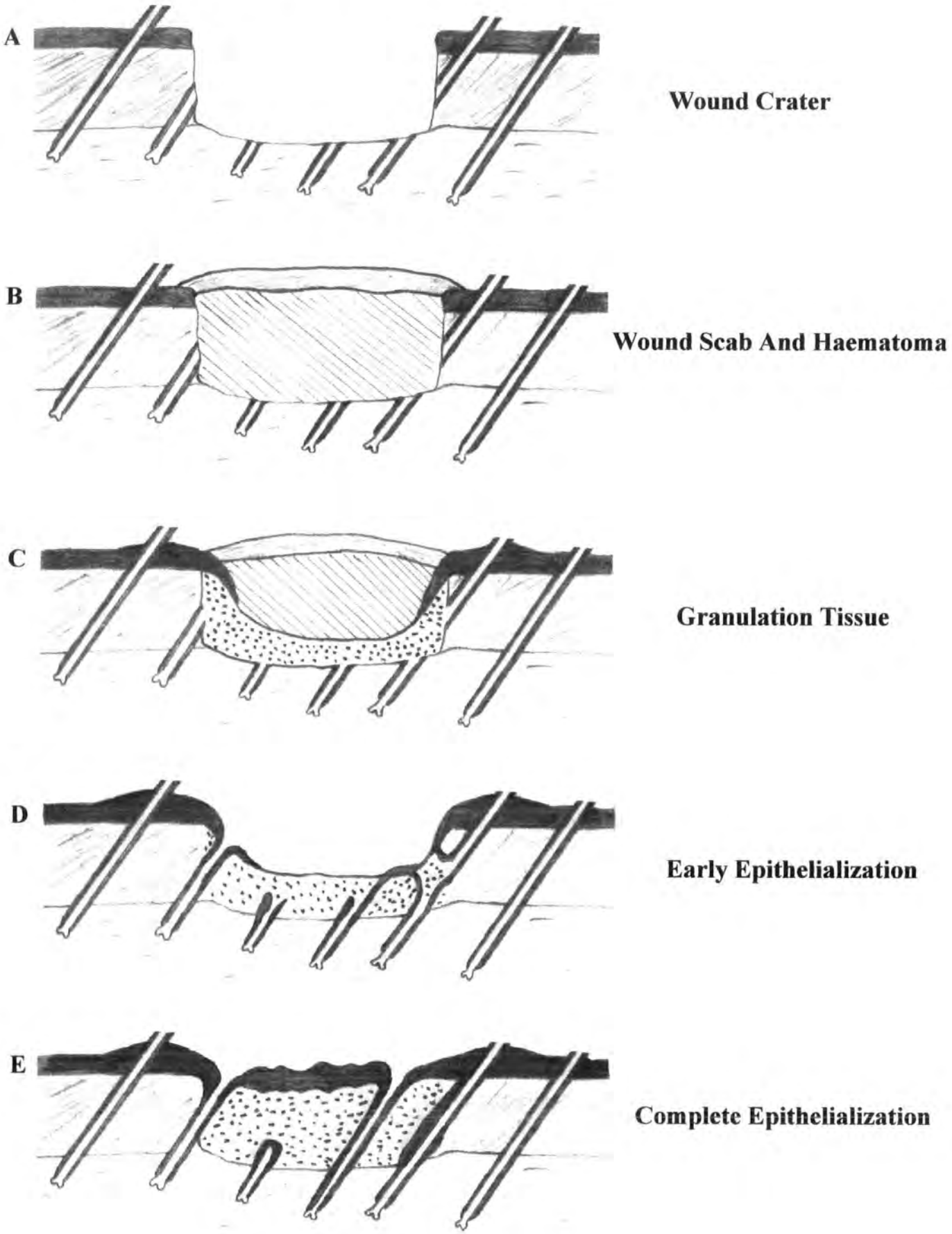
Types Of Wounds.

A wound may be defined as an interruption in the continuity of the structure of a tissue.In skin tissue specifically,a wound may be of an 'incisional type' or an 'excisional type' depending on whether the defect is in the form of an incision with virtually no tissue loss or an excision defect with tissue loss (Gillman et al,1955). The excisional type of tissue defect can be a full thickness skin tissue defect or a partial thickness defect. The nature of the defect affects the type of wound healing process that will ensue.

Brief Description Of The Wound Healing Process.

The wound healing process (Figure 3) starts very early after the trauma insult. The wound crater/tissue gap becomes filled with haematoma from injured blood vessels in the dermis and and subcutaneous tissue (Figure 3 B).The wound haematoma soon becomes infiltrated with macrophages and neutrophils which perform the early debridement of the wound. Fibroblasts and myofibroblasts eventually move into the haematoma framework and lead to granulation tissue formation (Figure 3 C). Epithelial cells at the edge of the wound and within remaining parts of skin appendages start to divide and regenerate new epithelial cells (Figure 3 D) that migrate over the granulation tissue and lead to epithelialization of the wound defect (Figure 3 E).

Figure 3. Diagramatic Representation Of The Wound Healing Process In A Full Thickness Skin Punch Biopsy Wound.



1:3 Phases Of The Dermal And Epidermal Wound Healing Process.

(Human Model).

The wound healing process can in general be divided into three phases (Forrester,1976). The immediate response to wounding is the triggering off of the haemostatic process which is followed by the inflammatory phase. During these first three to four days there is no detectable wound strength or contraction:*'Substrate Phase'*. Intense biological activity takes place at this stage,paving the way for fibroblast proliferation and extracellular matrix formation. The *'Phase of Proliferation'* starts at approximately the fifth day and is dominated by fibroblast and capillary proliferation leading to an increasing deposition of collagen and matrix with a corresponding increase in wound strength. The fibroplasia moderates after two to three weeks and is followed by the *'Phase of Maturation'*. During this phase the wound collagen is remodelled and the wound strength continues to slowly increase. The collagen turnover at this stage remains high.

Haemostasis

After wounding the Virchow's triad operates to achieve haemostasis. This involves the combined action of the blood vessels, the platelets and the coagulation system each leading in turn to vasoconstriction, platelet plug formation and blood coagulation (Shattil et al, 1980). The events in the haemostatic process can be divided into four phases (Cotran et al,1994):

*Contact Phase:*Exposure of the subendothelial,e.g. collagen,by the wounding process results in platelet adhesion,activation and degranulation leading to vasoconstriction and further platelet activation.Tissue trauma also exposes tissue factor which activates factor VII.

*General Activation:*Activated Factor VII (Factor VIIa) triggers the extrinsic coagulation pathway which together with the intrinsic pathway leads to the activation of Faxtors IX and X.Active Factor X (Factor Xa) binds to receptors (Va) on the surface of activated platelets and thus directs the coagulation cascade to the precise site of injury.

Stabilization: Factor Xa converts prothrombin to thrombin which in turn converts fibrinogen to fibrin.Fibrin is further stabilised by cross linking of the fibres in the presence of active factor XII and this leads to a stable haemostatic plug.

Remodelation: Once the bleeding has been stopped the blood vessels need to be remodelled so that their patency can be re-established.This involves the process of fibrinolysis which results in lysis of the occluding blood cloth by a carefully balanced action of plasmin and tissue plasminogen activator.

Inflammation.

Activated platelets liberate multiple biologically active peptides (Shattil et al,1980) that provide a chemotactic signal that attracts neutrophils,lymphocytes,mast cells and monocytes to the injured tissue thereby setting up the inflammatory response.

Neutrophils: are the first cells to arrive in the wound area and constitute the first line of defence against bacterial wound infection. Neutrophils release proteolytic enzymes that help in wound debridement (Davidson,1992).

Macrophages: Macrophages appear within the first 5 days and gradually replace the polymorphonuclear leucocytes.They have a relatively long life span and can function at low oxygen tension at the wound edge.Furthermore macrophages play a pivotal role in wound healing (Diegelmann et al,1981) as they are involved in the inflammatory process (Browder et al,1988),in lymphocyte activities,in tissue debridement and bactericidal activities as well as in tissue remodelling. They secrete a variety of important growth factors which perform several important functions:chemoattraction of many cellular components,stimulation of angiogenesis, stimulation of collagen synthesis and collagenase secretion, keratinocyte activities,as well as stimulating granulation tissue and extracellular matrix formation. Macrophages also release inhibitory factors which inhibit epithelial cells,fibroblasts and collagen synthesis.

Lymphocytes: Lymphocytes become prominent towards the end of the first week after

wounding,however their presence is very dynamic.They provide a regulatory role to wound healing via their regulatory influence on macrophages. An intact T-cell system is essential for normal wound healing (Barbul,1990 and Efron et al,1990)

*Mast cells:*Mast cells play an important function in dermal wound repair.They contain prominent granules, the contents of which e.g. heparin,histamine,chemotactic factors, and proteolytic enzymes,perform important functions in the wound healing process (Trabucchi et al,1988). Degranulation of the mast cells occurs within the first few hours after injury and leads to the release of chymase which helps to hasten the death of damaged cells. Chemotactic factors attract help to attract the components of the inflammatory infiltrate whilst histamine, heparin and tumour necrosis factor alpha help to stimulate fibroblast proliferation,angiogenesis and collagen synthesis.

Provisional Matrix Formation.

The blood clot inside the wound is remodelled into a matrix within the first 2 days. Platelets in the wound help in the formation of the matrix by releasing growth factors (Eckersley and Dudley,1988). The newly formed matrix is composed mainly of fibrin and hyaluronic acid.The latter provides stabilization to the matrix. The matrix thus provides a structural support for the budding capillaries as well as regulating the influx of inflammatory cells:neutrophils,monocytes,lymphocytes and fibroblasts.

Epithelial Regeneration.

Within hours after the injury resulting in a skin defect the epithelium at the edges of the wound becomes thickened (Ordman and Gillman,1966) as a result of increased cell division as demonstrated by the large number of mitotic figures. A layer of epithelial cells then starts to migrate from the edges towards the centre of the wound. This epithelial advancement is also helped by epithelial regeneration arising from the epithelial elements of any preserved skin appendages in the depth of the wound. The advancing epithelial sheets swiftly cover the wound and unite with one

another to complete a sheet of epithelial cover. After the second day the wound epithelium starts to thicken. The number of mitoses in the wound epithelium is however far fewer than the intense number of mitoses at the edges of the wound (Ordman and Gillmann 1966). The epithelium bridging the wound gradually starts to acquire the structural features of uninjured epithelium as remodelling proceeds. The superficial wound scab comes off when keratin has been laid down in the underlying superficial layer of epithelial cells.

Angiogenesis.

Macrophages in the wound area release fibroblast growth factor which in turn activates endothelial cells in capillaries in the immediate vicinity of the wound. Activated endothelial cells release plasminogen activator and procollagenase which lead to the formation of plasmin and collagenase respectively. Plasmin and collagenase in turn degrade the endothelial basement membrane and lead to the formation of holes in the membrane (Banda et al,1988 and Folkman and Brem,1992) .Under the influence of chemoattractants like fibronectin and heparin endothelial cells escape through the defects in the basement membrane using pseudopodia and migrate into the wound.The so formed capillary buds then acquire a lumen and blood flow is established.

Granulation Tissue.

Granulation tissue is the newly formed highly cellular and vascular tissue in the wound that acts to clear all the debris from the wound,provides a framework to fill the tissue defect and supports the regenerating epithelium (Dover and Wright,1991),and helps to nourish the newly forming mesenchymal tissue.It is composed of an extracellular matrix of a gel consistency supporting the newly formed capillaries and the recruited macrophages and fibroblasts. The matrix itself contains collagen,hyaluronic acid,fibronectin and other glycosaminoglycans. Fibronectin seems to appear in the wound before collagen and helps in the chemoattraction of the inflammatory cells(Lambert et al,1984). With the ingrowth of more and more capillaries the nutritional status and oxygenation of granulation tissue improves leading to a more efficient

fibroblast function with more collagen and extracellular matrix production (Hunt,1988). In newly formed granulation tissue the collagen fibres are orientated parallel to the capillaries whereas in mature granulation tissue the collagen fibres are perpendicular to the capillaries (Linares,1996).

Definitive Wound Extracellular Matrix.

The extracellular matrix is a complex dynamic macromolecular structure composed of four major components namely:collagen,elastic fibres,glycoproteins and proteoglycans. It surrounds the connective tissue cells and supports the superficial epithelium and will ultimately determine the nature of the scar produced.

Collagen: As well as providing mechanical support and being the major determinant of the wound tissue biomechanical properties collagen is a very active molecule (Hopkins,1992). It modulates cell migration and proliferation as well as specific gene expression. Collagen also promotes re-epithelialization and is important in wound contraction.

Elastic fibres: Help to maintain the tissue structure by providing a recoil force against stretching.They contain glycine and proline like collagen but virtually no hydroxylated amino acids.

Glycoproteins and Proteoglycans: Glycoproteins are extracellular matrix proteins that play a role in various cell-surface interactions.They are particularly involved in mechanisms of adhesion and anti-adhesion amongst the different cell types (Engel,1991 and Chiquet-Ehrismann,1991). Adhesion is mediated via cell surface receptors mainly integrins (Pignatelli and Vessey,1994). The following are some examples of matrix glycoproteins: fibronectin, chondronectin, tenascin, vitronectin and anchorin.

Proteoglycans function as cell surface receptors, as ligands and as growth factor antagonists. They also provide extracellular signals for cellular growth, differentiation and migration (Ruoslahti,1989). Proteoglycans are classified according to either their core protein (3 types) or according to their glycosaminoglycan chains (6 types). Some examples of proteoglycans

include dermatan sulphate, heparan sulphate, thrombomodulin and syndecan.

Basement membrane: The basement membrane is a specialised part of the extracellular tissue matrix that separates the epithelium from the underlying dermis.It is composed of glycoproteins, proteoglycans and isoforms of collagen IV (Ekblom et al,1998).The basement membrane is responsible for the tight attachment of the epithelial cells to the underlying dermis (Martin et al, 1983).

Fibroplasia

Fibroblast proliferation and extracellular matrix deposition become prominent after the fifth day post injury.The ultrastructure of fibroblast close to the wound and in the wound changes in particular ways.In fibroblasts close to the wound the golgi apparatus and the endoplasmic reticulum move closer to the nucleus and actin filaments form within the cytoplasm.Fibroblasts within the wound show cytoplasmic dispersion of the golgi body and the endoplasmic reticulum and produce large amounts of collagen.Some fibroblasts in the wound align the actin filaments in parallel to each other in the align of wound contraction.These fibroblasts constitute myofibroblasts that are characterised by expressing α SMA isoforms (Gabbiani,1971).

1:4 Molecular Basis Of Wound Healing With Special Reference To TGF-β.

The timed control on the various stages of the wound healing process is performed by cytokines. Cytokines are proteins that bind to cell membrane receptors and control cellular function.Cytokine function can be endocrine, paracrine,autocrine or intracrine. A particular cytokine may affect the function of different cells by more than one of these mechanisms. The effect on cell function is mediated via alteration of intracellular kinase activity and phosphorylation of important proteins which in turn leads to sequence of events leading to the altered cell function.

Table 1. Summary of the growth factors involved in the different stages of wound healing.

Wound Healing Capacity	*Cytokine*
Inflammatory cell migration	PDGF
	TGF-β
	TNF-α
Fibroblast migration	PDGF
	TGF-β
	EGF
Fibroblast proliferation	PDGF
	TGF-β
	EGF
	IGF
	TNF-α
	IL-1
Angiogenesis	bFGF
	aFGF
	TGF-β
	TGF-α
	EGF
	TNF-α
	VEGF
	IL-8
	PD-ECGF
Epithelialization	EGF
	TGF-α
	KGF
	bFGF
	IGF
	HB-EGF
Collagen synthesis	PDGF
	TGF-β
	bFGF
	EGF

A summary of the known cytokine activities in wound healing is shown in Table 1., above (Lawrence, 1998).TGF-β is by far the most interesting cytokine involved in wound healing and scar formation.It is found in high concentration in the α-granules of platelets and is released into the wound at the time of platelet degranulation in response to the injury.TGF-β is also found in a variety of mammalian cells e.g.:macrophages,lymphocytes,renal parenchyma and bone (Assoian et al,1983)).Five subtypes of TGF-β have been described,however only the first three subtypes are found in mammalian cells and the isoforms are very homologous (carboxy terminal with seven cysteine residues) and perform similar biological activities (Massague,1987)).Examples of members of the TGF-β superfamily include activins,inhibins and bone morphogenic proteins.They are released in a propeptide form that require cleavage of a TGF-β binding protein to make the molecule active (O'Kane et al,1997).Three TGF-β receptors have been well characterised (Miyazono et al,1993) and the relative amounts of the three mammalian isoforms of TGF-β in the wound may directly influence the specific biological role of the cytokine.TGF-β stimulates macrophage migration (Whal et al,1987) and macrophages are in turn a main source of TGF-β.Furthermore TGF-β regulates its own production by macrophages through an autocrine mechanism and stimulates macrophages to produce other cytokines such as PDGF,TNF,FGF and IL-1.In the next stage of wound healing (fibroplasia) TGF-β is chemotactic for fibroblasts, upregulates integrin receptors on the fibroblast cell membranes and stimulates fibroblast proliferation (Postlethwaite et al,1987,Assoian et al,1984 and Roberts et al,1985).It also stimulates angiogenesis in the healing wound (Roberts et al,1986).

TGF-β is probably the most potent stimulus for collagen synthesis and accumulation (by decreasing collagen lysis) (Ignotz et al,1986).Work done by Shah et al (1992) has shown that specific antibodies to TGF-β can reduce the amount of collagen laid down in the wound healing scar.The margins of healing dermal wounds in adult rats were injected with a neutralising antibody to TGF-β.These wounds healed with no scarring whilst wounds control wounds healed with scar tissue

formation.It was therefore concluded that anti-TGF-β antibody applied to early in the wound healing series of events can inhibit the autoinductive and autcatalytic cascades of TGF-β in the wound and reduce the amount of growth factors in the wound leading to reduced scar formation.

1:5 Differences In Wound Healing Amongst Different Types Of Wounds.

Incisional Wound Healing

After surgical incision and surgical reapproximation of the wound edges ,the wound space is very narrow and filled with a haematoma. The epithelial wound edges become hypertrophic and proliferate underneath the wound scab. By 48 hours the gap in the surface epithelium is fully bridged. Fibroblast proliferation starts very early and provisional matrix is soon laid down. During this process very little or no granulation tissue is laid down.

Full Thickness Excisonal Wound Healing

Healing in full thickness skin wounds with tissue loss takes a different course.It first involves the formation of granulation tissue, which then acts as a bed for epithelialisation to take place from the edges. This process is necessarily longer and leads to more scarring.

Partial Thickness Excisional Wound Healing

by definition in partial thickness wounds a variable part of the dermis containing skin appendages is left at the base of the wound. After haemostasis and debridement the epithelial components of the skin appendages proliferate and start to cover the exposed dermis. An amount of matrix formation and granulation tissue is also formed and this will eventually lead to a decrease in the depth of the defect. Healing of partial thickness wounds is usually associated with little or no scarring.

Wound Healing In Burn Injury.

Wound healing in burn wounds is slower from that in excisional wounds. The reason for this is the fact that burn wounds are associated with a lot of oedema as well as tissue

ischaemia and hypoxia. Furthermore burn wounds contain more necrotic tissue and are subject to progressive tissue damage. All these factors lead to a prolonged initial 'Lag Phase' as well as to delayed inflammmatory response, delayed epithelial migration and delayed intercellular communication.

1:6 Factors Affecting The Wound Healing Process.

Certain factors regularly affect the rate of wound healing after a surgical procedure or after accidental trauma:

Age of patient:Healing is more rapid in the young and tends to become slower as age increases.The vigorous rate of wound healing in the young may be related to hypertrophic scarring and keloid formation which are both commoner in younger subjects.

Nutritional status:Wounds heal slower in malnourished and debilitated patients.Plasma protein levels are in general a good guide to the nutritional status of the patient and optimal levels favour the healing process.Ascorbic acid and zinc are essential nutrients for wound healing;deficiency leads to slow healing and wound breakdown.

Vascularity of the injured tissue / area of the body:Parts of the body with a very good blood supply like the face and especially the scalp are well known to heal more rapidly than areas with a weaker blood supply.Tendon division heals very slowly after repair as tendons are relatively avascular. Similarly lacerated intra-articular cartilage,eg:knee joint menisci,have an extremely slow healing rate.Another factor related to the part of the body in which a wound occurs is the density and depth of the epithelial skin appendages in the area.In this respect areas of the scalp with the hair follicles in anagen phase wound provide a more favourable epithelial cell reservoir and hence more rapid re-epithelialization of the wound.The deeper the appendages are situated in the skin the better is the chance that the deeper parts of the appendages are preserved during a skin injury such as a burn; hence the advantage of deep seated anagen hair follicles.

Drugs:Drugs that inhibit the inflammatory response such as corticosteroids,retard the wound healing process.Antimitotic drugs can also have adverse effects on wound healing.

Wound infection and systemic sepsis:Local wound infection decreases collagen synthesis and increases collagen lysis,significantly delaying wound healing.Systemic sepsis also has an adverse effect on wound healing.Diabetes mellitus is associated with poor wound healing as a result of depressed neutrophil antibacterial activity and increased risk of wound infection.

Oxygen supply to the wound area:Oxygen is the most important wound nutrient.Oxygen is required for the respiration of the vigorously active cells of the healing wound as well by the peroxidase system utilised by macrophages to kill ingested bacteria.When wound capillary perfusion is impaired by either local factors such as tight sutures and tissue trauma (crush injury),or by systemic problems like cardiopulmonary failure,there is decreased oxygen and nutrient delivery to the wound which leads to delayed healing.

Wound Dressings:A moist environment is favourable for wound healing.Tissue desiccation leads to death of the wound healing cells.Dressings that keep the wound environment moist encourage wound healing.

1:7 Basement Membrane Morphology And Its Involvement In Wound Healing.

Basement membranes separate the skin epithelium and appendages from the underlying supporting mesenchymal tissue. They contain isoforms of collagen IV,noncollagenous glycoproteins such as laminin and nidogens as well as proteoglycans (Ekblom et al,1998). The basement membrane zone has four structural zones namely:basal cell plasma membrane,lamina lucida,lamina densa and sublamina. The main function of the basement membrane is to provide a tight attachment of the epithelial cells to the dermis underneath and provide histologically distinct compartments (Martin et al 1983).

Laminin is a glycoprotein ubiquitously present in all basement membrane zones in mammalian tissues.The laminin molecule is a large heterotrimeric protein structure composed of alpha,beta and gamma chains (Ekblom et al 1998) and is orientated in such a way that part of it lies in the lamina lucida and part in the lamina densa of the basement membrane zone (Horiguchi et al,1991).It is not yet established which cells specifically produce the basement membrane laminin,however it is very likely that at least part of it is produced by the epithelial cells (Fine,1994).More recent work has shown that that only approximately 20% of the basement laminin is produced by the epithelium whilst 80% is produced by the dermis (Fleischmajer et al,1998).At the start of basement membrane regeneration laminins attach to receptors on the keratinocyte plasma membrane and serve as foci for further polymerisation of laminin molecules (Fleischmajer et al,1998).

Collagen IV is also a ubiquitous extracellular matrix protein present in the basement membrane zone.It is mainly present in the lamina densa (Fine,1994) and is important in the attachment of epithelial cells to the basement membrane (Aumailley and Timpl,1986). Indirect immunoflourescent studies for collagen IV have revealed a continuous linear marking along the dermoepidermal junction of interfollicular skin and around hair follicles, sebaceous glands and blood vessels (Weber et al,1982).

In vitro experiments of fibroblast and keratinocyte cultures revealed that both keratinocytes and fibroblasts produced collagen IV however the mesenchymal cells were the major source.From these

studies it was further concluded that binding of collagen IV to keratinocyte cell-matrix integrins resulted in complexes that acted as nuclei for further polymerisation of collagen IV molecules resulting in basement membrane formation (Fleischmajer et al,1998).

Epithelial Basement Membrane Regeneration In Wound Healing.

In vitro and *in vivo* studies in the rat showed that basement membrane proteoglycans, perlecan, laminin 1 and collagen IV are of epithelial cell origin (Yamane Y. et al,1996).Olerud et al. (1988) showed that during epidermal repair of skin in elderly human subjects laminin and collagen IV appeared only at the lateral edge of the epithelial basement zone of the day 2,day 3 and day 5 wounds whilst they were present throughout the basement membrane zone of the 14-day wound. It can therefore be proposed that basement membrane regeneration in a wound starts at the perimeter of the wound and proceeds centripetally into the centre of the wound.A further interesting point relating to the capacity of keratinocytes to regenerate the basement membrane structure is the fact that keratinocytes autografts in the pig can restore basement membrane however its anchoring capacity is weaker than that of normal skin (Carver et al,1993).

Hair Follicle Basement Zone.

During fetal development in the rat laminin and collagen IV are present continually in the epithelial interfollicular and in the follicular basement membrane zone both before and after hair follicle morphogenesis.Later on they are also present in the dermal papilla (Westgate et al,1984).The latter finding was further investigated by Couchman (1986).Anagen hair follicle dermal papilla of postnatal rats had a matrix very rich in basement membrane components especially collagen IV and laminin.Messenger et al (1991)showed that laminin and collagen IV were expressed in the basement membrane of the outer root sheath and in the extracellular matrix of the dermal papilla in anagen and catagen follicles.Telogen follicles showed only outline staining of the dermal papilla cells as the matrix was very reduced in the telogen phase.Work done by Couchman and Gibson (1985) showed that there is increased deposition of laminin and collagen IV in the early anagen phase of

hair follicles and proposed that the constituents of membranes are important during the hair follicle cycle *in vivo.*

Upon review of the literature very little could be found relating to the repair or regeneration of the hair follicle basement membrane after wounding. Jahoda et al (1992) investigated the repair process *in vivo* of rat vibrissa follicles which had the lower follicle bulb amputated by microsurgical technique. It was noted that once the new hair follicle epidermis grew downwards,collagen IV and laminin were detected in the new basement membrane below the level of amputation. Furthermore following the assembly of the papilla forming mesenchyme, collagen IV and laminin were also detected in the newly regenerated papilla.

1:8 Wound Contraction,Fibroblasts,Myofibroblasts,Follicular Fibroblasts,Hair Follicle Dermal Sheath Cells,And α–Smooth Muscle Actin.

Wound Contraction.

Billingham and Medawar (1955) proposed the concept of wound closure by contracture and intussusceptive growth in the skin of mammals with 'loose skin'.They point out that in extensive wounds in the skin,the gap is first closed by a contraction force that moves the original wound edges closer to each other and reduces the defect.This then leads to a true growth of the surrounding skin (intussusceptive growth),to make up for the lost skin.They considered granulation tissue,migratory epithelium and fibrosis as temporary measures of repair,binding time until the intussusceptive growth achieves skin replacement.However it was pointed out that this mechanism can only function on mammals with a 'fully mobile integument'.Human skin is very closely attached to the underlying fascia and muscles and the panniculus carnosus is vestigial.Consequently contracture in human skin leads to detrimental outcomes as it causes restriction of movement and distortion.

The mechanism by which wound contraction takes place is still not fully understood and there is some controversy regarding the actual direct mechanism responsible for the contraction process.At present there are two main theories that attempt to provide an explanation:

Pull Theory:Myofibroblasts are suggested to be directly responsible for wound contraction (Majno et al,1971) and are mainly concentrated at the wound periphery.They make the wound contract in a 'picture-frame'fashion (Lawrence,1998).Myofibroblasts are only transiently present in the wound area and their presence correlates with the timing of wound contraction (Darby et al,1990).

Organisation Theory:This second theory suggests that fibroblasts reorganise the arrangement of the extracellular matrix and in so doing generate the force that produces wound contraction (Ehrlich et al,1986 and Ehrlich,1988). As the fibroblasts elongate and migrate through the collagen matrix *in vitro* they retract the collagen fibrils (Grinnell,1984). However this process is serum dependent (Guidry et al,1985).

The first theory is supported by electron microscopic and immunohistochemical observations of the morphology of myofibroblasts as described earlier. However the main support for this theory comes from animal experiments in which the centre or the periphery of a healing wound was excised and the outcome noted.Wounds with an excised centre still contracted whilst if the perimeter of the wound was excised no wound contraction occurred (Rudolph,1979). From this work it was concluded that the force of contraction was produced by cell contraction in the periphery of the wound,hence the function of myofibroblasts.Germain et al (1994) performed *in vitro* studies to examine the degree of collagen gel contraction produced by human wound healing fibroblasts (myofibroblasts) and dermal fibroblasts.As expected the wound healing fibroblasts contracted the gel far more than the dermal fibroblasts confirming the stronger contraction power of the myofibroblast as well as providing further evidence in favour of the myofibroblast phenotype as a separate entity.The work of Darby et al (1990) also supports the existence of the myofibroblast as a separate differentiation entity.

The second theory looks at looks at myofibroblast as simply fibroblasts at the wound perimeter with cytoplasmic actin stress fibers.The argument against myofibroblasts as a distinct entity is mainly supported by *in vitro* studies on collagen matrices. Fibroblasts on a collagen matrix *in vitro* develop stress fibres in their cytoplasm when exposed to mechanical stress.In other words they assume a myofibroblast appearance.When the stress in the matrix is removed the cell morphology reverts back to that of an ordinary fibroblast (Farsi and Aubin,1984).

Even though differences exist between the two theories,advocates of both theories agree that the contraction mechanism is cell-directed and that cell division is reqiured.However collagen synthesis does not seem to be a necessity (Lawrence,1998).

Fibroblasts And Myofibroblasts.

Abercombie et al (1956) postulated that cells in connective tissue generated the force required for wound contraction.Billingham and Russel (1956) named granulation tissue as 'the organ of contracture'. Further studies by Watts et al (1958) demonstrated that the contractile force originated

from the wound margins and not in the central part of the granulation tissue.Gabbiani et al (1971) elegantly demonstrated that isolated pieces of granulation tissue *in vitro* were able to contract and also showed that cells in the granulation tissue had some features of smooth muscle cells, in particular actin filaments arranged in bundles. The term 'myofibroblast' was therefore coined to describe the granulation tissue cells which were responsible for generating the contraction force (Skalli and Gabbiani,1987).They could be distinguished from fibroblasts under the electron microscope:- their cytoplasm contains actin microfilaments and abundant rough endoplasmic reticulum ;the nucleus is multilobulated.The question then was whether myofibroblasts were derived from dermal fibroblasts or from smooth muscle cells.Eddy et al (1988) and later Darby et al (1990) used cytoskeletal markers analysis to show that myofibroblasts are derived from fibroblasts and not from smooth muscle cells even though they express alpha smooth muscle actin.Fibroblasts that migrate into the forming granulation tissue differentiate into myofibroblasts (Darby et al,1990) and adhere tightly to the surrounding granulation tissue as suggested by the presence of fibroexus junctions (Singer et al,1984).The degree to which the wound resists contraction effects the timing of the change from fibroblast to myofibroblast (Darby et al,1990 and Welch et al,1990).Petroll et al (1993) further explained the mechanism which stimulates the differentiation of the fibroblast to myofibroblast. The fibroblasts at the wound edges start the force for wound contraction and as the resistance to contraction increases, the fibroblasts differentiate into myofibroblasts and the actin fibres become aligned along the lines of most resistance in order to contribute a greater force to contraction.

Parker (1932) described nine types of fibroblasts and suggested that fibroblasts are not a homogenous cell type.This was later confirmed by Harper and Grove (1976) who showed that skin fibroblasts from different depths in the dermis had different proliferation potentials.Furthermore rat fibroblasts aged *in vitro* differentiate along different particular cell lineages (Konterman and Bayreuther,1979). Morphological and biochemical evidence to show that human fibroblasts

differentiate along 'stem cell'/'pleuripontent cell-like' lineage was provided by the work of Bayreuther et al (1988). Further work by Franz et al (1989) attempted to identify markers relating to the type of differentiation pathway likely to be taken by fibroblast stem cells.All the above evidence points towards the heterogeneity of general skin fibroblasts

Follicular Fibroblasts And Dermal Sheath Cells.

Jahoda and Oliver (1981) isolated a special population of fibroblasts from the rat vibrissa follicle dermal papilla and described the morphological differences between skin fibroblasts and hair follicle derived dermal cells. Later on Messenger (1984) isolated an equivalent group of fibroblasts from the human hair follicle papilla.Horne (1987) isolated and cultured dermal sheath cells from the hair follicle.Dermal sheath cells were noted to induce hair fibre regrowth after implantation into the upper parts of hair follicles with an amputated papilla. Hair regrowth only re-started after the papilla was regenerated (Horne and Jahoda,1992).This study established the fact that dermal sheath cells can replace the dermal papilla cells and demonstrated their great regeneration potential.

Taylor et al (1992) reported that the glycosaminoglycans synthesised by dermal sheath cells and the dermal papilla cells were identical.Furthermore dermal papilla cells and dermal fibroblasts synthesised the same types of glycosaminoglycans with the only difference being that dermal papilla glycosaminoglycans were less sulphated than those produced by dermal fibroblasts.These findings highlight the similarities between the hair follicle dermal cells and the dermal fibroblast and further contribute to the view that these two types of cells may differentiate from one to the other under special influences e.g. wound healing.

Another similarity between hair follicle dermal cells and fibroblasts was noted by Messenger et al (1991).Cells cultured from human dermal papilla and cultured fibroblasts both showed positive staining for interstitial collagens ,collagen IV and laminin.This again shows the biochemical and structural similarity between hair follicle dermal cells and dermal fibroblasts. More recently,studies by Almond-Roesler et al (1997) showed that dermal papilla cells caused gel contraction via reorganisation of the extracellular matrix components *in vitro*; however this capacity was

significantly lower than that of dermal fibroblasts.

Alpha Smooth Muscle Actin.

Actin is expressed in mammals and birds as six isoforms (Garrels et al,1976, Vandekerckhove et al, 1978, Vandekerckhove et al,1979 and Vandekerckhove et al,1981). Four of the actin types are differentiation markers of muscle tissue whilst the other two types are found in most types of cells (Vandekerckhove et al,1979 and Vandekerckhove et al,1981).In non-muscle cells actin filaments are associated with other proteins to provide mechanical support and cell adhesion.They also perform other functions such as cell contraction (Fujiwara et al,1978 and Sanger et al,1983) and are responsible for the morphogenetic cell shape changes (Wessells et al,1971).Actin filaments are also responsible for maintaining integrity of epithelia and for pursestring wound contraction (Bement et al,1993).In smooth muscle tissue the relative proportion of the different actin isoforms is different in different organs.

Gabbiani and Skalli (1986) described a monoclonal antibody against alpha smooth muscle actin,the actin isoform expressed by hair follicle dermal sheath cells and wound healing myofibroblasts.In situ rat vibrissa hair follicle labelling for alpha smooth muscle actin shows positive marking only in the lower one third of the follicle dermal sheath (Jahoda et al,1991). Closer inspection of the lower end of the follicle revealed that there was no positive marking in the lowermost region of the dermal sheath (i.e. the part below and around the papilla) and the dermal papilla itself.However in human hair follicles the lowermost part of the dermal sheath is positive for alpha smooth muscle actin (Jahoda et al,1991).However *in vitro* cell cultures of dermal sheath and dermal papilla cells revealed positive staining by both types of cells.Furthermore skin fibroblast cultures were noted to have a lower number of positive cells.However fibroblasts derived from hair dense skin showed more positive marking than fibroblast from skin with less hair follicles (Jahoda et al,1991).It was therefore concluded that alpha smooth muscle actin was a good marker for follicular dermal cells in culture.These findings further support the view of fibroblast heterogeneity in the skin dermis with

special emphasis on the possibility that 'fibroblast-like cells',e.g.dermal sheath cells,may differentiate from one type to another and perhaps give rise to myofibroblasts during wound healing (Jahoda et al,1991).

Bjorkerud (1991) pointed out that alpha smooth muscle actin may be a differentiation specific marker so that the lower dermal sheath may possibly be more differentiated than the upper dermal sheath as the upper dermal sheath is less positive for alpha smooth muscle actin.Reynolds et al (1993) provided evidence pointing towards a differentiation pattern amongst dermal sheath cells and showed that dermal sheath cells from different regions of the proximodistal parts of the hair follicle expressed different amounts of alpha smooth muscle actin.The tissue from the lower part of the dermal sheath had the most positive cells (98%) in *in vitro* culture followed by tissue from the end bulb (85%). However tissue from the upper dermal sheath had a lower proportion (50%) of positively marked cells in culture. This differential expression of alpha smooth muscle actin could possibly be related to the specialised functions of the different parts of the hair follicle during the hair cycle and may even represent different differentiation potentials in terms of the possible phenotypic change to myofibroblasts in response to a wound healing environment.

1:9 Hair Follicle Repair And Regeneration And The Behaviour of Hair Follicle In Wound Healing.

It is well documented in medical and biological literature that skin appendages,in particular hair follicles,are involved in skin wound healing phenomena.Ordman and Gillman (1966) gave a comprehensive description of the healing of cutaneous wounds in the swine model.They dedicated part of their study to analysis of the outcome after the trauma sustained by the skin appendages as a result of a surgical incision using a scalpel blade.They start by mentioning that the damage can occur at any level along the length of hair follicle or the sebaceous or sweat glands and that the degree of trauma can range from injury to the connective tissue sleeve encircling the appendages,to dislocation of epithelial cells from the appendages,to complete transection of the appendages.Damaged sweat glands become infiltrated with neutrophils and their lumen may sometimes get sealed by epithelial growth resulting in a microscopic epidermoid cyst.Injury to the hair follicle results in new epithelial growth which may act to repair the damaged follicle or grow upwards towards the skin surface and join other epithelial elements or grow laterally or downwards to join the injured epithelial elements of other skin appendages.This new epithelial growth may sometimes seal off the transected end of the hair follicle and lead to the formation of a solid cyst which eventually resorbs. Furthermore it was pointed out that epithelial cells released as a result of the surgical trauma multiply and form scattered islands of abherrant epithelial cells. These cell islands provoke a full blown foreign body type reaction involving mononuclear,epithelioid and giant cells.The islands are then sealed off by a peripheral concentrically arranged fibrous tissue structure and are slowly resorbed over the weeks. Injury to the connective tissue coating the follicles results in a round cell reaction leading to fibrosis.Totally severed follicles are unlikely to reunite and reform the appendage. Whenever follicles are sealed off and obstructed either by the epithelial growth or

by fibrous tissue,a foreign body reaction ensues and the follicles tend to resorbe.

The lining epithelium of sealed follicles resorbes first and by 3 weeks all that remains is the hair shaft encircled in a foreign body giant cell reaction.They therefore concluded that the outcome of the damaged hair follicles in a surgical wound depended on the extent of the damage and whether that damage could result in good and functional healing or not.

Cohen (1961) first used the rat vibrissa follicle model for hair follicle dissection and study; and showed that parts of the follicle transplanted to other parts of the body were able to regenerate new whisker follicles. These findings showed that amputated and devascularized parts of hair follicles still retained their regenerative power provided that they were re-implanted into a suitable environment. Further work done by Oliver (1966) also demonstrated the reparative and regenerative capacity of the hair follicle when it was shown that generations of vibrissae can be produced following removal of the dermal papilla in the whisker follicles of the hooded rat.

Jahoda et al (1984a) studied the histological effects produced by wounding the lower part of the rat vibrissa follicle using a tungsten needle.Both the immediate and the long term effects (one year) were studied.Injury to the dermal papilla led cessation of hair fibre growth.Dermal papilla repair then took place,and on completion hair fibre regrowth resumed.It was also noted that the process of papilla healing produced no scar tissue.However in follicles that showed cell displacement post-injury,abnormalities in hair fibre growth were noted. The same group (Jahoda et al,1984b) also showed that wounding of the lower part of the hair follicle resulted in a 50% chance of the hair growth being longer than that in uninjured follicles provided that there was no cellular displacement the wounding process. However the actual growth rate of the hair fibre when altered was reduced. Jahoda et al (1992) further examined the effect of wounding on the rat vibrissa follicle when the lower follicle bulb was amputated and the outcome followed by immunohistochemistry and electron microscopy. Immediately after wounding the follicle

epidermis became hypertrophied and spread downwards below the level of the amputation. The dermal sheath cells of the follicle followed the epithelial component in its downward movement indicating that both the epidermal and dermal components are activated during hair follicle repair. The regeneration capacity of the upper part of the hair follicle after amputation of the bulb is not restricted to rat whisker follicles. Jahoda et al (1996) have recently shown that human hair follicles where able to regenerate a fibre-forming bulb after its amputation and implantation of the remnant into the nude mouse.

Inaba et al (1979) discussed hair follicle regeneration in the clinical context. They histologically examined the immediate effect produced by the subcutaneous tissue shaver, an instrument used to surgically shave off the sweat glands from the undersurface of the dermis as a radical treatment for hidradenitis suppuritiva, as well as the resulting skin after complete healing. Immediately after surgery it was noted that the shaver had removed the hair bulbs and most of the follicles up to a level close to the sebaceous duct.Hair re-growth from the follicle remnant occurred as long as the upper portion of the isthmus of the follicle was intact. It was further noted that epithelial pegs grew downward from the lower end of the follicle remnants and eventually the epithelial cells enclosed a mass of mesenchymal cells and formed a new papilla and bulb.This work further confirmed that the upper part of the follicle can regenerate the hair bulb.

The contribution of the hair follicle outer root sheath to interfollicular skin wound epithelium regeneration has long been established (Eisen et al,1955).More recently groups of outer root sheath cells with a long life span or even stem cell characteristics have been described (Rochat et al,1994).In vitro,in a submerged culture,outer root sheath cells show similar characteristics to interfollicular epidermal keratinocytes both morphologically and biochemically (Limat et al,1989). In coculture with dermal fibroblasts outer root sheath cells develop a stratified epithelium similar in appearance to regenerating epidermis (Lenoir et al, 1988).Limat et al (1995) grafted similar organotypic cultures of outer root sheath cells onto wounds

on nude mice and noted that a regular neo-epidermis was formed.

Martin (1997) discussed the contribution of basal remnants of injured hair follicles to the re-epithelialization of a wounded skin area and stated that the edges of injured hair follicles function as normal cut epithelial wound edges that grow out to form islands of cells from the follicle stump. All the above evidence further confirms the interaction and similarities between the outer root sheath cells and interfollicular keratinocytes during epithelial regeneration and supports the view that the outer root sheath provides an unlimited reservoir of 'stem cells' that give rise to epithelial keratinocytes. Furthermore outer root sheath cell organotypic cultures have been recently used with success in the clinical situation to help in the epithelialization of chronic venous ulcers (Limat et al,1996).Clauson et al (1982) investigated the proliferation characteristics of epidermal basal cells in resting hair follicles and in interfollicular dermis in mice. They found no heterogeneity in cell proliferation parameters between the two sites and concluded that both sites must have similar mechanisms responsible for maintaining growth equilibrium. However Yang et al (1993) showed that a subpopulation of keratinocytes derived from the upper part of the human scalp hair follicle had superior growth potential *in vitro* than epidermal keratinocytes,supporting the view that outer root sheath cells are better reservoirs of epidermal keratinocytes than the epidermis itself. Further interesting work, recently done by Ferraris et al (1997), showed that epidermal stem cells retain their embryonic pleuripotentiality during wound healing.Epidermal cells were noted to give rise to hair follicle buds and pilosebaceous units. More recently Miller et al (1998) investigated the contribution of the sweat apparatus and the hair follicle to epithelial wound healing.

They studied wounds of two depths:- a rather superficial type of wound which retained the hair follicles and a deeper type of wound that had only sweat glands and no hair follicles. The wounds in which the hair follicles were preserved healed faster and the wound epithelium resembled the adjacent unwounded epidermis both morphologically and biochemically (as proven by protein electrophoresis). The epithelium in the deeper wounds resembled either a mucosal epithelial lining or the plantar or palmar skin epithelium. It was therefore concluded that the sweat apparatus can

re-epithelialize a wound however the epithelium may not be the same as that of the adjacent non-wounded dermis.

Further data relating to the behaviour of hair follicles after wounding was supplied by the *in vitro* experiments performed by Williams et al (1994). Transection of the hair follicle above the level of the sebaceous gland led to growth of the hair shaft with no accompanying growth of the outer root sheath whilst transection of the follicle below the level of the sebaceous gland led to simultaneous growth of the hair shaft and the outer root sheath. This clearly showed that the level of amputation affected the reparative reaction of the hair follicle. Kim et al (1995) showed that grafted human scalp hair follicles from which the bulb and the dermal sheath had been removed regenerated new papillae and grew fresh normally pigmented hairs.

As one would assume epidermal wound healing is more essential to the organism than hair follicle development in terms of survival. This point is scientifically supported by the data from the experiments conducted by Guo et al (1996) where it was noted that keratinocyte growth factor was necessary for hair development but not for wound healing :- mice lacking keratinocyte growth factor were still able to heal their wounds effectively in spite of abnormal hair growth.

All the above evidence confirms the strong regeneration and repair potential of hair follicles. This can be extrapolated to suggest that hair follicles must have a permanent stem cell reservoir capable of repairing the follicles themselves,regenerating the follicles and contributing to the repair of the epidermis in a skin wound. By further extrapolation,since the hair follicle epidermis has such reparative powers it might well be that the hair follicle dermis has regenerative powers that help in dermal repair. Horne et al (1992) showed that the hair follicle dermal sheath can lead to new bulb formation and restoration of hair growth following implantation into the upper epidermal follicle cavity remnant after

amputation of the lower half of the follicle. This clearly shows the immense regenerative potential of the dermal sheath cells and suggests that the dermal sheath cells may be pleuripotent cells. More recently Matsuzaki et al (1996) further demonstrated the regenerative potential of the follicular dermal sheath by showing that the upper dermal sheath can regenerate small hair bulbs when implanted under the kidney capsule for eight weeks.The regeneration capacity of the upper dermal sheath was,however,found to be less than that of the lower dermal sheath.

During the healing of excised wounds in hair bearing skin,hair follicle buds are often observed in the wound tissue especially within the newly formed epidermis. The direct origin of these buds is still uncertain. Some may possibly arise from pleuripotent hair follicle cells implanted into the wound during the injury causing the wound. Whether or not the hypertrophic wound epithelium itself can regenerate a new follicle bud is not known. Rat low passage dermal papilla cells on their own can induce complete hair follicle neogenesis when re-implanted into heterotypic skin (Reynolds and Jahoda,1996). However cultured high passage dermal papilla alone and low passage dermal sheath cells alone do not stimulate hair follicle neogensis.They only induce hair follicle neogenesis when they interact with germinative epidermal cells (Reynolds and Jahoda,1996).Thus the hair follicle neogenesis (buds) observed in newly formed wound epithelium or within the wound tissue itself may arise from seeded dermal papilla cells acting on their own or from dermal sheath cells interacting with epithelial germinative cells.

1:10 Clinical Evidence For The Involvement Of Skin Appendages In Skin Wound Healing.

Holmes et al (1983) investigated the mechanism by which deep dermal wounds heal spontaneously.They mention that since the skin appendages (hair follicles,sweat glands and sebaceous glands) are situated deep in the dermis,enough of them will survive in deep dermal burns to supply epithelial cells for re-epithelialization. They consider the dermis –subcutaneous tissue interface as a key boundary :- if it is breached by the burn new connective tissue formation takes place and rapid granulation tissue formation covers the remaining epithelial elements in the appendages preventing them from helping in re-epithelialization. They also point out that new connective tissue arises from tissues deep to the dermis-subcutaneous tissue boundary and not from the remaining dermis. This work however lacks a clear comparison between histological findings and clinical findings or general outcome which would have possibly been very useful to support their views.

Burn injury delays re-epithelialization by three days (Holmes et al,1983 and Hell and Lawrence, 1979) when compared to a clean cut wound. Skin appendages are more sensitive to burn injury than the surrounding dermis thereby sustaining more severe damage. This could be one of the factors resulting in the delay in re-epithelialization of a burn wound.

In plastic surgery split thickness harvesting takes advantage of the fact that residual appendages left after removal of the epidermis are able to re-epithelialize the donor site. It would be reasonable to suggest that the higher the density of the appendages (mainly hair follicles) at the donor site the quicker would the donor area heal. Martinot et al (1994) conducted a prospective study comparing twenty nine cases of split skin graft harvested from the thigh with thirty seven grafts taken from the scalp. They looked for differences in the ease of the technique ,percentage graft take, graft quality and after-effects at the donor site.The main aim of the study was to evaluate the quality of the donor sites the real risk of adverse after-effects in chidren. Harvesting was found to be technically more difficult in the scalp as compared to the thigh however there was no statistically significant difference in graft quality between the two types of graft. The most important finding of this study

was the fact that significantly more healed scalp donor sites were invisible than thigh donor sites.This difference was statistically significant indicating that scalp skin heals better than thigh skin after split thickness skin harvesting.The main problem with this study was the fact that the observations were mostly subjective as well as the fact that it was not mentioned whether the same surgeon or group of surgeons performed the operation.

It is well established in the literature that split thickness skin can be harvested from the scalp on multiple occasions and with relatively short intervals in between (Crawford,1964,Taylor et al,1977 and Chih-Chun et al,1982).Crawford (1964) was the first to realise the donor site potential of hairy scalp skin in a case report describing how a nine year old boy with 60 % burns was treated using scalp split thickness skin grafts and suggested that this provided a solution to what appeared to be an insoluble problem at that time.It was also noted that scalp split thickness skin took rapidly and spread quickly to join the other grafts.Furthermore he noted good healing at the donor site with good hair re-growth and that very little hair had grown in areas grafted with scalp skin.

Taylor et al (1977) in a detailed review of 26 burns patients in which the scalp was used as a donor site concluded that scalp skin healing was rapid,even as short as seven days.They suggest that the time interval between re-harvesting was short enough to clearly show the rapidity of scalp skin healing.Furthermore it was noted that hypertrophic scarring did not occur in scalp donor sites and a patient that developed hypertrophic scarring in other donor areas did not develop hypertrophic scarring in the scalp donor site. They attributed the rapid healing to the rapid proliferation of epidermal elements deep within the dermis in particular the greater density of hair follicles in scalp skin.It was suggest that the rapidity of healing and the low incidence of infection may account for the lack of hypertrophic scarring observed at the scalp donor site. No transplantation of hair with the grafts was observed. This was attributed to the fact that most of the scalp hairs at any time are in anagen phase with the roots deep in the subdermal tissue and therefore unlikely to be transplanted

with split thickness skin. The lack of hypertrophic scarring in scalp donor sites suggests that scalp skin may heal in a different and better way than non-hairy skin.

Chih-Chun et al (1982) in describing their huge experience with extensive third degree burns emphasised that the scalp was a principal donor site in managing extensive third degree burns and note the strong healing power and resistance to infection of scalp skin. This was attributed to the thick dermis with deep seated hair follicles as well as to the rich blood supply of scalp skin. They carried out repeated harvesting at intervals of between five and seven days and pointed out that the scalp heals with no scarring and no interference to hair growth.

Zingaro et al (1988) examined the anatomy of the scalp looking for features that make it an advantageous skin donor site. The main factors noted were that the scalp has a thick dermis;abundant epithelial appendages; bases of hair follicles extending into the subcutaneous tissues and an excellent blood supply. They attributed the better healing in hairy skin to these four factors.

Whereas most authors claim that the incidence of complications after scalp split thickness skin harvesting was very low,Lesesne and Rosenthal (1985) had a different experience. They reviewed 113 patients and found that seven patients had postoperative alopecia,ten had exudative crusting,two developed staphyloccocal folliculitis and one patient developed significant hypotension during skin harvesting as a result of excessive blood loss. It was therefore recommended that the scalp may not be the preferred donor site in paediatric cases as it can lead to a relatively greater blood loss as the scalp represents a larger proportion of the total body surface area than in the adult. However they did agree with all the other advantages of scalp skin mentioned by the other authors specifically the decreased donor site visibility, the rapid healing time, the capacity for repeated cropping and the fact that it is potentially a large donor surface area.In their study it was also noted that scalp skin provided a better colour match for facial skin grafting.The main limitation with this article was the fact that the authors did not mention the thickness of the grafts they were taking and it could well be that they had problems because of thicker graft

harvesting.In fact they do admit that the postoperative alopecia was due to uneven split thickness skin graft harvesting.They also state that their review was the largest series of patients with scalp donor sites at that time,but unfortunately this was not so as three years earlier Chih-Chun et al (1982) published a much larger series and found no postoperative complications.

Two further groups of authors in separate communications (Brou et al,1990 and Engrav et al,1990) were somewhat concerned about the possible complications following scalp split thickness skin graft harvesting. Brou et al (1990) found a 13% incidence of alopecia in non-burned scalps in a series of 194 paediatric patients. This incidence was even higher if the donor scalps had been burned. There was a statistical correlation between the risk of alopecia and large burns and a high number of skin graft re-harvesting repeats. The interval between re-harvesting also appeared to correlate with the risk of alopecia. As a result they advocated meticulous donor site management especially when harvesting on multiple occassions. In spite of all the possible problems they still supported the view that the scalp is an acceptable donor site. Engrav et al (1990) reported their experience with 5 patients in whom the scalp donor site failed to re-epithelialize and developed a dried granulation tissue with embedded hairs. These areas were treated by removing the granulation tissue after which epithelialization progressed well.

Finucan et al (1984) reviewed 23 scalp grafts in 18 patients and performed biopsies on a series of cadaver scalps to assess the thickness of the scalp superficial to the bases of the hair follicles. They emphasised the convenience of scalp split thickness skin grafting for head and neck defects as well as the fact that it gave a good cosmetic match for the forehead,nose and eyelid skin. The scalp donor sites were found to be comfortable for patients, healed rapidly without infection and could be re-cropped every five to seven days.It was also suggested that the scalp should be used in elective procedures in elderly patients in whom other donor sites may heal poorly. An apparent absence of hypertrophic scarring and keloid formation was also noted in young black patients suggesting some protective mechanism in scalp skin against scarring. Scalp thickness measurements ranged between 2.51 mm to 2.72 mm with an average of 2.63mm demonstrating that relatively thick scalp grafts can

be taken before the integrity of the hair follicle is disturbed. However they gave no details of the number of cadavers they examined or their age.

Berkowitz (1981) also noted the lack of hypertrophic scarring after split thickness skin graft harvesting from the scalp donor site. It was also suggested that bald areas of the scalp should not be used as donor sites as they are slow to heal due to a low skin appendage population as well as the fact that the scarring was more visible. Further work done by Barnett et al (1983) also emphasised the importance of hair follicles in skin wound healing. They suggested that the rate of healing was proportional to the density of epithelial foci in the defect.The scalp being densely populated with epithelial foci is hence expected to heal rapidly. Moreover in a series of 250 scalps used as donor sites they observed no hypertrophic scarring.

A final interesting point is that brought up by a case report by Buckland et al (1986) whereby hair was noted to have regrown in the scar produced after a deep burn in previously bald human scalp. This can theoretically be interpreted in terms of the fact that the burn insult resulted in a deep wound which triggered the migration of fibroblasts/myofibroblasts into the wound. Keeping in mind the close morphological and biochemical properties of dermal fibroblasts and hair follicle dermal cells ,the dermal fibroblasts might have possibly differentiated into hair follicle dermal cells under the influence of the wound,which in turn gave rise hair follicle re-growth.It would have been very interesting if a representative histological analysis had been done.

1:11 Animal Models Of Wound Healing And The Effect Of The Hair Follicle Cycle On Skin Thickness Variation.

Hairy animals such as the mouse, rat and rabbit, used in experimental wound healing studies have a variable and rapidly changing skin thickness at any particular part of their skin (Zawacki and Jones, 1967). Animals from the same litter also differ greatly in the thickness of the skin in similar regions of their body (Geary 1952). The skin thickness varies in a wave-like cyclical pattern in association with the hair growth cycle (Zawacki and Jones,1967). The skin areas where the hair follices are in anagen phase are markedly thicker than skin areas with hair follicles in the telogen phase (Zawacki and Jones,1967). In the mouse,for example,anagen skin is three to four times thicker than telogen skin (Montagna,1962).This regional difference in skin thickness and the need to standardise the skin before setting up any wound healing experiments was later emphasised by Moserova and Houskova (1989).

The rapidly changing waves of anagen and telogen skin can be observed macroscopically by looking at the pattern of hair growth after shaving. Specifically the technique described by Zawacki and Jones (1967) involves shaving the skin area in question using clippers under a general anaesthetic. The animal is than allowed to recover from the anaesthetic and returned to its environment. After five days anagen skin areas show rapid hair re-growth whilst no hair re-growth occurs in telogen areas. Further confirmation can be obtained by microscopic examination if required.In this way skin areas can be standardised and a true comparison can be made amongst wounds in identical skin areas (skin in the same phase of the hair cycle). It is worth noting that for this standardisation procedure the hair should only be shaved with a clippers as using depilatory creams e.g. barium sulphide containing paste (Miles and Miles,1952) can stimulate hair growth in telogen areas and therefore change the skin thickness (Zawacki and Jones,1967) thereby confounding the experiment.

Section 2.

Background And Aims Of The Current Study.

2.1 Background

A review of the literature showed that since the publications by Ordman and Gillman (1966) there have been relatively few good histological wound healing studies. Furthermore most studies involved older histological techniques and little attention has been paid to the variation in skin thickness associated with the hair follicle cycle. Histological data on skin wound healing in the rat is relatively scanty in the literature and the specific effect of the hair follicle cycle on skin wound healing has only received some limited attention (Zawacki and Jones 1967 and Moserova and Houskova 1989). The behaviour and repair of the epithelial and the hair follicle basement membrane during wound healing in hairy skin has also received very little attention. Clearly there exists a need for more detailed studies using modern histological techniques of skin wound healing in hair bearing skin with special attention to the potential effects of the hair follicle cycle on the wound healing process.

Whereas considerable detailed work has been done investigating the contribution of the hair follicle outer root sheath (Eissen et al,1955 and Limat et al,1995) to skin wound healing,much less has been done to investigate the role of the hair follicle dermal cells in skin wound healing. As discussed above,it is well known that hair follicle dermal cells exhibit a lot of similarities to fibroblasts and myofibroblasts (Almond-Roesler et al,1997 and Messenger et al,1991). The dermal sheath of surgically injured hair follicles healed without scarring (Jahoda and Oliver,1984). When the base of a hair follicle is amputated,the follicle regenerates a new hair bulb (Oliver,1966). The new bulb epidermal matrix cells are derived from the remaining outer root sheath cells in the upper part of the follicle and the new dermal papilla is created from the remaining dermal sheath cells in the upper par of the follicle. This illustrates the fact that just as the outer root sheath cells act as a reservoir of epithelial cells for both hair follicle regeneration and skin wound epithelial repair, the dermal sheath

cells also have as a reserve or 'stem cell-like' function in hair follicle repair and regeneration and possibly in wound healing. Moreover, Jahoda et al (1991) and Reynolds et al (1993) have specifically postulated that hair follicle dermal sheath cells may act as a source of myofibroblasts during the wound healing process.

The rapid repair of hair bearing skin after split thickness skin graft harvesting can be attributed in part to the regenerative capacity of the outer root sheath of the hair follicles,however the dermis heals without scarring (Chih-chun et al,1982) and involvement of the dermal sheath cells is a likely possibilty. The above evidence highlights the need to investigate in further detail using modern histological techniques the contribution of the hair follicle components,especially the dermal cells,to skin wound healing.

2.2 Aims Of The Current Study

General Aim:

- To study the healing of excisional wounds in the PVG rat both macroscopically and histologically.

Specific Aims:

- To look for any difference in the rate of wound contraction between anagen skin and telogen skin.
- To investigate any differences in the wound healing process in anagen and in telogen skin.
- To investigate the dermo-epidermal interactions occuring during the healing of excised wounds.
- To investigate the role of the hair follicle dermal cells in skin wound healing.
- To investigate the activities of hair follicle α-smooth muscle actin during skin wound healing.
- To study the behaviour as well as the regeneration process of the hair follicle and skin epithelium basement membrane during the wound healing process.

Section 3.

Methods And Materials.

3.1 Animals

Adult PVG rats of both sexes,aged between 4 and 6 months (mean age: 4.8 months) were used under Home Office licence approval and guidance. All rats were from the same Durham University LSSU inbred strain and were fed a standard pellet diet and given water *ad libitum*. They were kept in their usual standard environment in separate individual cages with regular standard cleaning of the cages.

3.2 Wound Site Preparation And Wounding Procedure.

Rats were anaesthetised using a mixture of halothane and oxygen and after ensuring a surgical level of anaesthesia an area measuring four by six centimetres centred on the middle of the back was shaved using electric clippers until the skin felt and looked completely hairless.At the end of the procedure the animals were allowed to recover in a warm environment and monitored regularly.

The shaven area on the back was inspected after five to seven days to determine areas of definite active hair growth (anagen skin) and areas of no active hair growth (telogen or catagen skin).When it was clear that a distinction in the hair growth cycle could be made the rats were anaesthetised as described above and four standard wounds were applied using a 2mm diameter biopsy punch (manufactured by Stiefel). Two wounds were applied in anagen skin whilst another two wounds were applied to telogen skin.The punch biopsy wounds were made through the full thickness of the skin down to the panniculus carnosus. Slight bleeding was noted from the wounds,which stopped spontaneously.No dressings were applied to the wounds (Figure 4).

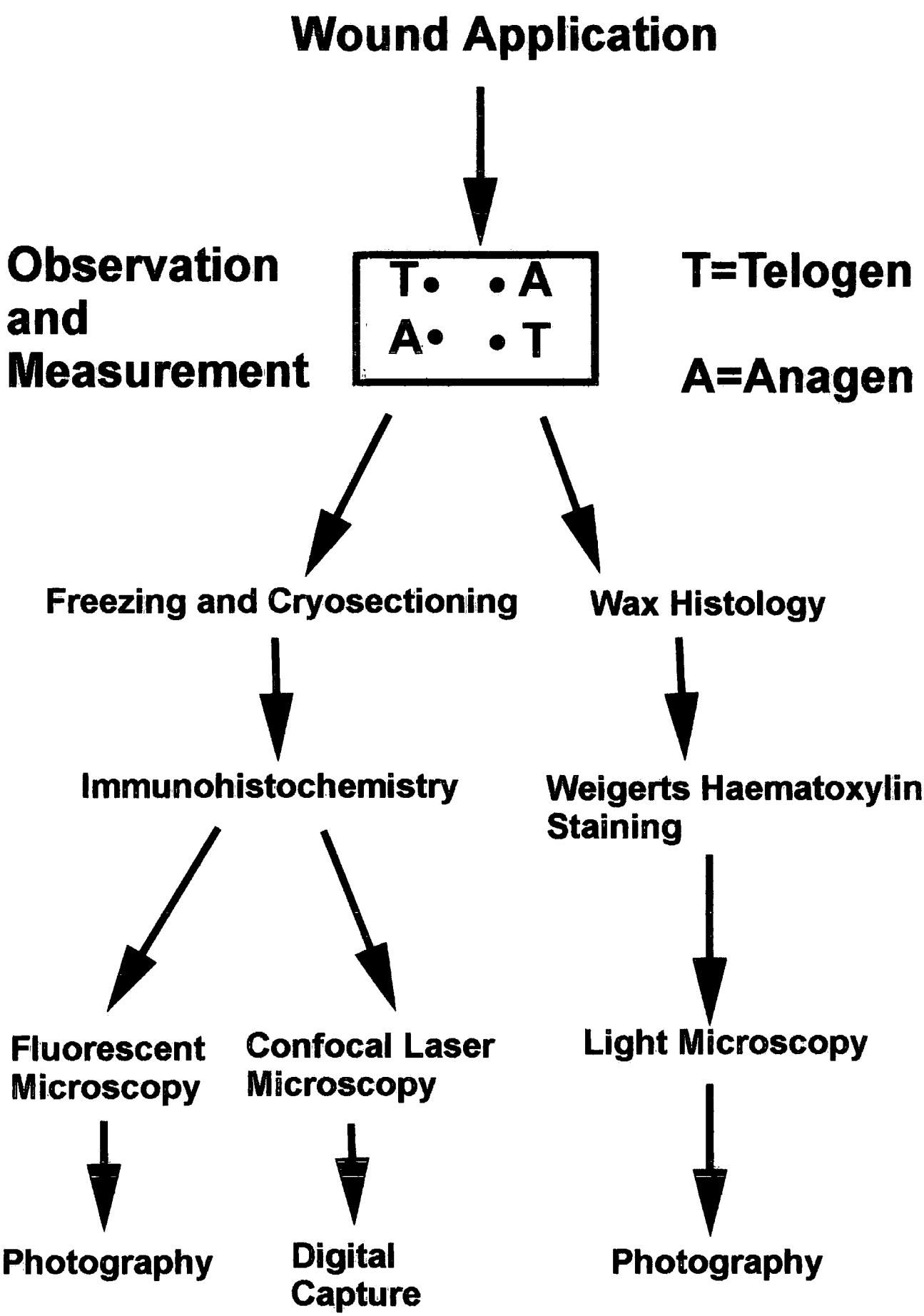
3.3 Specimen Treatment And Analysis.

Twenty one rats were divided into seven groups of three rats each.Each of the groups were sacrificed using a Schedule I procedure (knocked unconscious followed by cervical dislocation) at

Figure 4. The Dorsal Aspect Of The PVG Rat Showing The Shaved Area Of Skin Containing Four Punch Biopsy Wounds.

Excisional punch biopsy wounds on the back of the PVG rat.Two wounds are situated in anagen skin and the other two are in telogen skin.(This photograph was taken during preliminary work when 4mm punch biopsies were being tested.)

Figure 5. Summary Of The Processing Of The Wound Tissue Blocks



2,3,5,7,8,10 and 15 days after wounding.An outline of the procedure is shown in Figure 5. Immediately after the rats were sacrificed the wounds were photographed using a 35mm SLR camera mounted on an operating microscope (Zeiss,West Germany) with a Kodak Gold 200 print film.Each wound was photographed at X10 and at X20 magnification. The diameter of the wounds in parallel with the vertebral spine of the rat was then measured to the nearest 0.05mm using Vernier calipers under the operating microscope at X10 magnification. All measurements were taken from the true skin edges and not from the advancing edge of the migratory epithelium. A note was also taken of the state of the skins surrounding the biopsy site. The wounds were then excised down to the deep fascia under magnification as a full thickness square of skin of approximately 7x7 mm in size with the wound located centrally. Excision was performed using a scalpel mounted with a number 15 blade. A fresh blade was used for each wound to produce clean cut sharp incisions with minimal distortion of the wound.

From each rat four blocks of tissue each containing a wound were obtained. Two wounds were from anagen skin and two wounds were from telogen skin. One telogen and one anagen wound were frozen for cryosectioning, and the other two were placed in 4% formol saline for later paraffin embedding.

3.3.1. Paraffin Embedding,Cutting and Staining of Tissue Blocks.

Paraffin Embedding.

The blocks previously fixed in formol saline were embedded in paraffin using the following procedure. Fresh wax (paraplast) was placed in a clean beaker and allowed to melt in the oven at 60^{o} C overnight. The tissue blocks were transferred from the formol saline into 70% alcohol for one and a half hours. The alcohol solution was changed three times during that time period.The 70% alcohol was then changed to 90% alcohol and allowed to stand for one hour with three changes of the alcohol during that period of time. The 90% alcohol was then changed to absolute alcohol and left to stand for two hours again with three changes of the alcohol during that period. The absolute

alcohol was then changed to histoclear,which was allowed to stand for three hours and changed three times during that time. The histoclear was then changed to molten paraffin wax and allowed to penetrate into the tissue blocks at 60° C in the oven overnight. The next morning the tissue blocks were embedded lying on their side in molten wax in glass trays previously warmed to 60°C and their inside smeared with glycerine-albumin to prevent the wax from adhering to the glass.A label was also attached to each block. After the correct orientation of the tissue block was ascertained the wax blocks were rapidly cooled down in cold water and allowed to fully solidify. When the blocks had solidified they were taken out of the glass trays and prepared for sectioning.

Wax Block Cutting.

The wax blocks containing the embedded tissue were first trimmed in size using a razor blade and then fixed onto wooden blocks using molten wax and a hot spatula.Correct orientation of the tissue block was ensured at all times. The wooden block was then fasten into the chuck of a Leica tissue microtome and correct orientation of the chuck achieved. Disposable Bright microtome blades mounted on a Bright microtome blade-holder were used to cut the sections.A fresh blade was used for each cutting session.The microtome was adjusted to cut sections at 8 micrometer thickness and the sections were allowed to come out as a ribbon.The ribbon containing the sections was transferred onto a nearby glass non-stick surface using hypodermic needles.End frosted glass slides were smeared with a minute amount of glycerine-albumin so as to allow the wax sections to adhere better to the slides. The ribbon was then cut into lengths equal to the transparent length of the slide and each length was transferred to a slide using hypodermic needles. Two drops of air-free distilled water (prepared by boiling the distilled water for some time and allowing it to cool down) were then added to each slide to allow the ribbon to stretch.The slides were then placed on a hot plate at 50°C and allowed to dry overnight.

Staining.

The wax sections were then stained with Weigert's Haematoxylin. The Weigert's haematoxylin was prepared by mixing two solutions,A and B,in equal proportions(Appendix 3.). A fresh mixture was used for each staining session. The complete staining procedure also involved two further stains namely Alcian Blue and Curtis Ponceau S, (Appendix 3.).

The staining procedure started by removing the wax from the sections by washing in Histoclear and then transferring through alcohols of decreasing concentration to distilled water. This was then followed by staining in Alcian Blue followed by a wash in distilled water before staining in Weigert's Haematoxylin. The sections were then dipped in 1% acid alcohol to differentiate the nuclei. The final part of the staining procedure then involved a short dip in Ponceau S. The full details of the staining procedure are shown in Appendix 1.

3.3.2. Embedding,Cryosectioning and Indirect Immunofluorence Labelling.

Embedding.

One of each of the anagen and the telogen blocks were placed on their side in aluminium foil boats and Tissue-Tek (Miles Scientific), a water-based embedding compound,was poured into the boats.A label was then attached to each boat. The boats were then floated on the surface of liqiud nitrogen in a flask and allowed to set.The frozen blocks were then stored in a - 80° C freezer for later cryosectioning.

Cryosectioning.

The frozen blocks were taken to the cryostat (Bright) where the excess mountant was first trimmed and then the blocks were mounted on a chuck. The temperature of the cryostat was kept at -25° C and a freshly sharpened knife was used. Sections were then cut at 6 micrometer thickness.An anti-roll plate was used and the sections were collected on poly-l-lysine coated slides. Consecutive sections were collected in turn on three to four separate slides to allow comparative staining with different antibodies. Three to four sections were picked up on each slide. The poly-l-lysine slides were prepared by immersing plain glass slides with frosted ends in diluted (1:10) poly-l-lysine in a

staining tray for five to ten minutes. The poly-l-lysine was then poured out and the slides allowed to dry in an oven at 60° C for one to two hours. The slides were then allowed to cool to room temperature before being used.During the cutting process toluidine blue staining was used on some sections to help detect the wound area and allow representative sections to be collected from specifically important parts of the blocks. After collection on the poly-l-lysine slides the sections were allowed to dry in air at room temperature for one hour. The sections were then fixed in acetone at -25° C for two minutes and again allowed to dry in air at room temperature for two hours. The sections were then ready for indirect immunoflourescence staining.

Indirect Immunoflourescence Labelling.

The sections were then washed in staining trays containing filtered phosphate buffered saline (PBS) (Appendix 4.) for ten minutes.The washing procedure was repeated three times.The slides were then taken out of the staining trays and the excess PBS was wiped off from around the sections in preparation for the application of the primary antibody.Three primary antibodies were used on separate slides, Table 2. below.

Having dried (using tissue paper) the extra PBS from around the sections after the third wash, 13 microlitres of the diluted primary antibody were added to each section ensuring that the sections were completely covered with antibody in order to allow the reaction to occur in the whole section as well as prevent the sections from drying up.The sections were then placed in humidified chambers and allowed to incubate for two hours at room temperature.After the incubation period was over the sections were then washed three times in filtered PBS,allowing ten minutes for each wash.Thorough cleanliness of the glassware was ensured at every stage.The extra PBS around the sections was then dried using tissue paper in preparation for the addition of the secondary antibodies.

Table 2. Summary of the various antibodies used for indirect immunoflourescence staining.

	Primary Antibody	**Secondary Antibody**
α-Smooth Muscle Actin	Mouse anti-alpha smooth muscle actin monoclonal antibody. Dilution: 1:10 Source: G.Gabbiani Department of Pathology, University of Geneva.	Rabbit anti-mouse FITC labelled monoclonal antibody. Dilution: 1:30 Source: DAKO.
α-Laminin	Rabbit anti-laminin monoclonal antibody. Dilution: 1:100 Source: GIBCO.	Swine anti-rabbit FITC labelled monoclonal antibody. Dilution: 1:30 Source: DAKO.
Collagen Type IV	Mouse anti-collagen type IV monoclonal antiboby Dilution: 1:10 Source: GIBCO.	Rabbit anti-mouse FITC labelled monoclonal antibody. Dilution: 1:30 Source: DAKO.
Controls	Nil.	The relevant FITC labelled secondary antibody.

The secondary antibodies used are shown in Table 2.,above. A trace of Evans blue was added to each secondary antibody preparation as a counter stain. Exposure to bright light was avoided during preparation and the phials were wrapped in aluminium foil.

After the excess PBS had been wiped off the slides 13 microlitres of the relevant secondary antibody were added to each section ensuring complete cover of the sections. The slides were then placed in humidified chambers and incubated in a dark room for one hour at room temperature. After completion of the incubation period the sections were again washed three times in PBS, this time allowing 15 minutes for the first wash and 10 minutes for each of the remaining two washes.

Excess PBS was then drained off the slides and two 13 microliters of Mowiol anti-fading mountant (see Appendix 2.) were added to each slide followed by a coverslip, taking care to avoid entrapment of air bubbles. The slides were then allowed to dry overnight in a drawer away from direct light. The slides were then examined under ultra violet light generated from a mercury lamp in an Zeiss Axiophot photomicroscope (Carl Zeiss Inc.,Oberkochen,FRG). The sections were analysed under X5,X20 and X40 objectives and photographs of representative areas were taken on a high quality Fuji ISA 400 35mm slide film. A diagrammatic record of all the photographed areas was also made. The sections were also examined in further detail under a Computerised Confocal Laser Microscope (BIORAD) under X20 and X40 objectives and representative images were stored in digital form (TIFF) on CD-ROM.

Section 4.

Observations And Results.

4.1 Observations On The Pattern Of Hair Regrowth After Shaving In The PVG Rat.

Hair growth above the skin surface was noted to resume as early as the second day after shaving in areas of actively growing hair (anagen). However the pattern of hair regrowth was very patchy with areas of thick hair growth interspersed amongst areas with no (telogen) or very little hair growth. There was a transitional zone between the anagen and the telogen skin regions where the hair follicles had grown less fibre than those in anagen regions,but more fibre than follicles in telogen areas.

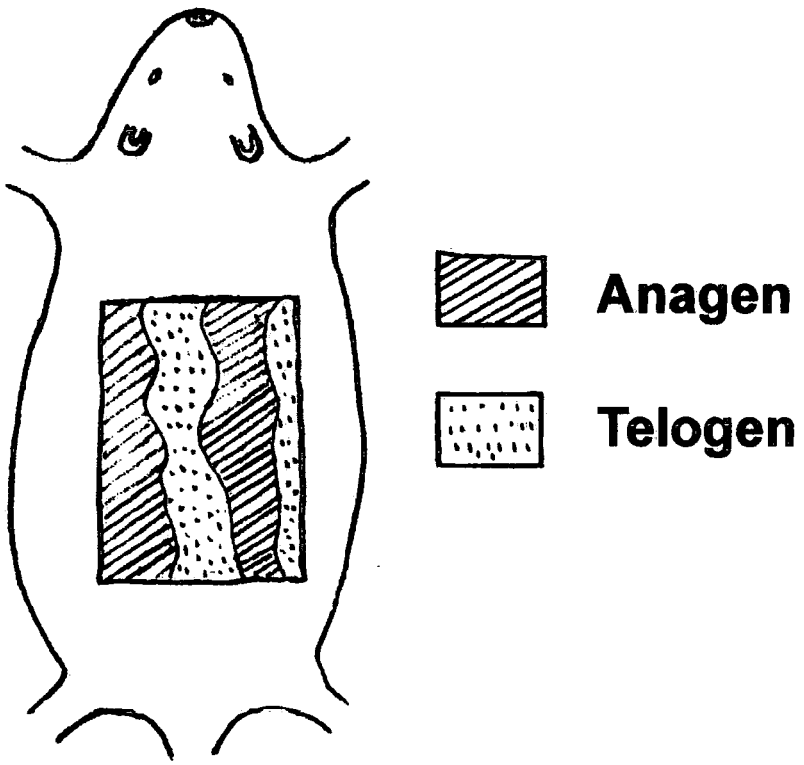
The pattern of distribution of the anagen and telogen regions was in general irregular, however in some cases it was arranged in a longitudinal pattern along the back of the rat constituting a 'wave-like' pattern as described by Zawacki and Jones (1967).In other cases the pattern of growth was patchy and irregular.The two general patterns of hair re-growth are illustrated in Figure 6. The wave type pattern of hair re-growth was characterised by the patches being long and narrow and aligned in line with the vertebral spine.The random patchy pattern had no regularity at all.These two patterns occurred with almost the same frequency:10 rats showed the patchy random pattern whilst 11 rats had the wave longitudinal pattern. In both types of hair re-growth the anagen areas blended smoothly with telogen areas across the transition zone.

Another interesting observation was the fact that not all rats from the same litter had nearly similar pattern of hair regrowth after shaving. This observation has also been previously noted by Moserova and Houskova (1989).

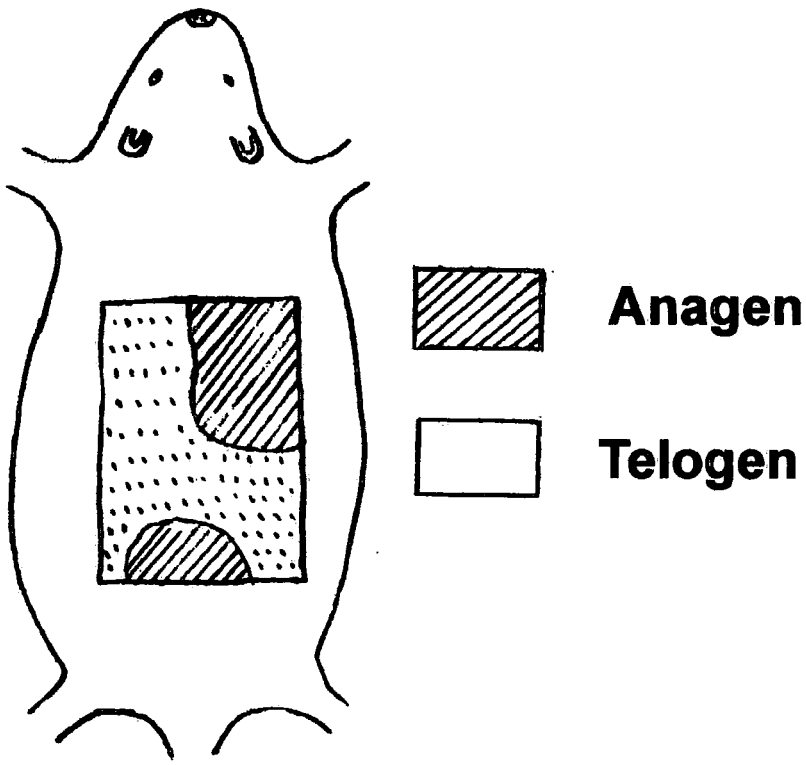
In general,inspection of the shaved areas revealed areas of overt hair growth (anagen) interspersed with areas in which there was relatively less hair growth (catagen or early telogen) or no hair growth at all (telogen). It was impossible to differentiate between areas of very slow hair growth and areas with even slower hair growth or no growth at all. For the purpose of the

Figure 6. Patterns Of Hair Regrowth.

1. 'Wave-type' Pattern Of Hair Regrowth.



2. Random Patchy Pattern Of Hair Regrowth.



experiment all these very slow growing areas had to therefore be considered as telogen. Furthermore, some areas of very slow hair growth changed their rate of hair growth in the period following wounding and created a situation where wounds that were originally applied in telogen skin were in anagen or partly anagen skin when harvested for histological examination.These observations were later verified on histological examination,where anagen follicles were noted in skin originally classified as telogen.This degree of overlap of anagen areas over telogen areas was deemed to be due to the wave-like pattern of hair regrowth (Zawacki and Jones 1967). This phenomenon made it necessarily difficult to label harvested telogen wounds as definitely telogen or telogen in the transitional phase of changing to anagen.

4.2 Wound Measurements.

The diameter of the wounds measured in parallel to the vertebral spine using the Vernier calipers to the nearest 0.05mm are shown in Table 3. below.

Table 3. Wound diameters for anagen and telogen wounds at different timescales.

PVG RAT CODE	DAY POST WOUNDING	ANAGEN WOUND 1	ANAGEN WOUND 2	TELOGEN WOUND 3	TELOGEN WOUND 4
1	2	2.1 mm	1.7 mm	2.1 mm	2.2 mm
2	2	1.8 mm	1.7 mm	2.2 mm	2.0 mm
3	2	1.7 mm	1.75 mm	2.0 mm	2.1 mm
4	3	2.0 mm	2.0 mm	2.0 mm	2.0 mm
5	3	2.0 mm	2.2 mm	1.8 mm	1.8 mm
6	3	1.8 mm	2.1 mm	1.8 mm	1.9 mm
7	5	2.0 mm	2.0 mm	2.0 mm	2.0 mm
8	5	2.1 mm	1.9 mm	2.0 mm	2.0 mm
9	5	1.75 mm	1.9 mm	1.9 mm	2.0 mm
10	7	1.0 mm	0.9 mm	1.6 mm	1.7 mm
11	7	1.3 mm	1.1 mm	1.9 mm	1.8 mm
12	7	1.0 mm	0.9 mm	1.2 mm	1.4 mm
13	8	1.0 mm	1.0 mm	1.5 mm	1.5 mm
14	8	1.4 mm	1.5 mm	1.5 mm	1.7 mm
15	8	0.8 mm	0.7 mm	1.8 mm	1.5 mm
16	10	0.75 mm	0.8 mm	1.25 mm	1.4 mm
17	10	1.0 mm	1.1mm	1.5 mm	1.6 mm
18	10	1.4 mm	1.1 mm	1.5mm	1.3 mm
19	15	0.9 mm	0.9 mm	1.2 mm	1.5 mm
20	15	0.9 mm	0.8 mm	1.1 mm	1.0 mm
21	15	0.8 mm	0.9 mm	0.9 mm	1.0 mm

Statistical analysis.

The above data was analysed statistically. The mean wound diameter for every type of wound (anagen or telogen) was calculated for each time period and the data was plotted into a histogram,Figure 7. Further detailed statistical analysis was then performed to establish whether the observations shown in Figure 7. were of any statistical significance. A 2-way ANOVA test using STATISTICA computer software was employed looking for any significant difference between the different groups. For this purpose the mean diameter of the two anagen wounds and the two telogen wounds in each rat were calculated separately and the data was used to construct a 2-way ANOVA test.A statistically significant difference between the wound diameter of anagen and telogen wounds was found.In order to establish how significant the difference was and where the significant difference was a Tukey Honest Significant Difference post hoc comparison test was performed. It was noted that the difference in wound diameter between anagen and telogen wounds was statistically significant on day 7 ($p = 0.012$) and on day 8 ($p = 0.024$). The differences in days 2,3,5,10 and 15 were not statistically significant.These results are summarised in Figure 8.

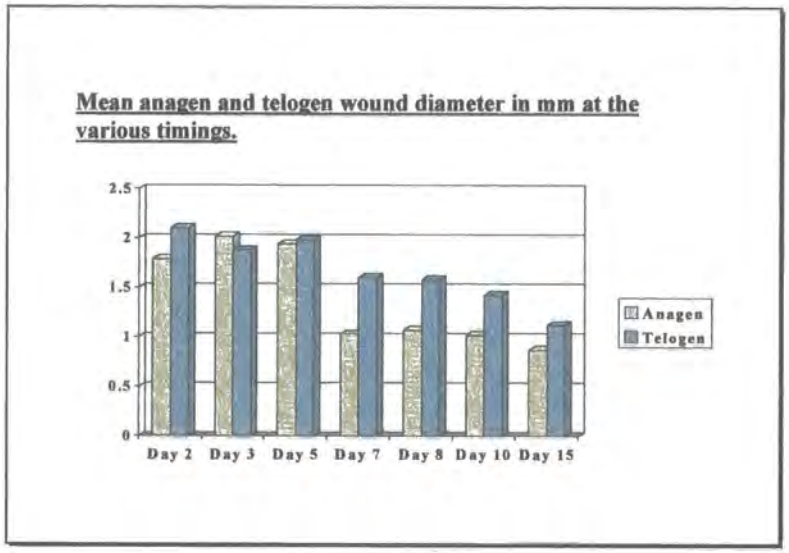


Figure: 7.

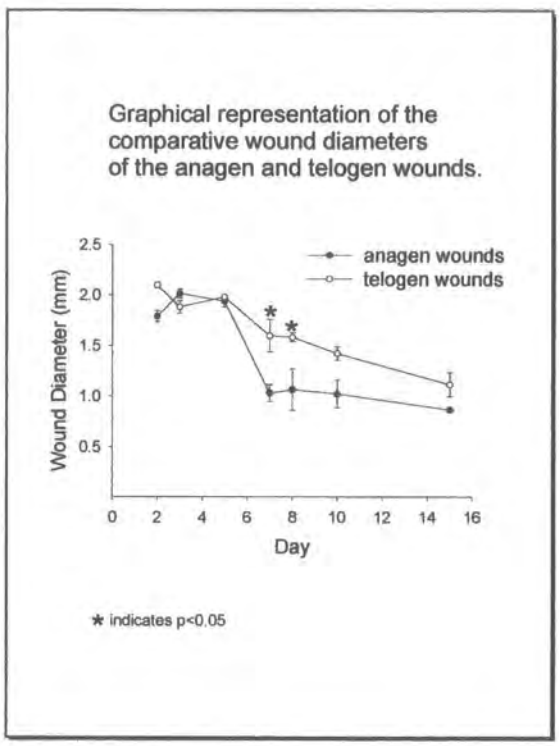


Figure: 8.

4.3 Macroscopic Observations Of The Healing Wounds.

Summary

The early wounds appeared to have a dark red scab in the depth of the cavity and the wound edges were sharp and well delineated. An area of skin erythema was also noted around the wound edges.With time the wounds appeared to become smaller in size,both in terms of diameter and in terms of the depth of the concavity.The wound edges became smoother and sloped gently into the wound crater. By day 10 the scab was shed in all the wounds and a shiny white newly formed epithelium was noted covering the still slightly depressed wound area.At this point the wounds were significantly smaller than the original wound and the edges blended smoothly with the adjacent skin and were hard to delineate.A most interesting observation was the fact that no hair follicles were observed in the centre of the mature wounds;hair follicles were observed growing into the edges of these wounds.The only macroscopic difference that was noted between anagen and telogen wounds was the fact that in the later stages of wound healing the anagen wounds showed a greater amount of hair regrowth in the outer periphery of the wounds as compared to telogen wounds.

Day 2 Wounds.

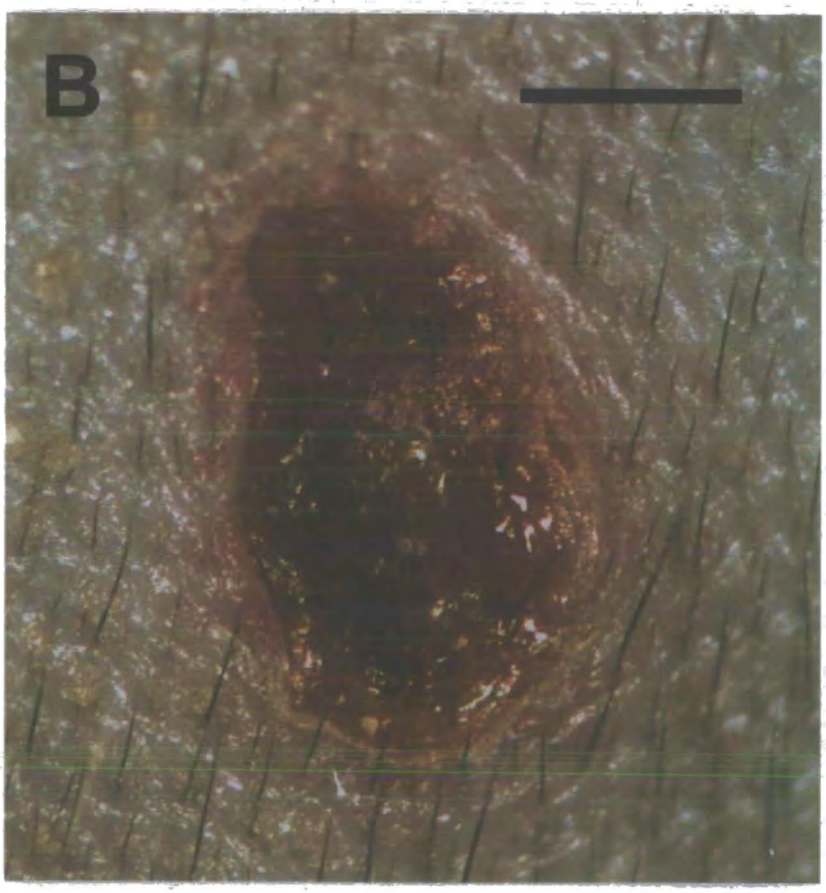
At day 2 the wound area was covered with a bright red scab filling the depths of the cavity. There was no visible difference in size or shape between wounds in anagen and wounds in telogen skin. The wound edges were sharply delineated from the adjacent healthy skin and dropped down steeply to the base of the wound crater.At this stage the wounds were as deep as the full thickness of the skin.There was erythema in the skin around the wound indicating the presence of an inflammatory reaction.The only observed difference between anagen and telogen wounds at this stage was the fact that in anagen skin thick hair fibres were present right to the edge of the wound (Figure 9 A) whilst in telogen wounds the relatively thin fibres were generally not visible as close to the wound edge (Figure 9 B).

Figure 9. Macroscopic Appearance Of Day 2 Wounds.

A. Day 2 wound in anagen skin showing the wound scab and hair fibres right at the edge of the wound. (Scale bar = 500μ).

B. Day 2 wound in telogen skin showing the wound scab and lack of hair fibres at the edge of the wound. (Scale bar = 500μ).
(The small hair follicle end bulbs in the wound as well as the surrounding fine hairs suggest that this area of skin is going into early anagen).


A


B

Day 3 Wounds.

At day 3 the wound scab was darker red in colour.The wound edges still appeared sharp and well delineated from the adjacent unwounded skin.However the shape of the wound crater was becoming more sloping and more concave in shape as compared to the steep edges observed at day 2.The depth of the wound was still as deep as the full thickness of undamaged skin.No surrounding erythema was noted at this stage and there was no macroscopic difference in size,shape or colour between wounds in anagen skin (Figure 10 A) and wounds in telogen skin (Figure 10 B).

Day 5 Wounds.

At day 5 the peripheral parts of the scab were noted to have been shed and replaced by the shiny newly formed epithelium.The central part of the scab remained dark red in colour.The wound edges were now less distinct and had started to blend into the surrounding unwounded skin.Furthermore the wounds were noted to be less deep and the slopes of the concavity were smoother.
No macroscopic difference in wound size was noted in comparison to wounds of earlier stages.There was no hair regrowth in the outer peripheral newly epithelialized areas of the wounds in both anagen (Figure 10 C) and telogen wounds (Figure 10 D).No macroscopic differences were noted between the latter wounds.

Day 7 Wounds.

At day 7 the central red scab part of the wound had decreased in size as the white epithelialized peripheral area became larger.In general the crater of the wound was noted to be shallower than in earlier wounds with the wound edges having even smoother slopes.The wound edges blended in smoothly with the surrounding skin.It was also noted that hair follicle regrowth had started to recover at the peripheries of the wounds.This was most noticeable in anagen wounds (Figure 10 E) as compared to telogen wounds (Figure 10 F).

Figure 10. Macroscopic Appearance Of Day 3,Day 5 And Day 7 Wounds.

A. Day 3 wound in anagen skin showing the dark red wound scab. (Scale bar = 500μ)

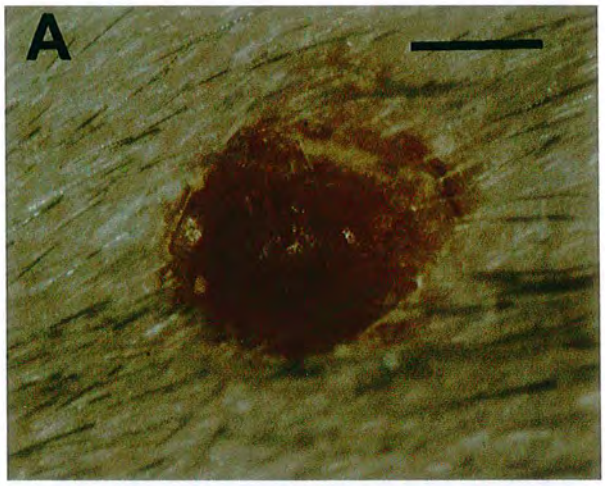
B. Day 3 wound in telogen skin showing the dark red wound scab. (Scale bar = 500μ)

C. Day 5 wound in anagen skin showing a dark red central part and shiny newly formed epithelium at the edges. (Scale bar = 500μ)

D. Day 5 wound in telogen skin showing a dark red central part and shiny newly formed epithelium at the edges.. (Scale bar = 500μ)

E. Day 7 wound in anagen skin showing a smaller central scab,a shallower crater and smooth merging of the wound edge with the surrounding skin. (Scale bar = 500μ)

F. Day 7 wound in telogen skin showing a smaller central scab,a shallower crater and smooth merging of the wound edge with the surrounding skin. (Scale bar = 500μ)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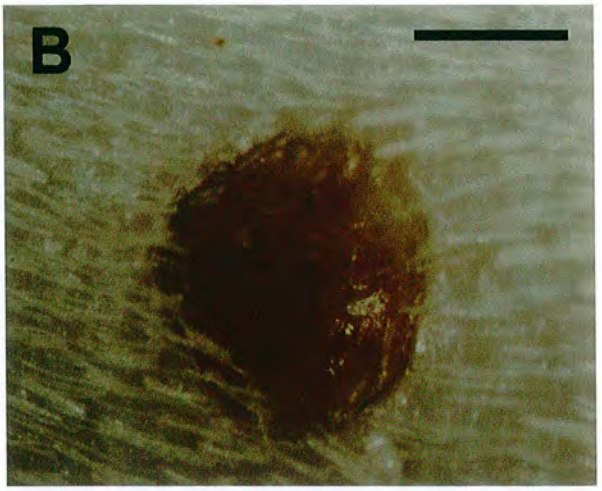
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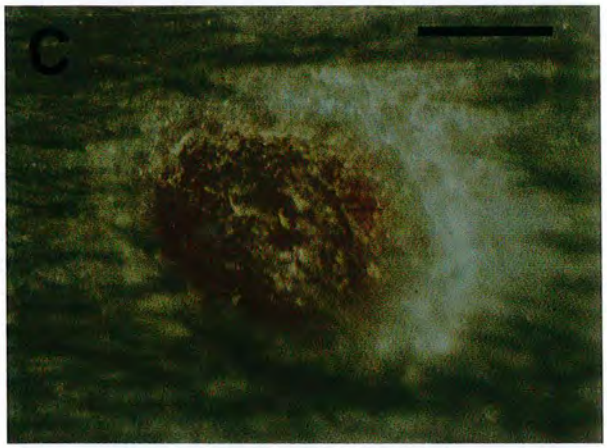
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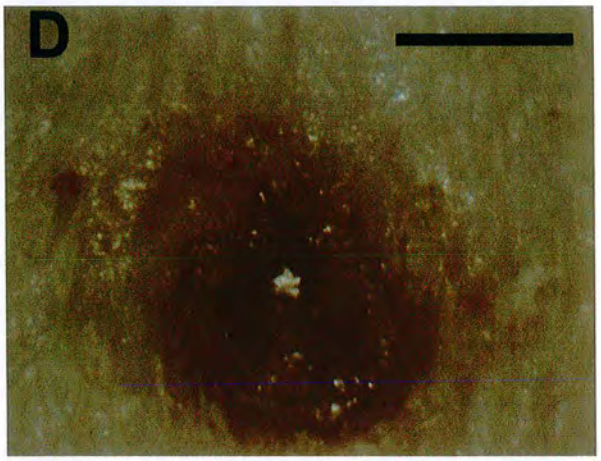
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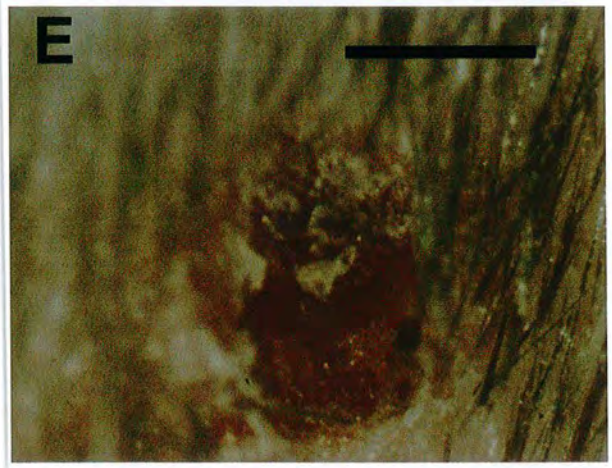
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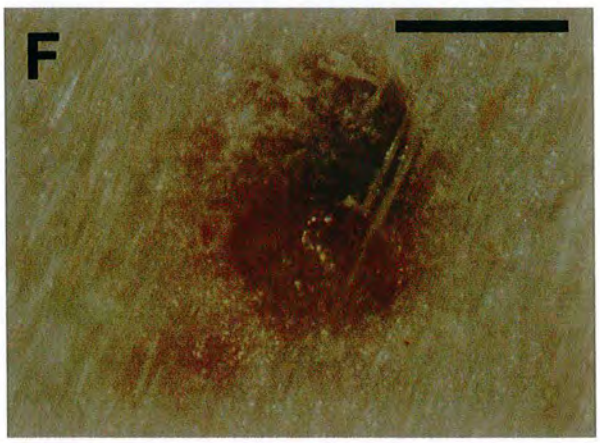
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Day 8 Wounds.

The day 8 wound had an even smaller lighter red scab in the centre and a larger shiny white halo.The wound was now only a shallow depression and the edges were smooth and merged into the surrounding skin.A decrease in wound size as compared with wounds of earlier timescales was now macroscopically obvious.At this stage no macroscopic morphological difference was observed between anagen (Figure 11 A) and telogen (Figure 11 B) wounds.

Day 10 Wounds.

By day 10 the wound scab had all been shed and the entire wound surface was covered with the new epithelium.The wound was now shallower and the wound edges were hard to distinguish from the adjacent skin.Hair regrowth in the outer peripheral parts of the wound was now more pronounced especially in anagen wounds (Figure 11 C) as compared to telogen wounds (Figure 11 D). These hair follicles appeared to be sprouting out of the white newly formed epithelium and were therefore follicles regenerating from injured follicles at the edge of the wound or arising de novo.

Day 15 Wounds.

At day 15 the wound area was difficult to locate with the naked eye,however it was relatively easy to identify under the operating microscope at X10 magnification.The healed wound area was now level with the adjacent skin and had no central depression.The size of the wound now appeared significantly smaller than at earlier stages.Hair regrowth in the outer peripheral parts of the wound was even more pronounced,and was significantly more in anagen wounds (Figure 12 A) compared with telogen wounds (Figure 12 B).

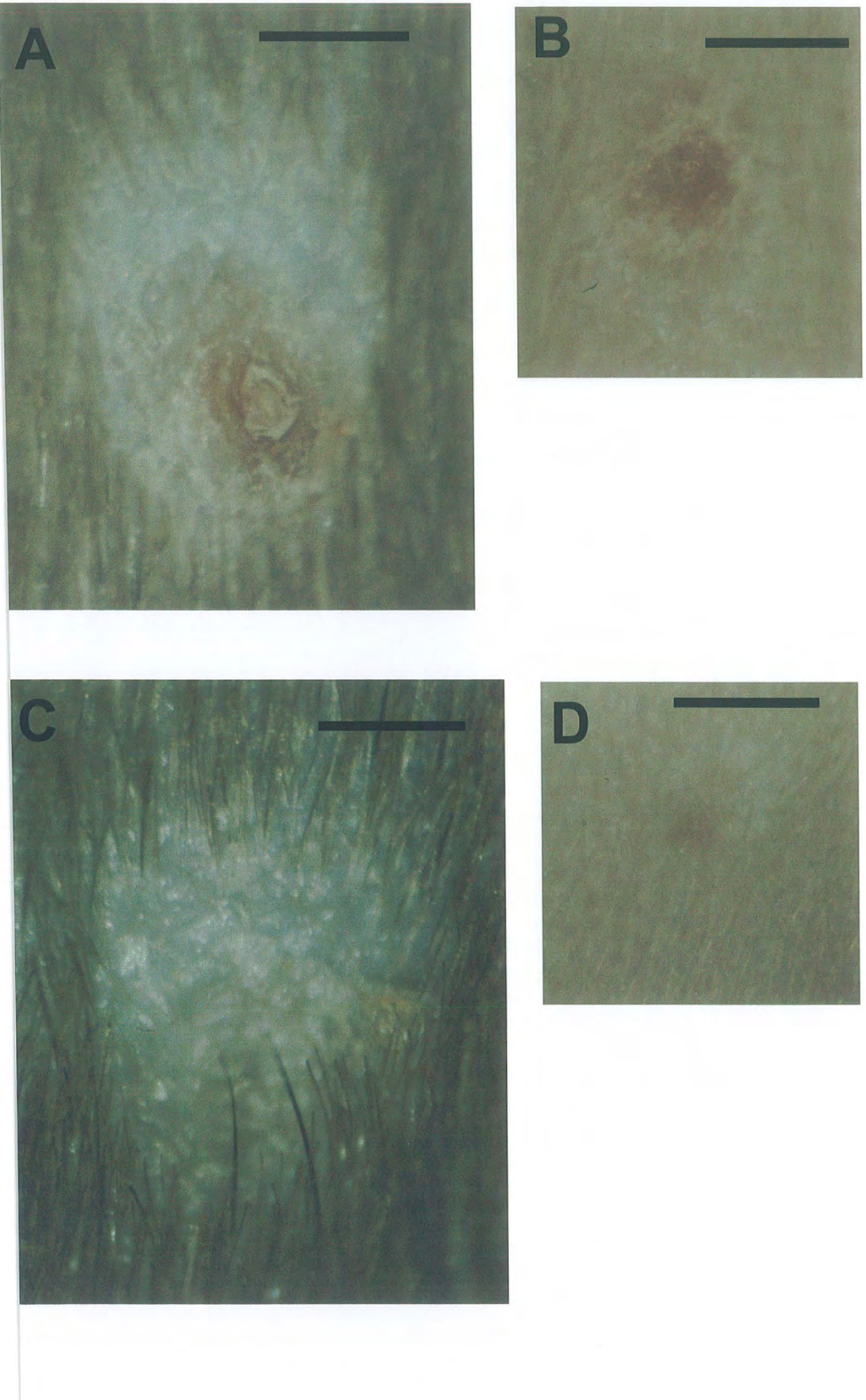
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Figure 11. Macroscopic Appearance Of Day 8,And Day 10 Wounds.

A. Day 8 wound in anagen skin.The wound is now smaller in size and has a lighter red scab in the centre and a larger shiny white halo at the periphery (Scale bar = 500μ).

B. Day 8 wound in telogen skin.The wound is now smaller in size and has a lighter red scab in the centre and a larger shiny white halo at the periphery (Scale bar = 500μ).

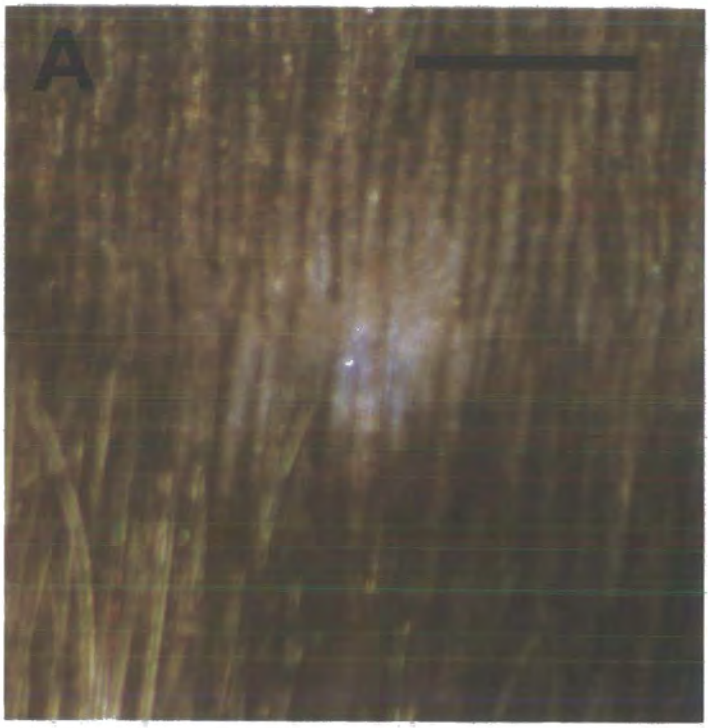
C. Day 10 wound in anagen skin.All the wound scab has been shed and the surface of the wound is now covered by the newly formed epithelium.Note the hair regrowth at the periphery of the wound (Scale bar = 500μ).

D. Day 10 wound in telogen skin.All the wound scab has been shed and the surface of the wound is now covered by the newly formed epithelium (Scale bar = 500μ).

Figure 12. Macroscopic Appearance Of Day 15 Wounds.

A. Day 15 wound in anagen skin.The wound is now greatly reduced in size and has no central depression (Scale bar = 500μ).

B. Day 15 wound in telogen skin.The wound is now greatly reduced in size and has no central depression (Scale bar = 500μ).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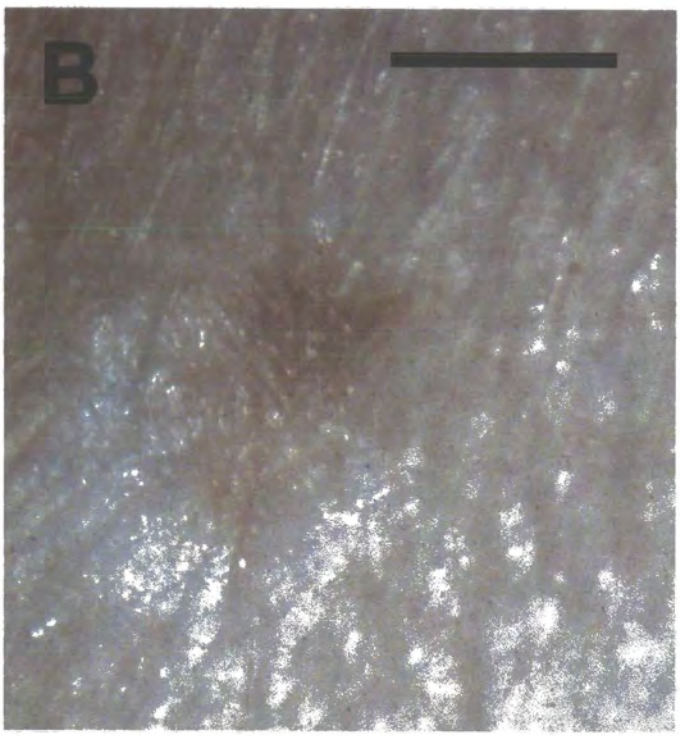
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4.4 Observations On Weigert's Haematoxylin Stained Histological Sections.

Summary

Microscopic examination of the sections stained with Weigert's Haematoxylin revealed no detectable histological difference in the wound healing mechanism between the healing in wounded anagen skin and telogen skin.However some observations were made that differed in the two types of skin.Anagen skin was noted to be significantly thicker than telogen skin and the difference in thickness was even evident on naked eye inspection of the histological sections.Furthermore it was noted that in anagen skin the hair follicle bulbs were significanly larger and situated deeper in the dermis than in telogen skin (the latter may be due to the difference in skin thickness).

Day 2 Weigert's Haematoxylin Staining.

The most superficial layer of the wound was composed of a fibrin scab (Figure 13 A).The texture of the wound infiltrate at this point was very fragile and friable and it was very difficult to cut sections preserving the wound infiltrate.The skin epithelium at the edge of the wound was noted to be hypertrophied when compared to the epithelium away from the wound.This corresponded with an increase in mitotic figures. At day 2 the wound area was noted to be largely filled with red blood cells,from the original wound haematoma, and an infiltrate of mononulear cells (Figure 13 B).In the dermis the inflammatory infiltrate was irregularly scattered amongst the red blood cells of the original wound haematoma (Figure 13 C).Moreover there was a higher density of inflammatory mononuclear cells at the wound edge;the infiltrate being most intense at the base of the wound (Figure 13 C).The inflammatory infiltrate was made up of a heterogeneous mixture of cells namely mast cells (stained light blue),macrophages and lymphocytes (Figure 13 A).The fibrin network of the wound haematoma stained light pink and was irregularly distributed within the wound.In the depths of the wound,the fibrin network seemed to merge smoothly with the connective tissue of the panniculus carnosus (Figure 13 D).At a more superficial level in the wound it was noted to merge with the dermal connective tissue at the edge of the wound.The hair follicles at the edge of the

Figure 13. Day 2 Histological Sections Stained With Weigert's Haematoxylin.

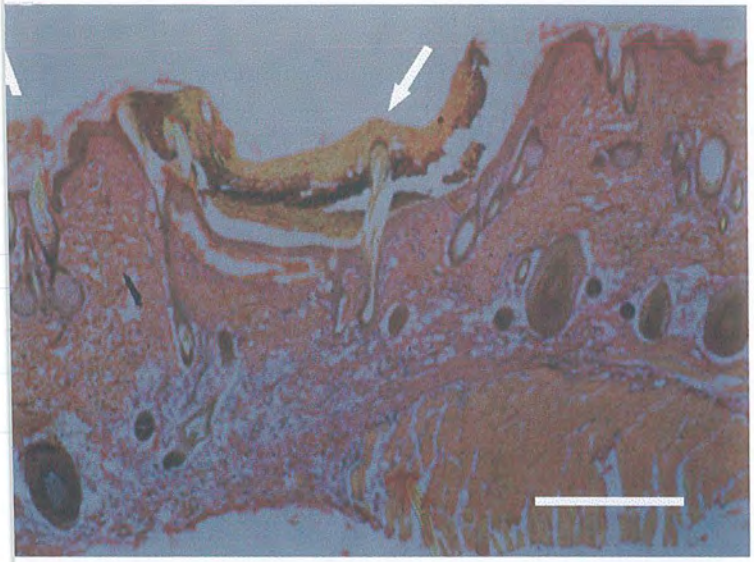
A. Low power histological section through a day 2 wound to show the general architecture of the wound at this stage.Arrow pointing at wound scab. *Weigert's Haematoxylin staining.* (Scale bar = 400μ).

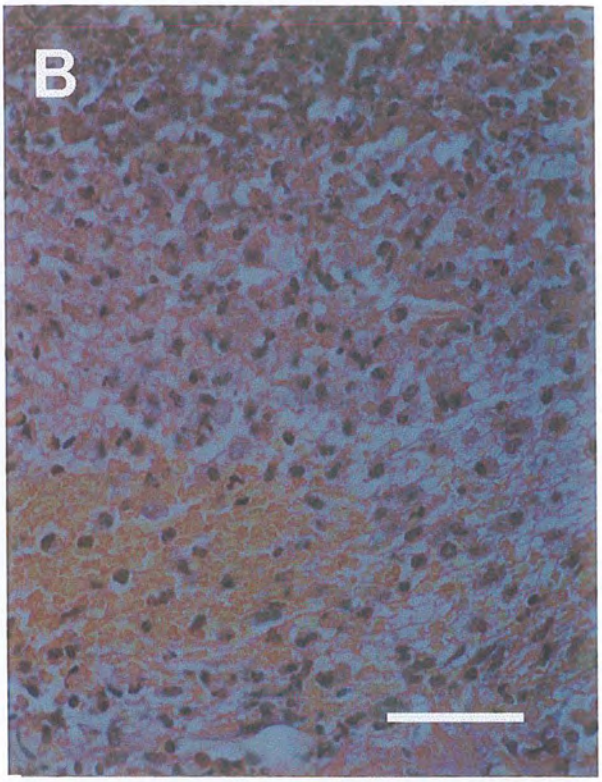
B. High power section through wound tissue showing the wound haematoma and mononuclear cells. *Weigert's Haematoxylin staining.* (Scale bar = 50μ).

C. Section through the dermis adjacent to wound to show the inflammatory infiltrate. *Weigert's Haematoxylin staining.* (Scale bar = 100μ).

D. High power section through the deeper parts of the day 2 wound and underlying panniculus carnosus to show the cellular infiltrate at this level. *Weigert's Haematoxylin staining.* (Scale bar = 50μ).

E. Section through a hair follicle at the edge of the wound showing normal hair follicle architecture. *Weigert's Haematoxylin staining.* (Scale bar = 100μ).

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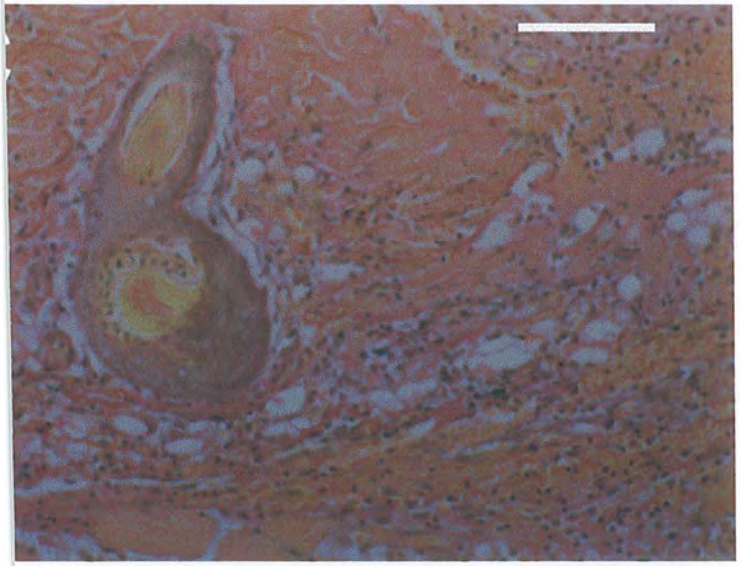

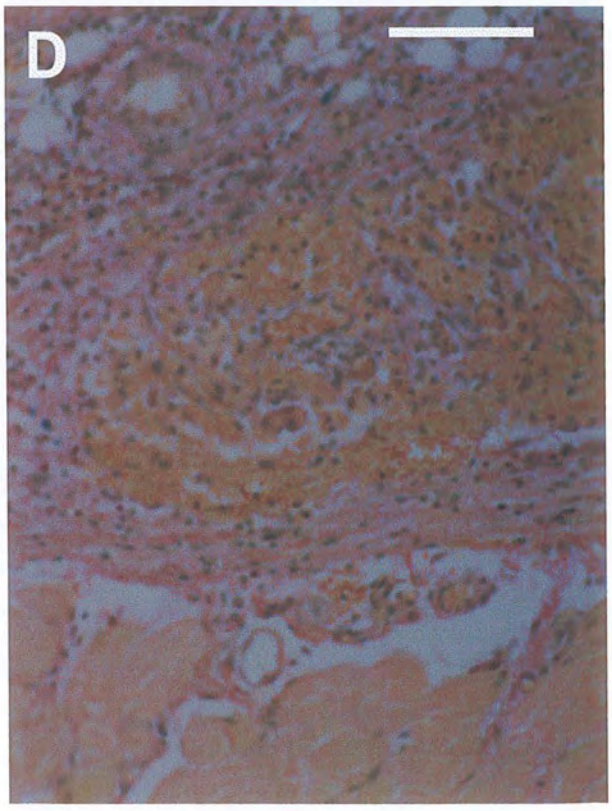
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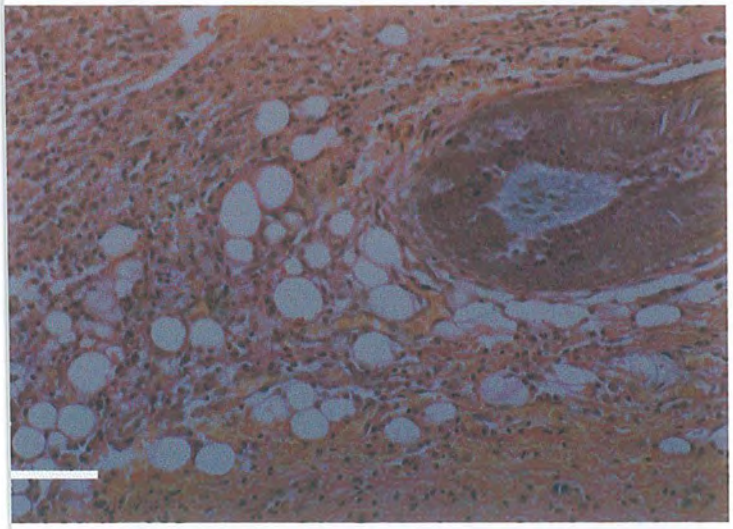


Figure 14. Day 2 Histological Sections Stained With Weigert's Haematoxylin.

A. Histological section through the bulb/base of a hair follicles at the wound edge to show the surrounding mononuclear cell infiltrate.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

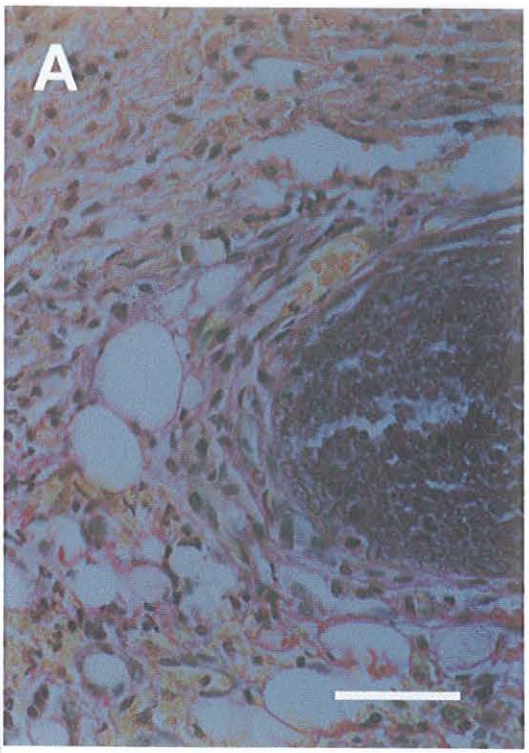
B. Section showing hair follicle elements/fragments (arrow) in the wound itself.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

C. Section through a hair follicle fragment with the wound tissue to show the intact dermal sheath (arrow).
Weigert's Haematoxylin staining. (Scale bar = 100μ).

wound at this stage were essentially similar to hair follicles away from the wound (Figure 13 E).However a mild to moderate mononuclear inflammatory cell infiltrate was noted around these follicles.On closer inspection this infiltrate seemed to be more concentrated around the bulb/base of the follicles (Figure 14 A).No anatomically intact hair follicles were noted in the wound area;however some follicle fragments or bases of follicles the upper parts of which had been amputated during the wounding process were observed (Figure 13 C and Figure 14 B).The follicle remnants had a normal dermal sheath (Figure 14 C) which was surrounded by connective tissue.This was in turn surrounded by a dense monocuclear inflammatory infiltrate.The dermal sheath was identified as a separate structure from the surrounding inflammatory infiltrate.Again,an increase in the intensity of the inflammatory infiltrate around these follicle fragments was noted.No signs of new blood vessel formation were noted within the wound area.The only blood vessels observed were situated at the base of the wound.

Day 3 Weigert's Haematoxylin Staining.

A day 3 the epithelium was noted to lie underneath the superficial scab (Figure 15 A) which was still prominent.The epithelial covering of the wound had advanced significantly,covering the entire wound surface (Figure 15 B). The epithelium at the edge of the wound (Figure 15 A) was noted to be more hypertrophied than in the day 2 wound.The epithelial component of the hair follicles (outer root sheath) at the wound edges (Figure 15 C) was also hypertrophied.The cellular infiltrate remained predominantly mononuclear, however it was more intense and there were fewer red blood cells left from the original haemostatic plug.The wound mononuclear cell infiltrate had a general grey background appearance as a result of the cytoplasm staining grey (Figure 15 A).The nuclei were very prominent and stained dark (black).Mast cells remained prominent at the base of the wound (Figure 15 D) and around the bases of hair follicles adjacent to the wound.There was a similar blending of the wound fibrin network with the connective tissue of the wound edges (Figure15 B) and base,as noted in the day 2 wound.Follicles or follicle elements within the wound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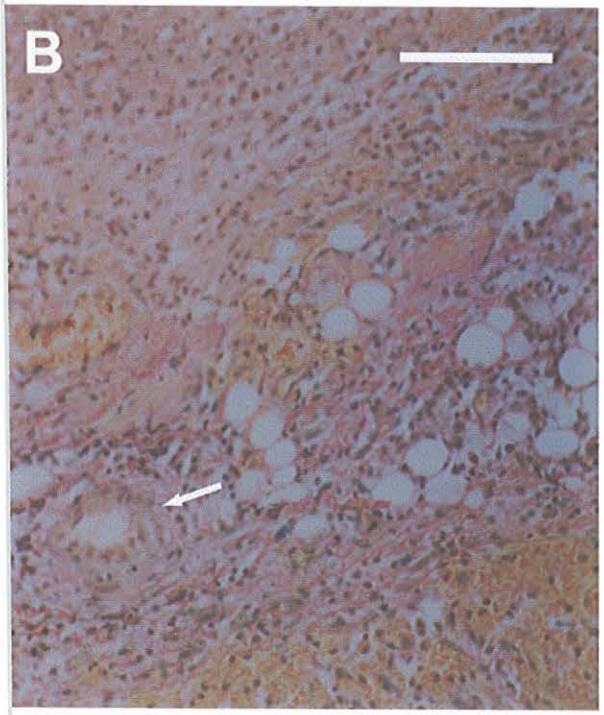
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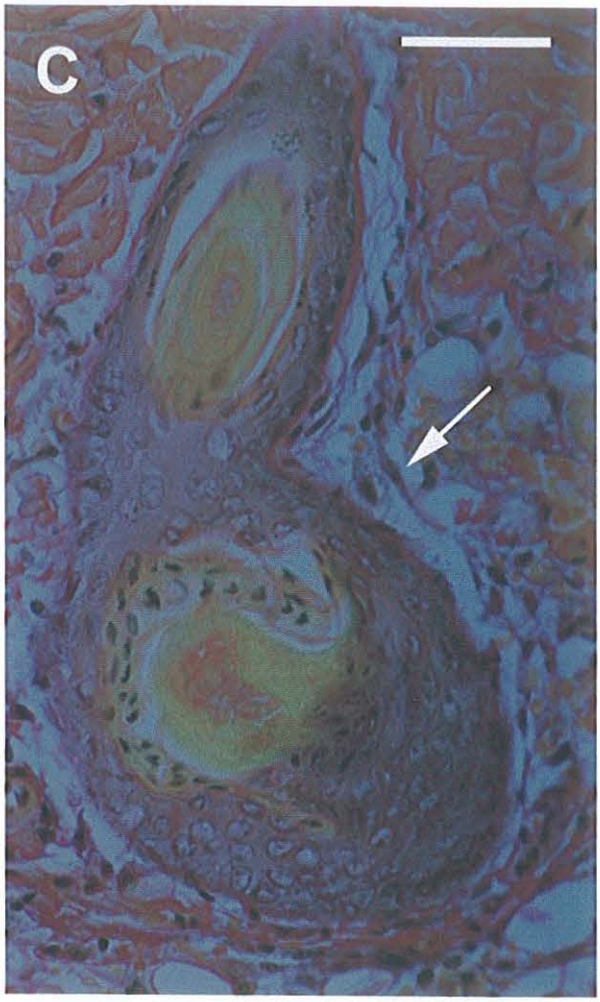
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Figure 15. Day 3 Histological Sections Stained With Weigerts' Haematoxylin.

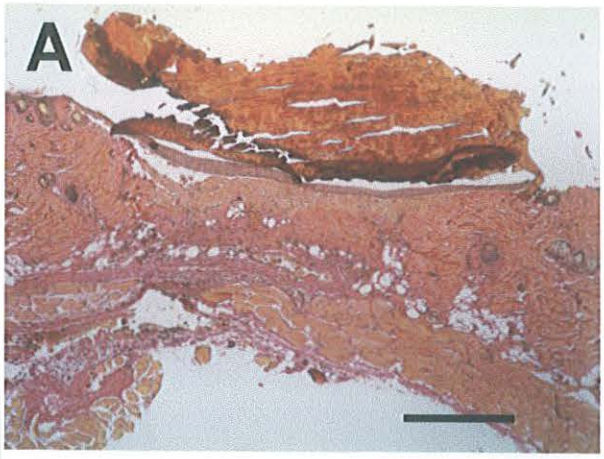
A. Low power section through a day 3 wound showing the newly formed wound epithelium underneath the scab.
Weigert's Haematoxylin staining. (Scale bar = 400μ).

B. Histological section showing the new wound epithelium in further detail and the blending of the wound collagen fibrils (pink) with the dermis at the wound edge.
Weigert's Haematoxylin staining. (Scale bar = 100μ).

C. Section showing hair follicle elements at the wound edge with hypertrophied outer root sheath.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

D. High power section through the base of the wound showing mast cells.(indicated by the arrows).
Weigert's Haematoxylin staining. (Scale bar = 50μ).

E. High power transverse section through a hair follicle within the wound substance. Note the early signs of blending of the dermal sheath with the neighbouring fibroblast network.
Weigert's Haematoxylin staining. (Scale bar = 50μ).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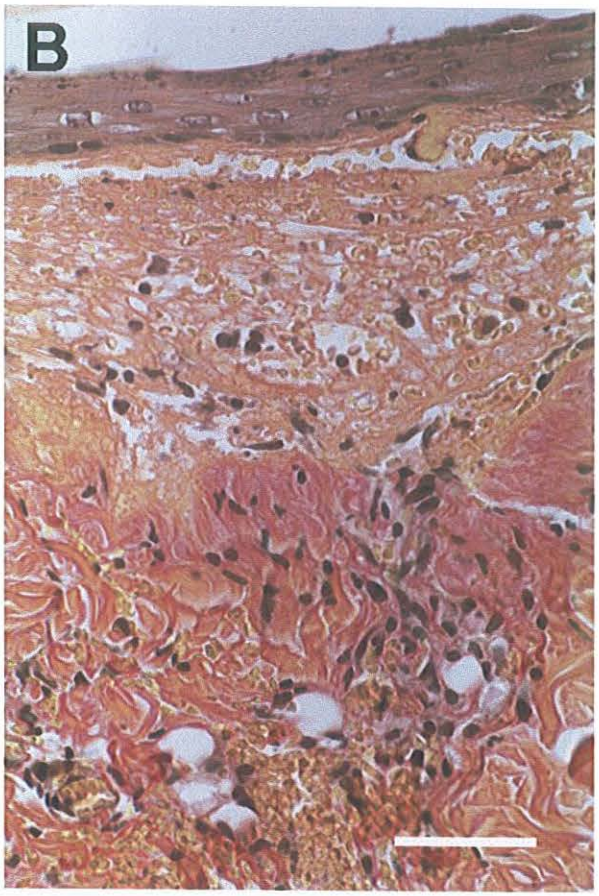
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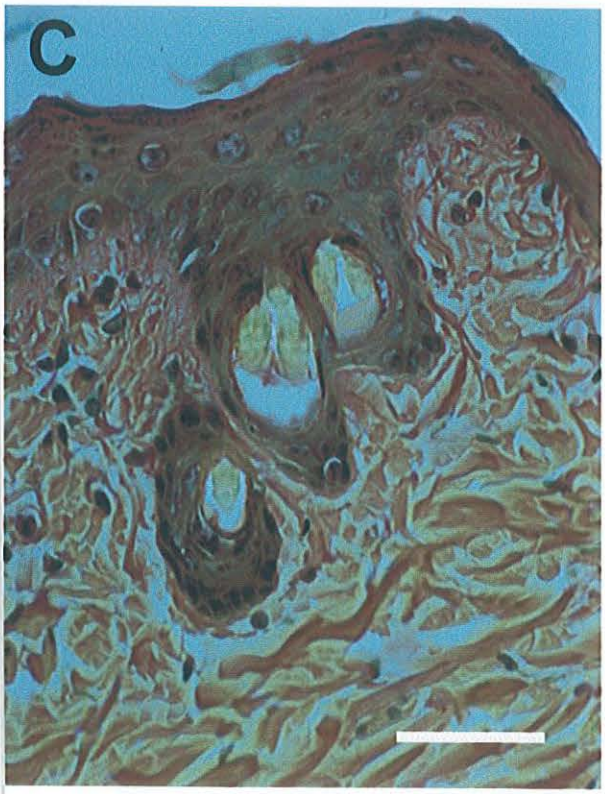
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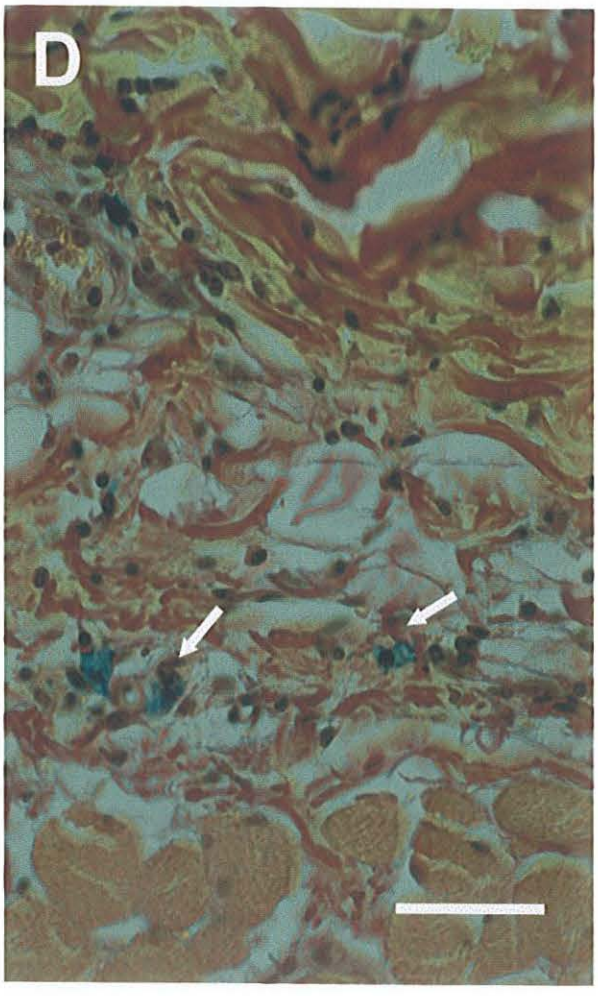
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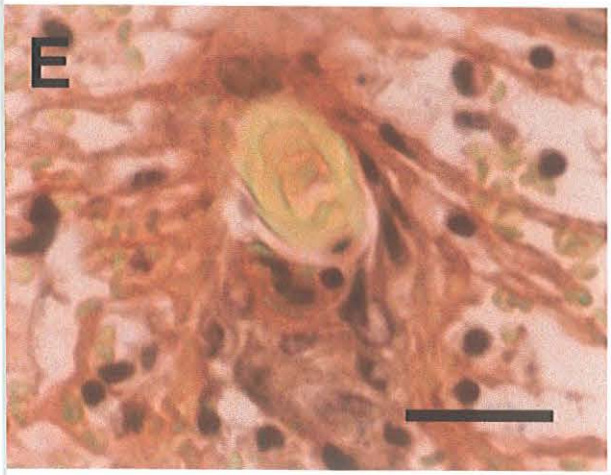
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showed early signs of the dermal sheath blending /merging with the surrounding wound fibroblast network (Figure 15 E).

The lower part of the dermal sheath of hair follicles near the wound was hypertrophic and composed of multiple layers of pink staining cells (Figure 16 A).Furthermore the dermal sheath (pink staining) appeared to have possibly radiated into and blended with the adjacent connective tissue (Figure 16 B).In hair follicles in contact with the wound edge the dermal sheath was more hypertrophied with multiple layers of cells radiating and blending into the adjacent connective tissue and merging into the wound infiltrate (Figure 16 C).The wound mononuclear infiltrate was more intense around hair follicles at the edge of the wound (Figure 16 D).In contrast,follicles away from the wound showed a regular dermal sheath (Figure 16 E) composed of one layer of cells with no signs or tendency to radiate out and there was no surrounding mononuclear infiltrate.

Day 5 Weigert's Haematoxylin Staining.

At day 5 the wound tissue was less friable in texture and relatively easier to cut.The epithelial cover of the wound was complete (Figure 17 A) though the wound epithelium still looked very irregular and hypertrophic (Figure 17 B).The hair follicles close to the wound area were noted to have hypertrophied outer root sheath in continuity with the hypertrophied wound epithelium (Figure 17 C).The superficial scab had become thinner at this stage was partly shed in some wounds.The cellular wound infiltrate was now very intense with a general homogeneous appearance (Figure 17 D and E).The cellular component was largely composed of spindle shaped cells,with a pink to grey staining cytoplasm and a dark nucleus, and of round mononuclear cells with a grey cytoplasm and a prominent black staining nucleus.The latter cells had a high nucleus to cytoplasm ratio.Moreover the spindle shaped cells were arranged in a regular pattern constituting a uniform network (Figure 17 F). Practically no red blood cells were present in the wound infiltrate at this stage.Further microscopic examination revealed a good number of hair follicle fragments or injured hair follicles in the substance of the wound itself (Figure 17 F).A striking and constant

Figure 16. Day 3 Histological Sections Stained With Weigert's Haematoxylin.

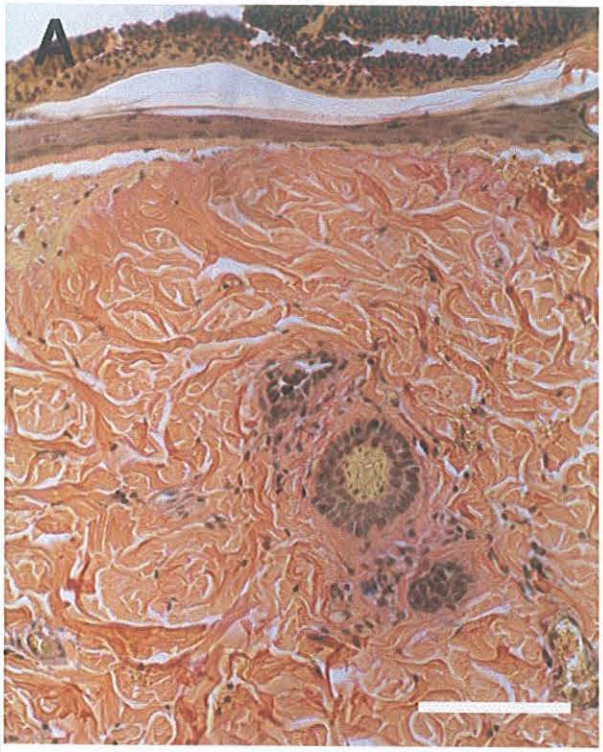
A. Section through dermis close to the wound edge to show the hypertrophied dermal sheath in follicles close to the wound.
Weigert's Haematoxylin staining. (Scale bar = 100μ).

B. High power section showing the hair follicle shown in **A.** to indicate the hypertrophied dermal sheath possibly radiating into the adjacent connective tissue of the dermis.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

C. Section showing hair follicles at edge of wound (w) with hypertrophied dermal sheath.
Weigert's Haematoxylin staining. (Scale bar = 100μ).

D. Section showing the intense mononuclear infiltrate around follicles at edge of Wound (w).
Weigert's Haematoxylin staining. (Scale bar = 100μ).

E. Histological section of a follicle well away from the wound to show the regular normal dermal sheath for comparison purposes.
Weigert's Haematoxylin staining. (Scale bar = 50μ).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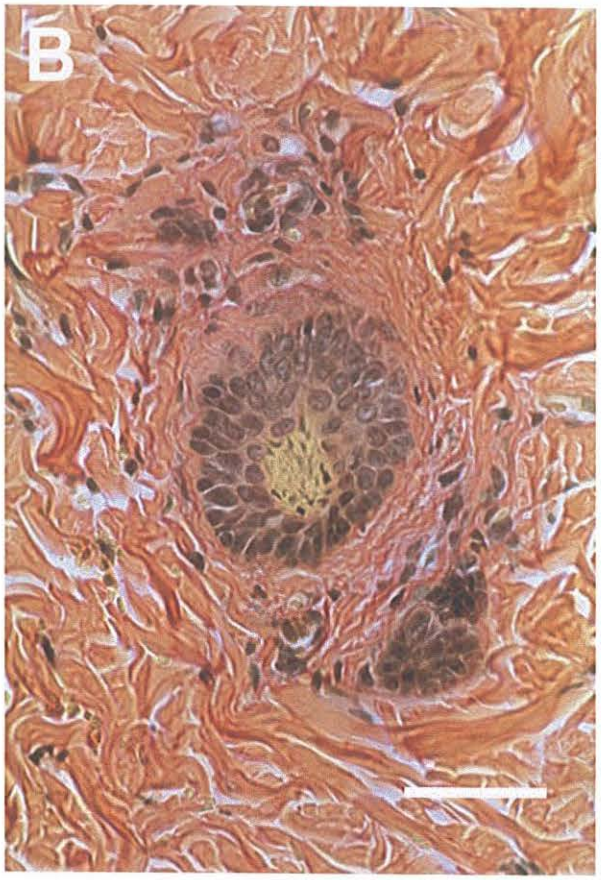
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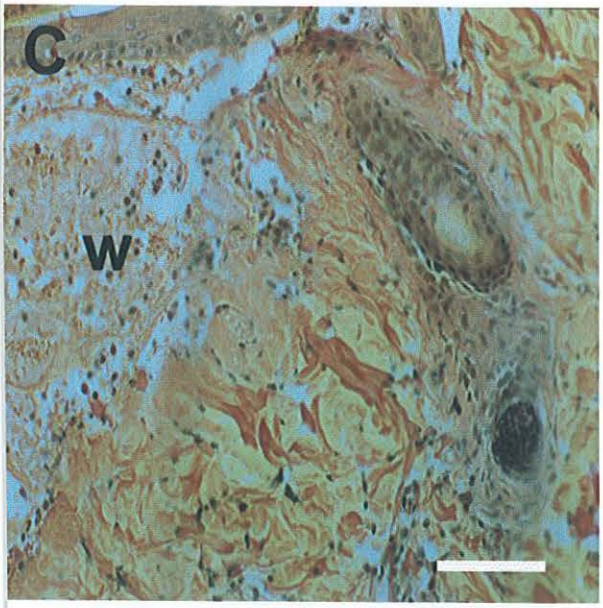
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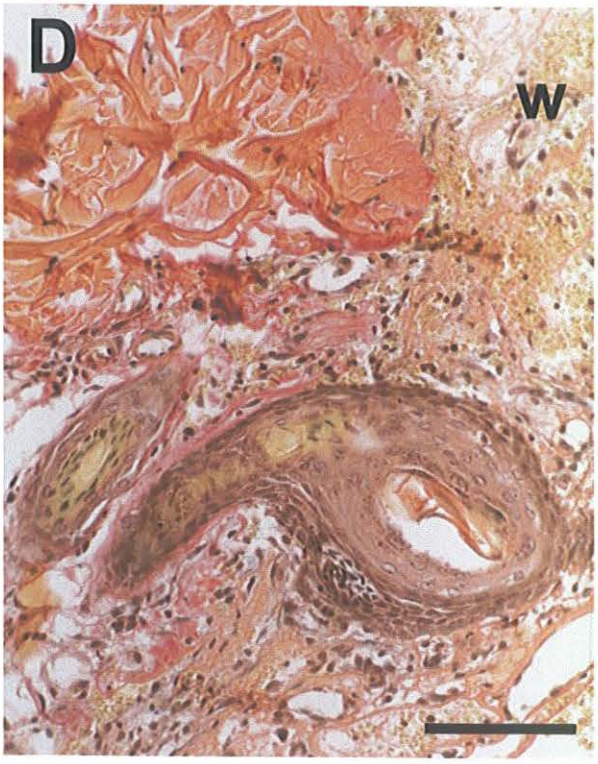
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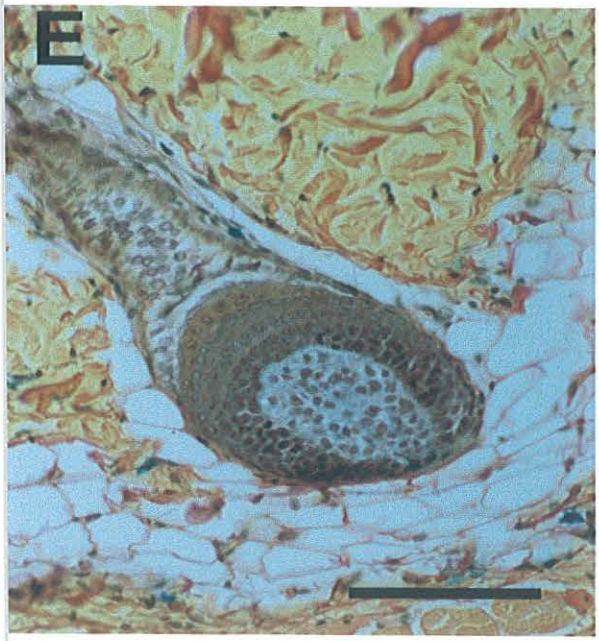
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Figure 17. Day 5 Histological Sections Stained With Weigert's Haematoxylin.

A. Low power section to show the general architecture of the day 5 wound.
Weigert's Haematoxylin staining. (Scale bar = 400μ).

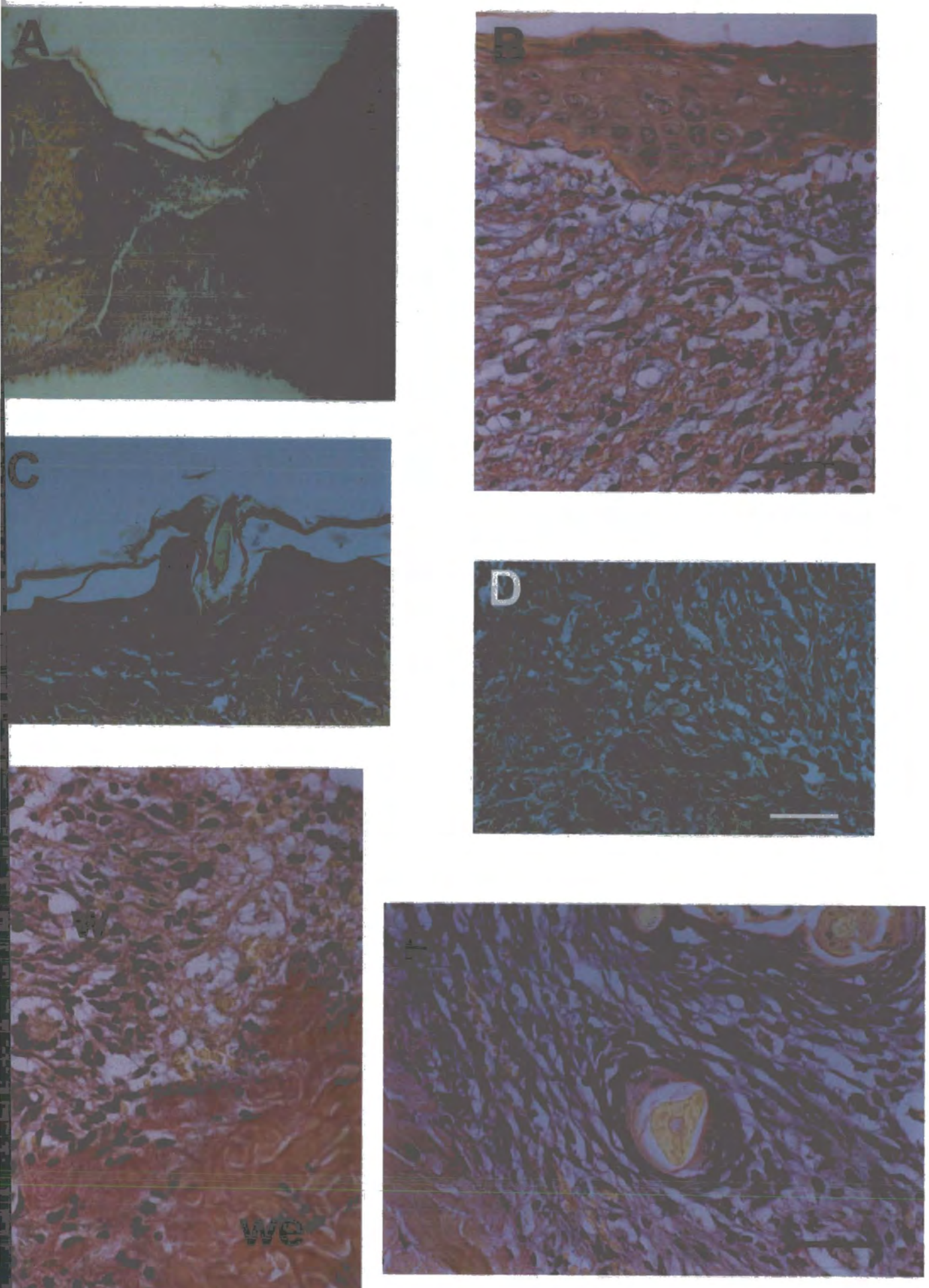
B. High power view of the day 5 hypertrophied wound epithelium.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

C. Section showing a hair follicle element at edge of wound with hypertrophied outer root sheath in continuity with the hypertrophied wound epithelium.
Weigert's Haematoxylin staining. (Scale bar = 100μ).

D. A high power section through the wound infiltrate to show mononuclear cells and fibroblasts.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

E. A high power view of the wound (w) infiltrate close to the wound edge (we).
Weigert's Haematoxylin staining. (Scale bar = 50μ).

F. Section through a hair follicle fragment within the wound surrounded by dermal sheath cells/fibroblasts in a uniform pattern.
Weigert's Haematoxylin staining. (Scale bar = 50μ).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feature was that the dermal sheath of hair follicles or follicle fragments deep within the wound substance was now merged and integrated with the cellular network of the wound tissue (Figure 17 F and Figure 18 A and B).The dermal sheath cells and the wound fibroblasts around the follicles appeared very similar in morphology,however the arrangement of the cells around the hair follicle was quite distinct from the arrangement of the wound fibroblasts in the rest of the wound.Infact the cells around the follicle were arranged in a concentric or possibly spiral pattern highly suggestive of the possibility that these cells were dermal sheath cells that have multiplied and radiated out into the wound tissue.The concentric pattern in which the dermal sheath cells or wound fibroblasts (as it was not possible to differentiate morphologically between the two) aligned themselves around the hair follicles in the deeper parts of the wound is reminiscent of hair follicle dermal sheath patterning (Figure 18 A and B).Follicles deep within the wound,whether in the centre of the wound substance or in the base of the wound,showed this constant typical appearance.In hair follicles at the edge or close to the edge of the deep parts of the wound, the dermal sheath was hypertrophied and showed a tendency to radiate into and or blend with the adjacent tissue (Figure 18 C).Follicles precisely at the edge of the wound displayed a very distinctive pattern.On the side of the hair follicle in contact with the wound tissue,the dermal sheath was not distinguishable anymore as it blended with the wound fibroblasts whilst on the side of the follicle in contact with the uninjured tissue the dermal sheath was hypertrophied and possibly radiating in pattern (Figure 18 D).At the edge of the wound a concentration of pink staining fibrin fibres (Figure 18 E) linking the wound infiltrate with the adjacent tissue was observed.

Day 7 Weigert's Haematoxylin Staining.

At day 7 the wound was shaped like an hour glass (Figure 19 A).This indicated that the forces of wound contraction have started to work at this stage.The wound surface epithelium remained hypertrophic but was smoother and more regular than in earlier wounds.The inflammatory infiltrate showed a decrease in density of mononuclear cells whilst there

Figure18. Day 5 Histological Sections Stained With Weigert's Haematoxylin.

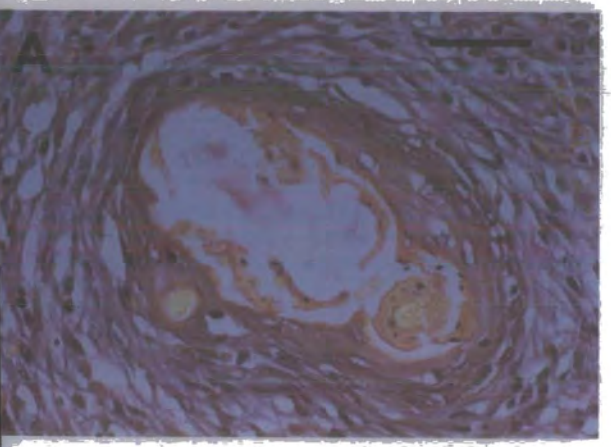
A. High power section showing a hair follicle deep in the wound surrounded by dermal sheath cells/fibroblasts arranged in a concentric pattern.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

B. Low power view of hair follicle in the wound shown in **A.** surrounded by dermal sheath cells/fibroblasts aligned in a concentric pattern.
Weigert's Haematoxylin staining. (Scale bar = 100μ).

C. Histological section showing a hair follicle at the edge of the wound.Note that the dermal sheath (arrows) is blended with the adjacent connective tissue.
Weigert's Haematoxylin staining. (Scale bar = 100μ).

D. Section showing a hair follicle right at the edge of the wound (w) with the dermal sheath merged with the wound fibroblasts and with the dermis of the wound edge.
Weigert's Haematoxylin staining. (Scale bar = 100μ).

E. High power section showing pink staining fibrin fibres at edge of the wound linking the wound infiltrate with the adjacent dermis.
Weigert's Haematoxylin staining. (Scale bar = 50μ).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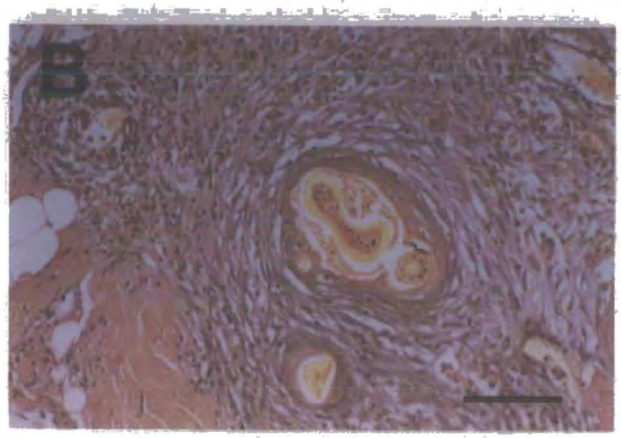
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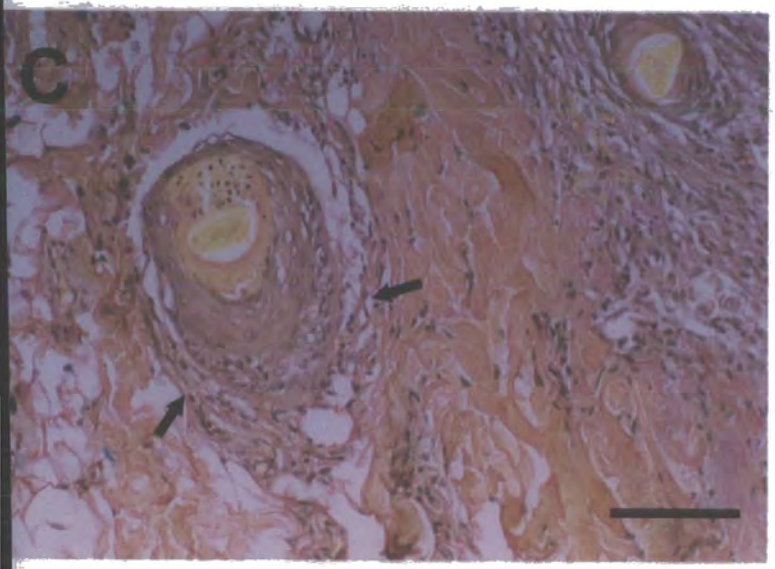
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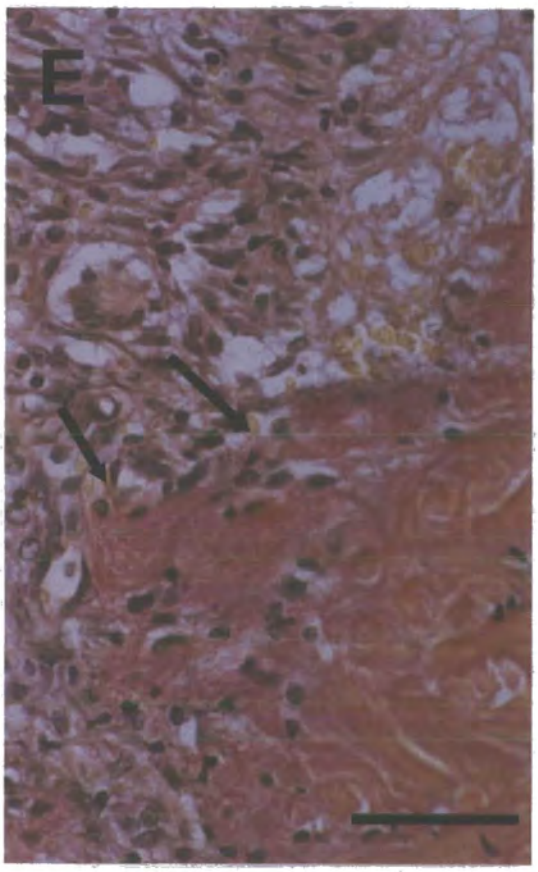
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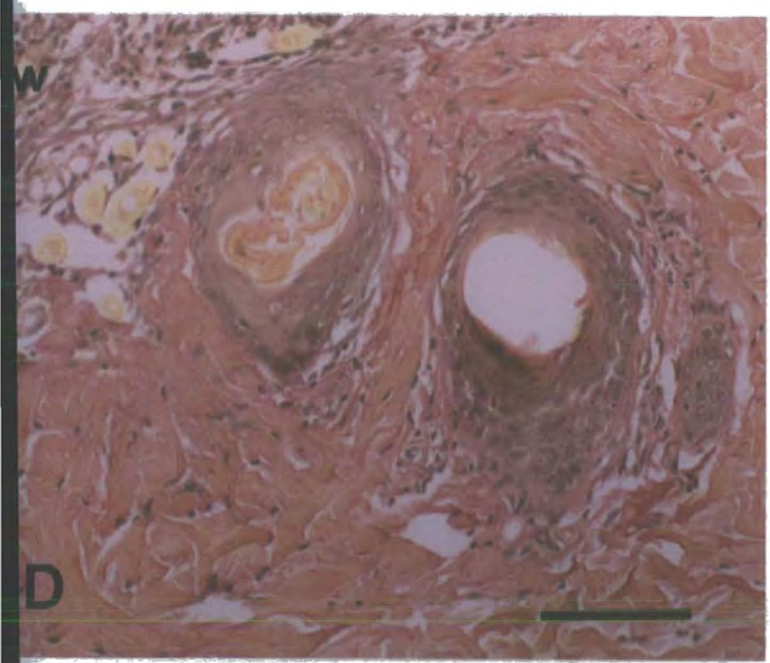
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Figure 19. Day 7 Histological Sections Stained With Weigerts' Haematoxylin.

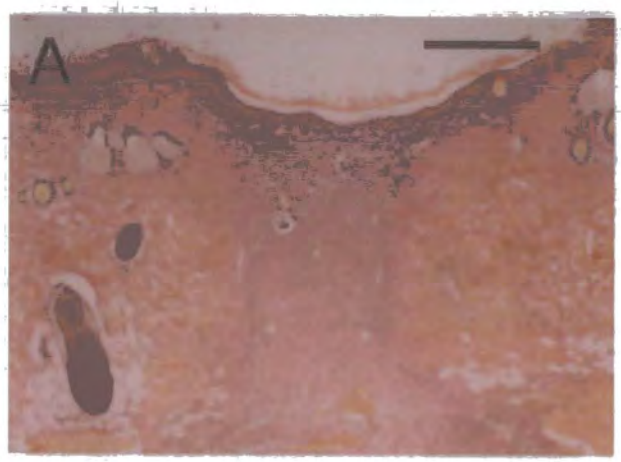
A. Low power section showing the general appearance of the wound at day 7.
Weigerts' Haematoxylin staining. (Scale bar = 400μ).

B. High power section showing mast cells at the base of the wound.
Weigerts' Haematoxylin staining. (Scale bar = 50μ).

C. Section showing the junction between the wound (W) and dermal (D) edge of the adjacent skin.
Weigerts' Haematoxylin staining. (Scale bar = 100μ).

D. High power section showing newly formed blood vessels (arrows) in the wound substance.
Weigerts' Haematoxylin staining. (Scale bar = 50μ).

E. High power section showing the hypertrophied outer root sheath at edge of the wound and the fir-tree pattern arrangement of the dermal sheath cells or fibroblasts around the hair follicle.
Weigerts' Haematoxylin staining. (Scale bar = 50μ).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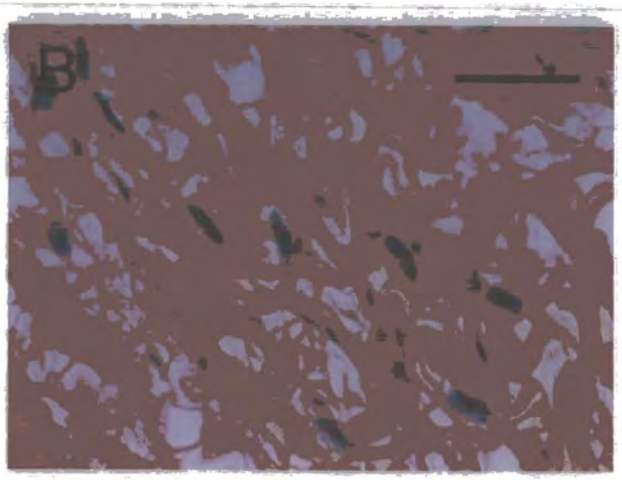
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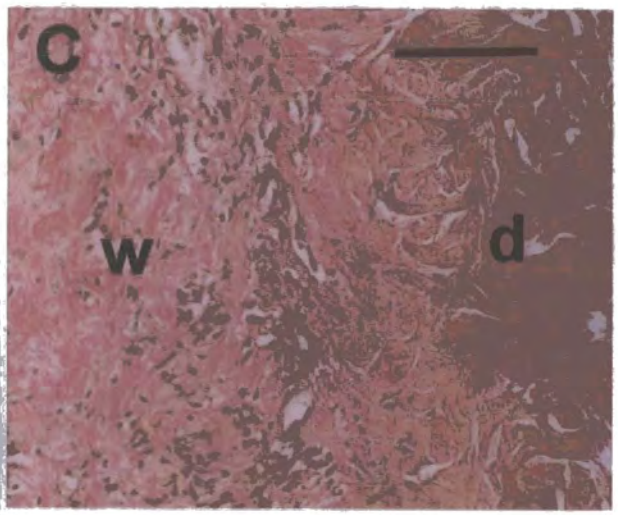
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D

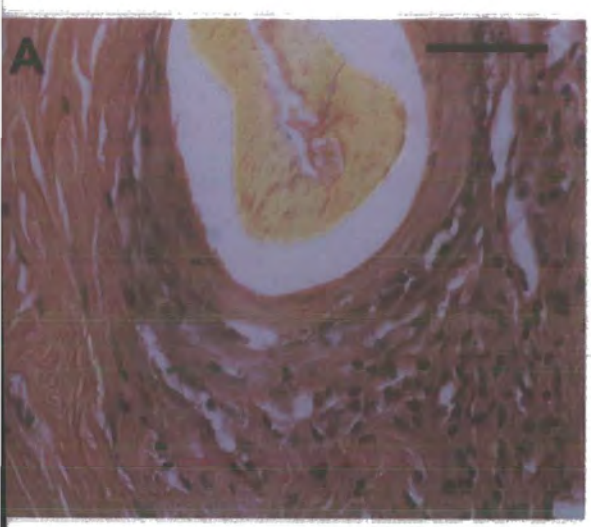

E

Figure 20. Day 7 Histological Sections Stained With Weigerts' Haematoxylin.

A. Section through a hair follicle at the edge of the wound showing the dermal sheath merged with the wound fibroblast network.
Weigerts' Haematoxylin staining. (Scale bar = 100μ).

B. High power section through a hair follicle fragment in the wound epithelium possibly undergoing repair or regeneration (or possibly being expelled from the skin).
Weigerts' Haematoxylin staining. (Scale bar = 50μ).

C. High power section through hair follicle element within the wound substance possibly undergoing repair or regeneration.
Weigerts' Haematoxylin staining. (Scale bar = 50μ).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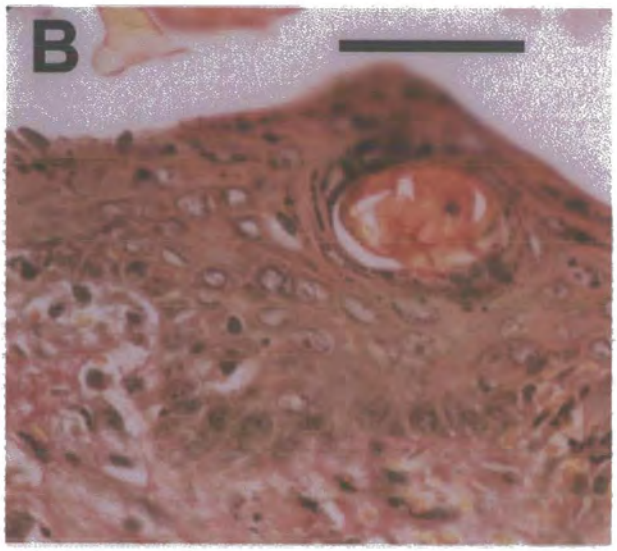
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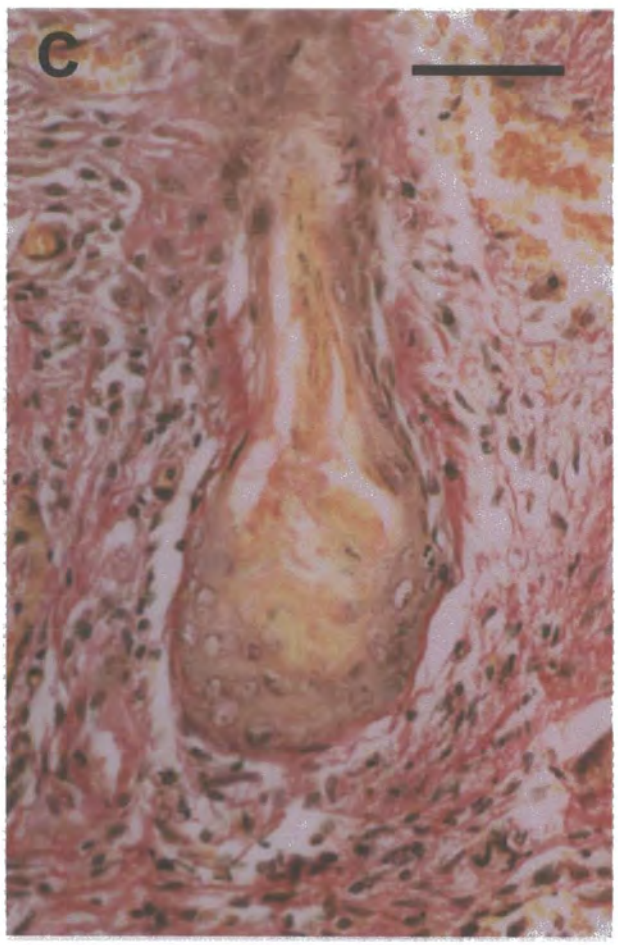

C

was a steady increase in fibroblasts.Mast cells were noted at the base of the wound (Figure 19 B). There was an increase in pink stained collagen laid down and intermingled within the fibroblast network (Figure 19 C).The collagen network was seen to blend deeply into the adjacent dermis of the wound edges (Figure 19 C).Neovascularization was noted within the wound substance; capillaries and larger vessels became apparent (Figure 19 D).The outer root sheath of the hair follicles at the wound edge was still very hypertrophied and made up of multiple layers of cells (Figure 19 E) containing mitotic figures.Similar to the observations noted at day 5,the fibroblast network was orientated in a fir-tree pattern (Figure 19 E) around some hair follicles in the wound that were sectioned longitudinally and in a concentric pattern around some follicles that were sectioned transversely.Hair follicles at the edge of the wound were still surrounded by a dense mononuclear infiltrate (Figure 20 A).The infiltrate was noted to be most intense around the bulbs of the hair follicles.The dermal sheath of the hair follicles in the wound was blended with the adjacent fibroblast network (Figure 19 E) and could not be identified even at high power. In hair follicles at the edge of the wound,the dermal sheath blended into the fibroblast network on the side of the follicles in contact with the wound (Figure 19 E and Figure 20 A).Further inspection of the day 7 Weigert's Haematoxylin stained sections also revealed some follicles and follicle fragments apparently in the process of repair and regeneration (Figure 20 B and C).

Day 8 Weigert's Haematoxylin Staining.

The observations at day 8 were similar to those at day 7.The wound epithelium and the outer root sheath were still hypertrophied (Figure 21 A).The fibroblast network has now practically replaced completely the mononuclear inflammatory infiltrate except for some areas at the base of the wound where mononuclear cells were still prominent.Hair follicles in the wound or at the edge of the wound still showed merging of the dermal sheath with the surrounding fibroblast network (Figure 21 A and B).There was also a high cell density in the vicinity of these follicles (Figure 21 B).Some hair follicles in the wound were noted to be surrounded by fibroblasts

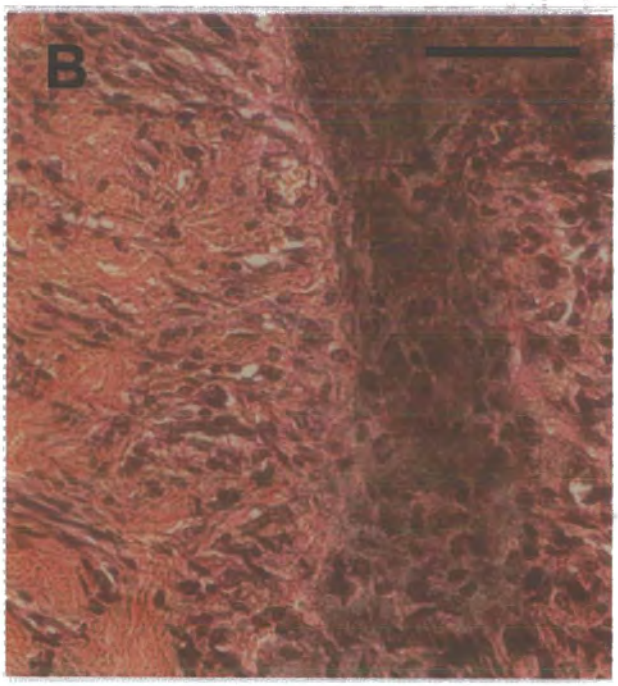
Figure 21. Day 8 Histological Sections Stained With Weigert's Haematoxylin.

A. Section through a hair follicle in the wound.Note the hypertrophied wound epithelium and the blending of the hair follicle dermal sheath with the surrounding fir-tree fibroblast architecture.
Weigert's Haematoxylin staining. (Scale bar = 100μ).

B. Section through a hair follicle in the wound showing the dermal sheath blending into the surrounding wound fibroblast network.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

C. High power section showing a dense mononuclear infiltrate around the base of a follicle in the wound.
Weigert's Haematoxylin staining. (Scale bar = 50μ).


A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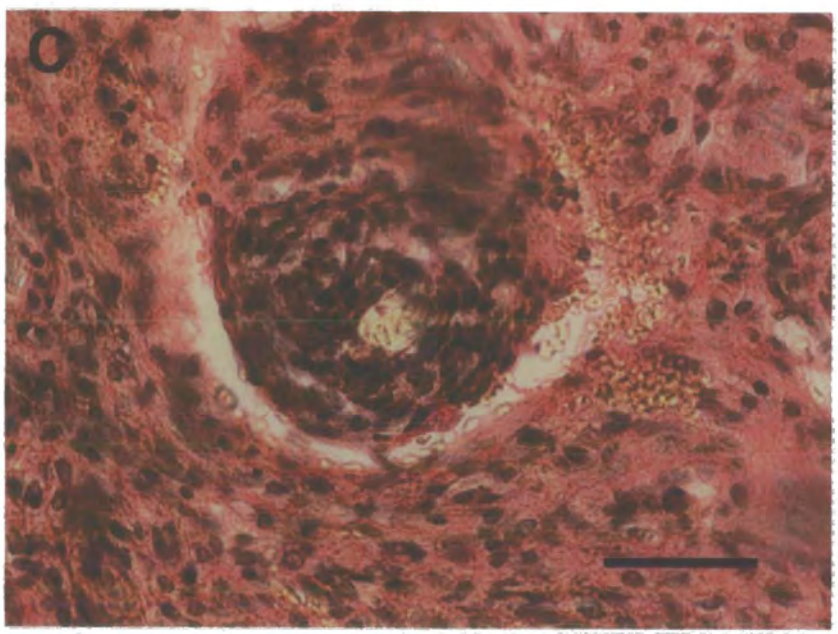
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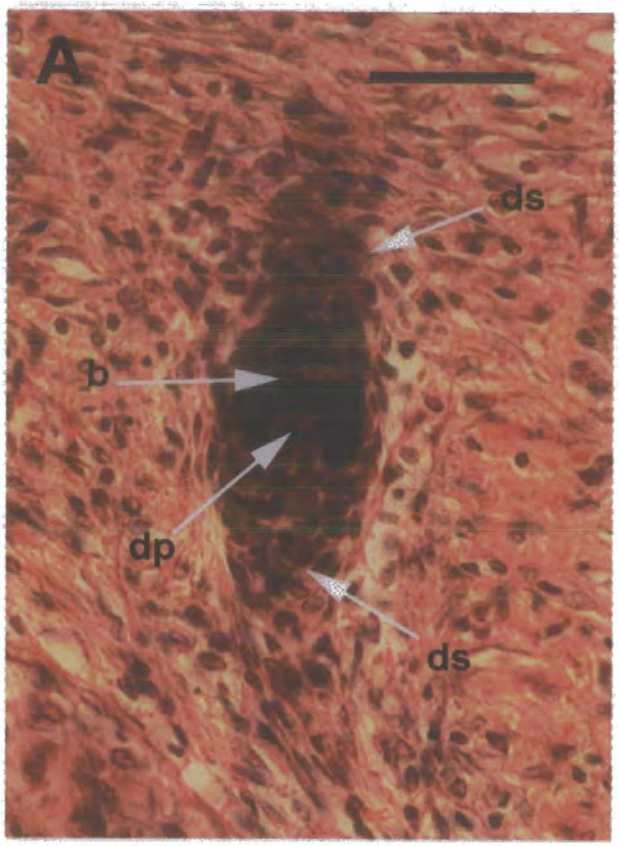
Figure 22. Day 10 Histological Sections Stained With Weigert's Haematoxylin.

A. High power section of a regenerating hair follicle within the wound tissue.Note the newly formed bulb (b),dermal papilla (dp) and the dermal sheath (ds).
Weigert's Haematoxylin staining. (Scale bar = 50μ).

B. High power section showing a hair follicle at the edge of the wound (w) with a relatively hypertrophied area (arrow) of the outer root sheath in contact with the wound as compared to the side of the follicle in contact with the dermis (d) at the wound edge.
Weigert's Haematoxylin staining. (Scale bar = 50μ).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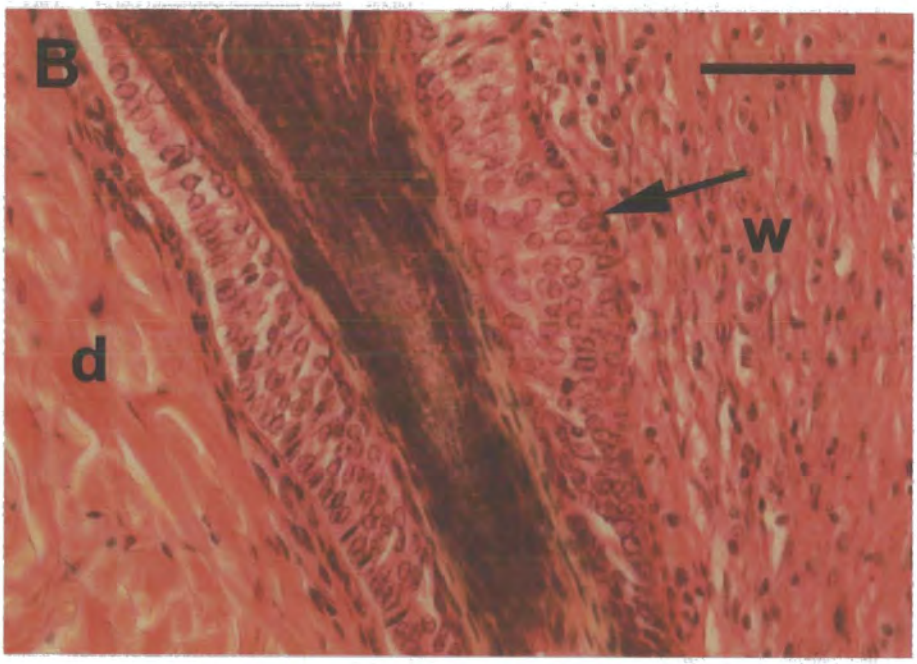
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orientated in a fir-tree pattern when examined in longitudinal section (Figure 21 A). Follicles in the depth of the wound were surrounded by a dense mononuclear cell infiltrate and fibroblasts (Figure 21 C).

Day 10 Weigert's Haematoxylin Staining.

At day 10 the wound epithelium was still hypertrophied however it was more regular and smooth in pattern.The wound infiltrate was entirely formed of a regular network of fibroblasts which changed pattern of orientation around hair follicles.Mononuclear inflammatory cells were now seen only at the base of the wound in the subcutaneous tissue.At this stage of wound healing,hair follicles in various stages of repair or regeneration were encountered in the wound tissue itself (Figure 22 A).The outer root sheath of follicles at the edge of the wound on occasions showed a larger number of layers of cells on the side in contact with the wound than on the side in contact with the dermis at the wound edge (Figure 22 B) and the dermal sheath was identified as a fine layer of cells covering the outer margins of the outer root sheath.Examination of hair follicles within the wound showed that the fibroblasts or dermal sheath cells surrounding the follicles were no longer orientated in any particular pattern and no definite concentric or fir-tree patterns could be identified (Figure 23 A and B).They now aligned themselves in a pattern similar to the rest of the wound fibroblast network. This may have possibly been due to the return of the dermal sheath cells back to the hair follicles or their possible phenotypic transformation to wound fibroblasts resulting in loss of the concentric spiral pattern of cellular arrangement around the hair follicles in the wound.

The wound infiltrate surrounding follicles in the wound or at the edge of the wound was now mostly composed of fibroblast or dermal sheath cells and very few mononuclear cells (Figure 23 A and B).

Day 15 Weigert's Haematoxylin Staining.

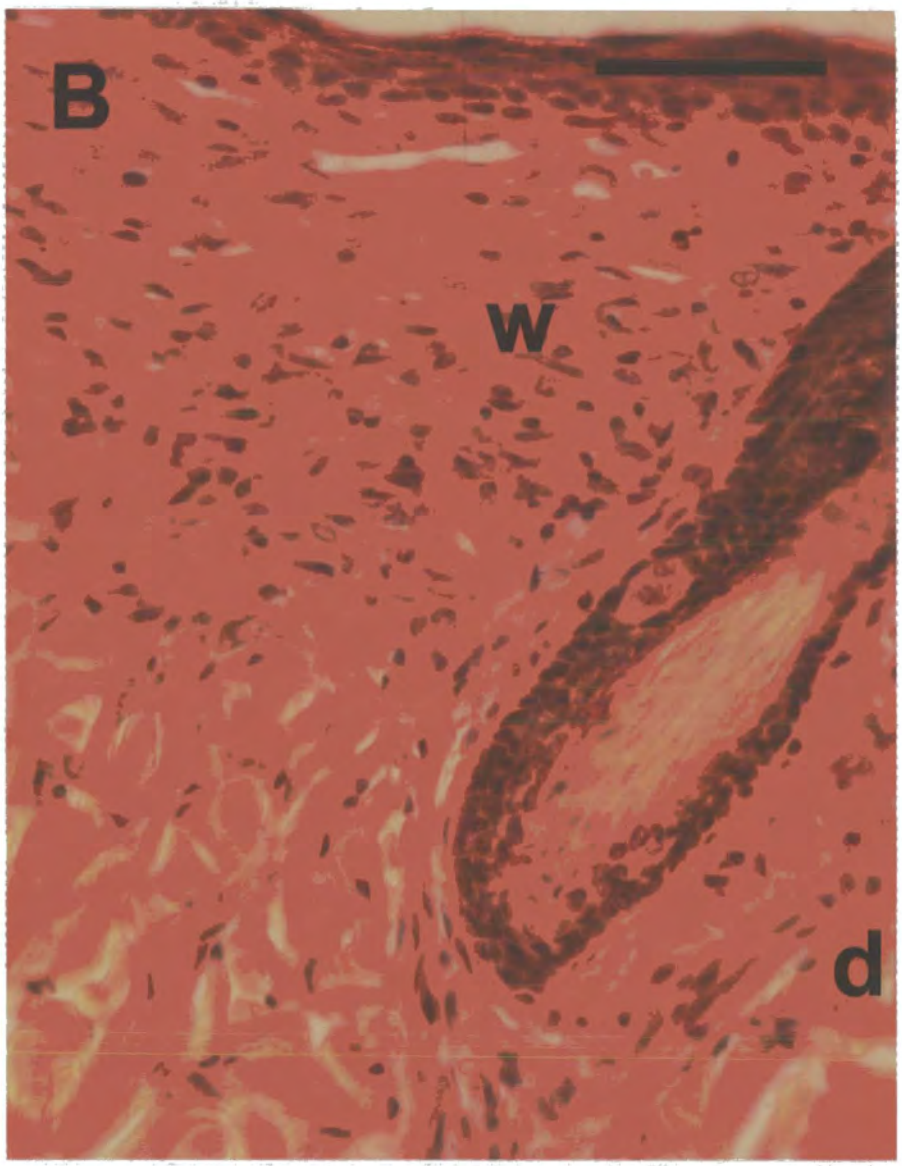
At day 15 wound contraction was noted to be even more prominent with the part of the wound at the level of the panniculus carnosus being most contracted.The wound had now taken the shape of an inverted cone with significantly narrowed deeper parts and wider at the top (Figure 24 A).

Figure 23. Day 10 Histological Sections Stained With Weigert's Haematoxylin.

A. Section of a hair follicle within the wound substance.Note that the fibroblast network around the follicles in the wound was no longer arranged in a clearly concentric pattern.
Weigert's Haematoxylin staining. (Scale bar =100μ).

B. High power longitudinal section through a hair follicle in the wound substance.Note that the pattern of fibroblast or dermal sheath cell arrangement around the hair follicle is no longer in a clear fir-tree pattern.
Weigert's Haematoxylin staining. (Scale bar = 50μ).


A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The wound epithelium was still hypertrophied,however it was more regular in pattern (Figure 24 B) when compared to earlier wounds.It also showed areas of possible hair follicle formation.The wound infiltrate at this stage was very homogenous and was composed almost entirely of fibroblasts with only few mononuclear inflammatory cells.The fibroblast lattice blended well into the adjacent normal dermis at the wound edges.At high magnification the pink staining collagen network ramifying into the adjacent dermis was noted,anchoring firmly the wound tissue with the rest of the integument (Figure 24 C).By day 15 there were no remants of the original wound haematoma neither in the wound tissue nor underneath the wound infiltrate.

The dermal sheath of the hair follicles situated within the wound substance was now distinct from the surrounding fibroblast network and no longer appeared blended into the adjacent wound fibroblast network (Figure 24 D).In some follicles deep in the wound however the dermal sheath was still indistinct and blended into the surrounding fibroblast lattice.Furthermore the presence of hair follicles in the wound tissue did not affect the pattern of orientation of the fibroblast network.

The fibroblast or dermal sheath cell arrangement around hair follicles in the wound did not show any specific pattern of orientation.In hair follicles adjacent to the edge of the wound the dermal sheath was often still indistinct at the sides of the follicles in contact with the fibroblast network whilst well distinct on the sides of the follicles in contact with the dermis at the edge of the wound (Figure 24 E).Hair follicle regeneration within the wound tissue was prominent at this stage.Hair follicles were observed budding from within the central part of the hypertrophied wound epithelium as well as at various stages of regeneration and repair within the wound substance (Figure 24 F).

Figure 24. Day 15 Histological Sections Stained With Weigert's Haematoxylin.

A. Low power section showing the general architecture of the day 15 wound (w).
Weigert's Haematoxylin staining. (Scale bar = 400μ).

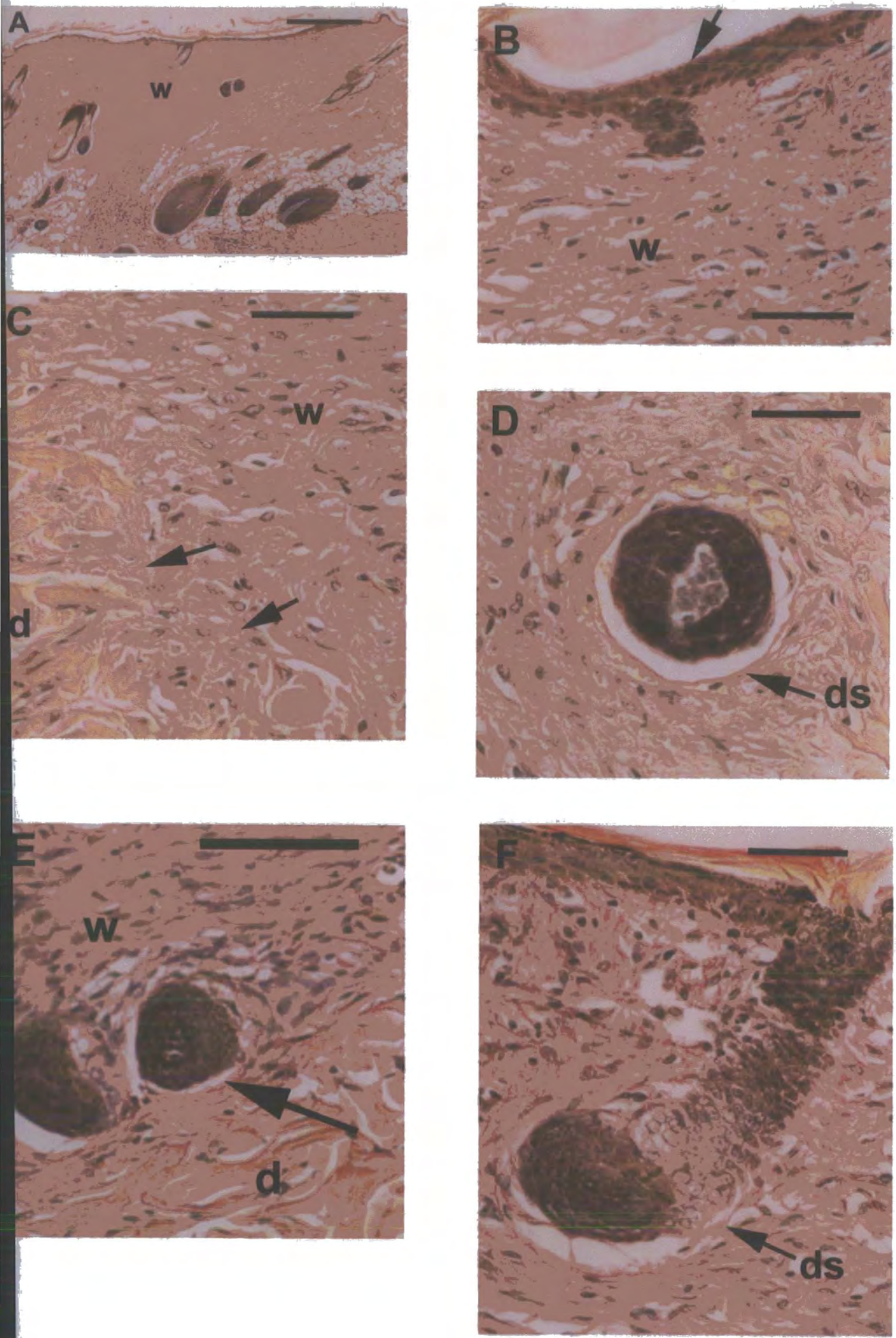
B. High power section through part of the wound (w) epithelium (arrow) that was still hypertrophied.Arrow indicates an area of possible new hair follicle formation.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

C. Section showing the pink staining collagen (arrows) network within the wound (w).
Weigert's Haematoxylin staining. (Scale bar = 50μ).

D. High power section through a hair follicle deep in the wound to show that at this stage it was again possible to identify the dermal sheath (ds) around the hair follicles as a distinct layer of cells.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

E. Hair follicle at edge of wound (w).Dermal sheath was clear on the side of the follicle in contact with the dermis (d) of the wound edge,however it was still difficult to identify on the side in contact with the wound.
Weigert's Haematoxylin staining. (Scale bar = 50μ).

F. High power longitudinal section through a regenerating hair follicle in the wound substance with an identifiable dermal sheath (ds).
Weigert's Haematoxylin staining. (Scale bar = 50μ).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4.5 Collagen IV And Laminin Basement Membrane Staining.

Summary.

Indirect immunoflourecence staining was performed in order to follow more closely the general architecture of the hair follicle during the wound healing process.However it also proved to be particularly useful in helping to follow the basement membrane formation of the newly formed wound epithelium as well as to observe the neovascularisation taking place in the healing wound.It also helped to demonstrate the various stages of wound epithelialization in general as well as the contribution of the hair follicles outer root sheath to wound epithelial repair.The wound tissue was very fragile in day 2 and day 3 tissue blocks.This made the cryosectioning technically more difficult,however good sections were still obtained.A point of interest was the fact that telogen follicles were observed in sections from blocks that were originally anagen and vice versa.This further increased the difficulty of trying to compare anagen with telogen wounds as wounds of purely one type (anagen or telogen) were hardly seen.

In the early stages the wound cavity was fully epithelialised.The wound epithelium was hypertrophic and irregular.The epithelial hypertrophy was in continuity with hypertrophy in the outer root sheath of hair follicles at the wound edge.At day 2 and day 3 no definite positive collagen IV or laminin staining was seen in the wound epithelium basement membrane at low magnification (X5 magnification).However at higher magnification (X20 and X40) collagen IV and laminin basement membrane expression was detected at the periphery of the newly formed wound epithelium as early as day 2 and progressed slowly towards the middle of the wound.It was also observed that laminin expression started de novo at day 2 in the middle of the wound independent from the expression at the sides of the wound.As the wound matured the positive staining gradually increased in intensity and became more and more regular.

The laminin wound epithelium basement membrane staining was all the time stronger than the collagen IV staining up to and including day 8, after which both were expressed at equal intensity.Hair follicles at the edge of the wound retained both the collagen IV and the laminin basement membrane staining throughout the whole process of wound healing.However hair follicles or hair follicle fragments in the substance of the wound either retained the basement membrane staining or lost it in part or in total.Neovascularisation occurred early in the wound tissue and gradually became more and more intense as the wound matured.During the process of the above observations no difference in staining pattern or wound healing characteristics could be identified between anagen and telogen wounds.

Day 2 Collagen IV And Laminin Staining.

At day 2 the wound was noted to be generally fully epithelialised with a hypertrophied epithelium The epithelial hyperplasia was in continuity with the hyperplasia in the outer root sheath of hair follicles at the edge of the wound.Collagen IV and laminin expression in the basement membrane region of the newly formed epithelium first appeared close to the wound edges (Figure 25 A) and in the centre of the wound (Figure 25 B) although in some wounds no basement membrane expression of collagen IV or laminin was observed.In general the basement membrane positive staining was of low intensity and irregular with occasional bright speckles.

Hair follicles at the edge of the wound retained regular undistorted normal positive basement membrane staining both in the case of laminin (Figure 25C) and in the case of collagen IV (Figure 25 A).Follicles well away from the wound showed perfectly normal laminin and collagen IV staining (Figure 25 D).Hair follicles and hair follicle fragments in the wound substance in general behaved in one of two ways:they either retained their basement staining or lost the basement membrane staining (Figure 25 E). Some follicle elements incorporated into the newly forming epithelium also seemed to have lost their basement membrane staining (Figure 25 E).

No difference in staining patterns could be encountered whilst studying anagen and

Figure 25. Collagen IV And Laminin Day 2 histological sections.

A. Section showing collagen IV staining of the wound epithelial basement membrane (arrow) at edge of wound.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

B. Section showing laminin epithelial basement membrane staining (arrow) in middle of wound (w).
Laminin indirect immunofluorescence staining. (Scale bar = 50μ).

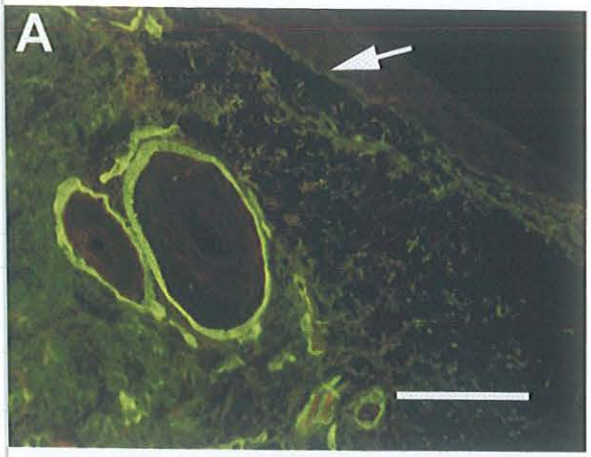
C. Histological section showing laminin hair follicle basement membrane (arrow) staining at the edge of the wound (w).
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

D. High power section showing collagen IV basement membrane staining of hair follicle away from the wound.
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).

E. Sections through hair follicles (arrows) in the wound (w) and in wound epithelium (e) that have lost the laminin basement membrane staining.
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

F. Section showing a telogen follicle in previously anagen skin.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

G. Section showing collagen IV staining of subcutaneous blood vessels at the base of the wound.
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).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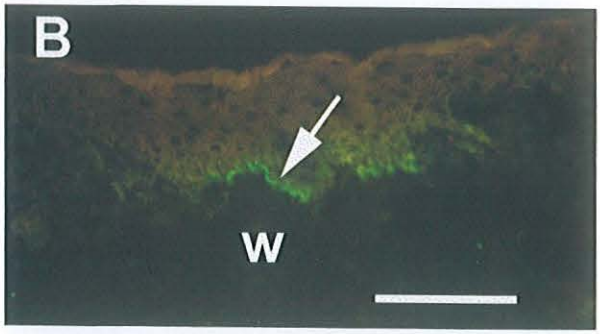
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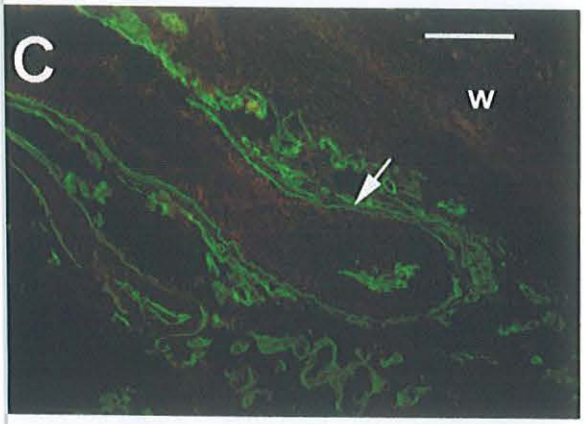
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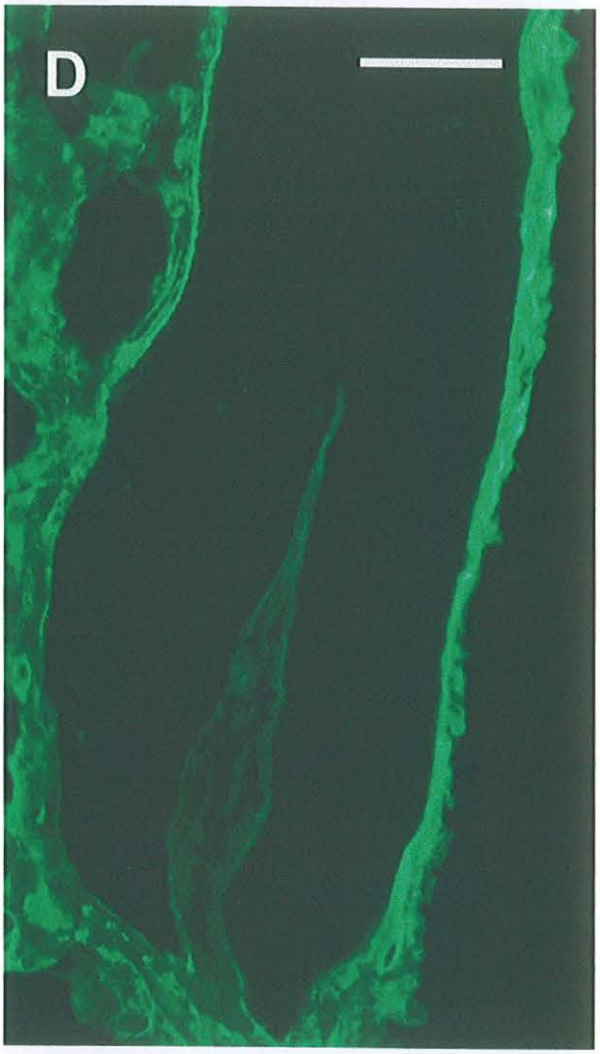
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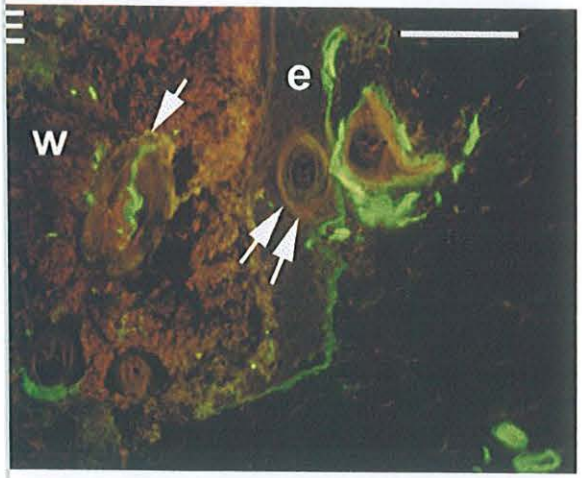
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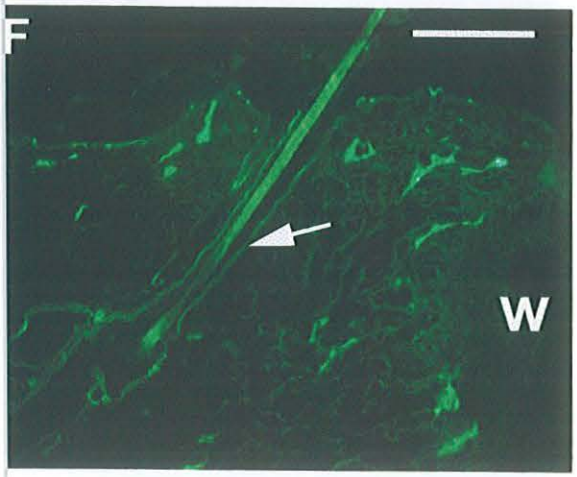
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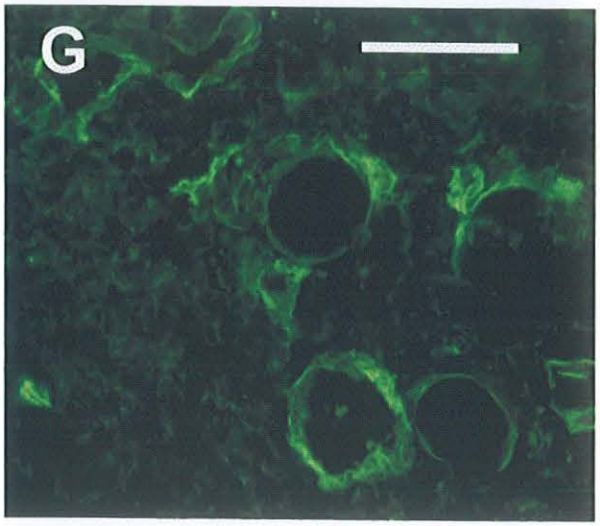
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telogen day 2 wounds.During the study of these sections telogen follicles could be identified in what was originally a macroscopically anagen wound (Figure 25 F).This further illustrates the difficulty encountered in trying to compare anagen wounds with telogen wounds and vice versa. Blood vessels in the subcutaneous tissue at the base of the wound stained well for both collagen IV and laminin (Figure 25 G).

Day 3 Collagen IV And Laminin Staining.

At day 3 the wound epithelium was more regular in shape,however it was still very hypertrophic.Laminin epithelial basement membrane expression had generally advanced further towards the centre of the wound (Figure 26 A).However collagen IV expression in some sections had not spread as much towards the central part of the wound (Figure 26 B).In general the laminin epithelial basement membrane expression was of stronger intensity (Figure 26 C) than the collagen IV expression (Figure 26 D).However this could have been due to the difference in affinity between the two antibodies used.Speckled collagen IV positive staining was also seen in the central part of the wound epithelium (Figure 26 E) suggesting the early stages of collagen IV deposition.

Hair follicles at the wound edges showed clear strong basement membrane expression which was mostly regular and only sometimes irregular.Hair follicles and hair follicle fragments in the wound behaved in a similar manner to the day 2 wound.In some sections the follicle fragments retained their basement membrane (Figure 26 F) whilst in other sections there was partial (Figure 26 G) or complete loss of the basement membrane expression.

At this stage the wound vascularization had become intense especially at the base of the wound and in the level of the panniculus carnosus (Figure 26 H).

Day 5 Collagen IV And Laminin Staining.

At day 5 the wound epithelium was still hypertrophic,however it was more regular in shape as compared to wounds of earlier timescale.At this stage both collagen IV (Figure 27 A

Figure 26. Collagen IV And Laminin Day 3 Histological Sections.

A. Section of the wound edge showing that the laminin epithelial basement membrane staining (arrow) had advanced further towards the centre of the wound (w).
Laminin indirect immunofluorecence staining. (Scale bar = 100μ).

B. Section showing collagen IV epithelial basement membrane staining at the edge of the wound (w).
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

C. High power view of the laminin wound (w) epithelial basement membrane staining.
Laminin indirect immunofluorecence staining. (Scale bar = 50μ).

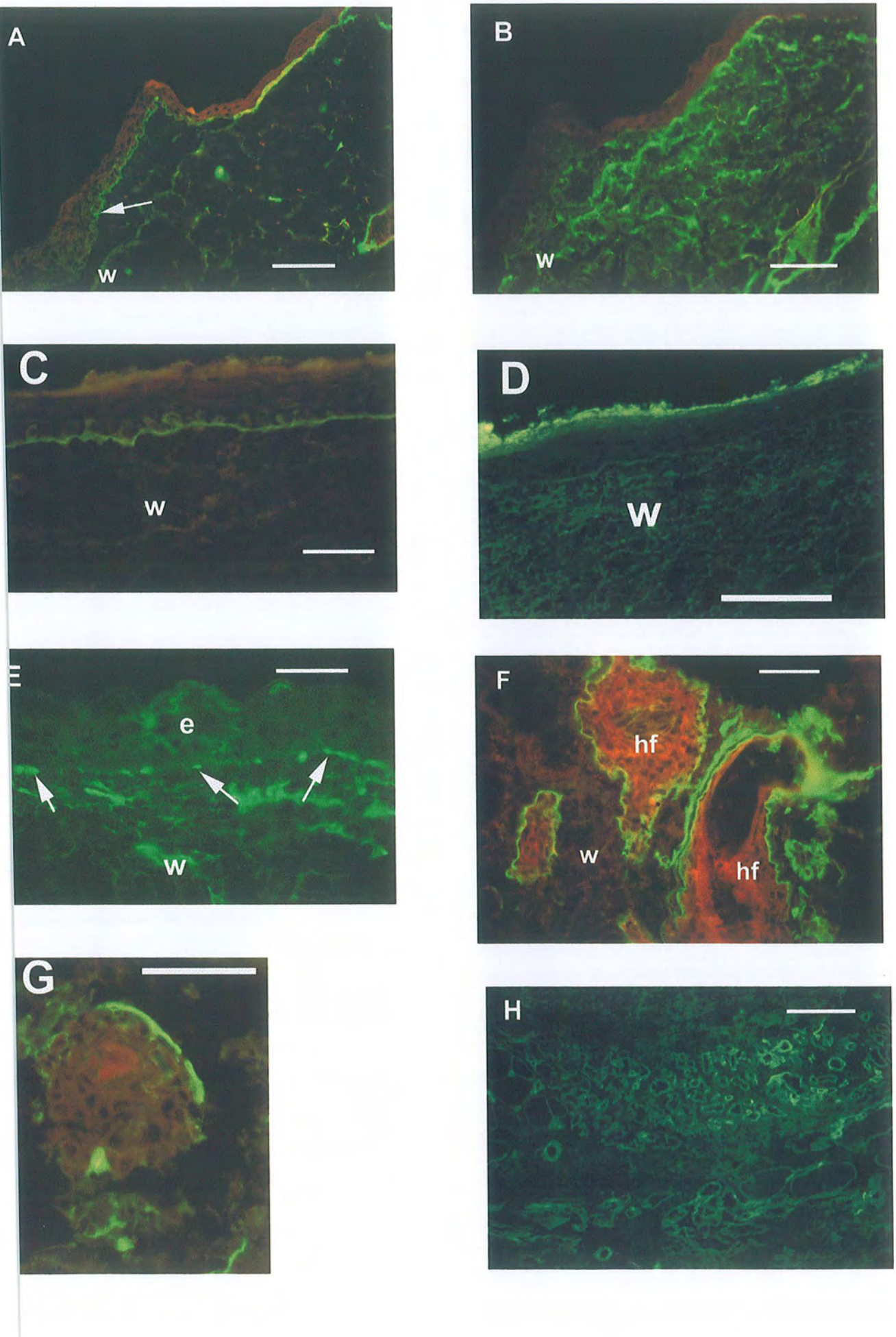
D. Section showing collagen IV wound epithelial basement membrane staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

E. High power section showing the speckled collagen IV basement membrane staining (arrows) in the middle of the wound (w) epithelium (e).
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).

F. High power section through hair follicle (hf) elements within the wound (w) that have retained the laminin staining.
Laminin indirect immunofluorecence staining. (Scale bar = 50μ).

G. Higher power view of hair follicle elements within the wound substance that have partially lost the collagen IV staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).

H. Section showining collagen IV staining of blood vessels at the base of the wound.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).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and B) and laminin (Figure 27 C) staining were expressed throughout the entire wound epithelium basement membrane and was now stronger than in the day 3 wound.The laminin staining was however stronger than the collagen IV staining (Figure 27 B and C).The basement membrane staining,though in general smoother than in earlier wounds,on occasions showed spikes of positive staining extending into the underlying wound tissue (Figure 27 D) representing anchoring fibrils. Hair follicles adjacent to the wound edge showed strong basement membrane expression of both collagen IV (Figure 27 E) and laminin (Figure 27 F) in continuity with the wound epithelium basement membrane.Hair follicles within the wound substance itself again behaved in a varied way.In some instances hair follicles deep in the wound lost both the collagen IV staining (Figure 28 A) and the laminin expression (Figure 28 B).Interestingly some of these follicles retained some α SMA staining (Figure 28 C).Other hair follicles retained some collagen IV staining (Figure 28 D) and laminin staining (Figure 28 E) and these same follicles had a radiating pattern of alpha smooth muscle actin staining (Figure 28 F).Follicle fragments with different staining characteristics could be seen adjacent to each other in the wound tissue.Blood vessel formation was even more advanced at this stage.

In general,no difference in staining characteristics or patterns could be identified between sections from anagen wounds and telogen wounds.

Day 7 Collagen IV And Laminin Staining.

At day 7 it was noted that the wound epithelium was even more regular but still hypertrophic.The collagen IV and laminin wound epithelial basement membrane staining was complete and bright (Figure 29 A and B).The wound epithelial basement membrane was in continuity with the basement membrane of the hair follicles at the wound edge (Figure 29 C).In general the collagen IV basement membrane staining (Figure 29 D) was weaker than the laminin staining (Figure 29 C).Furthermore the laminin staining seemed to be more irregular,showing spikes radiating into the underlying wound tissue (Figure 29 C).This may

Figure 27. Collagen IV And Laminin Day 5 Histological Sections.

A. Low power view of the day 5 wound (w) stained for collagen IV.
Collagen IV indirect immunofluorescence staining. (Scale bar = 400μ).

B. High power section showing collagen IV wound (w) epithelium (e) basement membrane staining (arrow).
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).

C. High power section showing laminin wound (w) epithelium (e) basement membrane staining (arrow).
Laminin indirect immunofluorescence staining. (Scale bar = 50μ).

D. Section showing an area of wound (w) epithulium (e) basement membrane staining with irregular spikes of bright staining (arrow).
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

E. Histological section showing collagen IV hair follicle (hf) basement membrane staining (arrow) in continuity with the wound (w) epithelial (e) basement staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

F. Section illustrating laminin hair follicle (hf) basement membrane staining (arrow) in continuity with the wound (w) epithelial (e) basement staining.
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

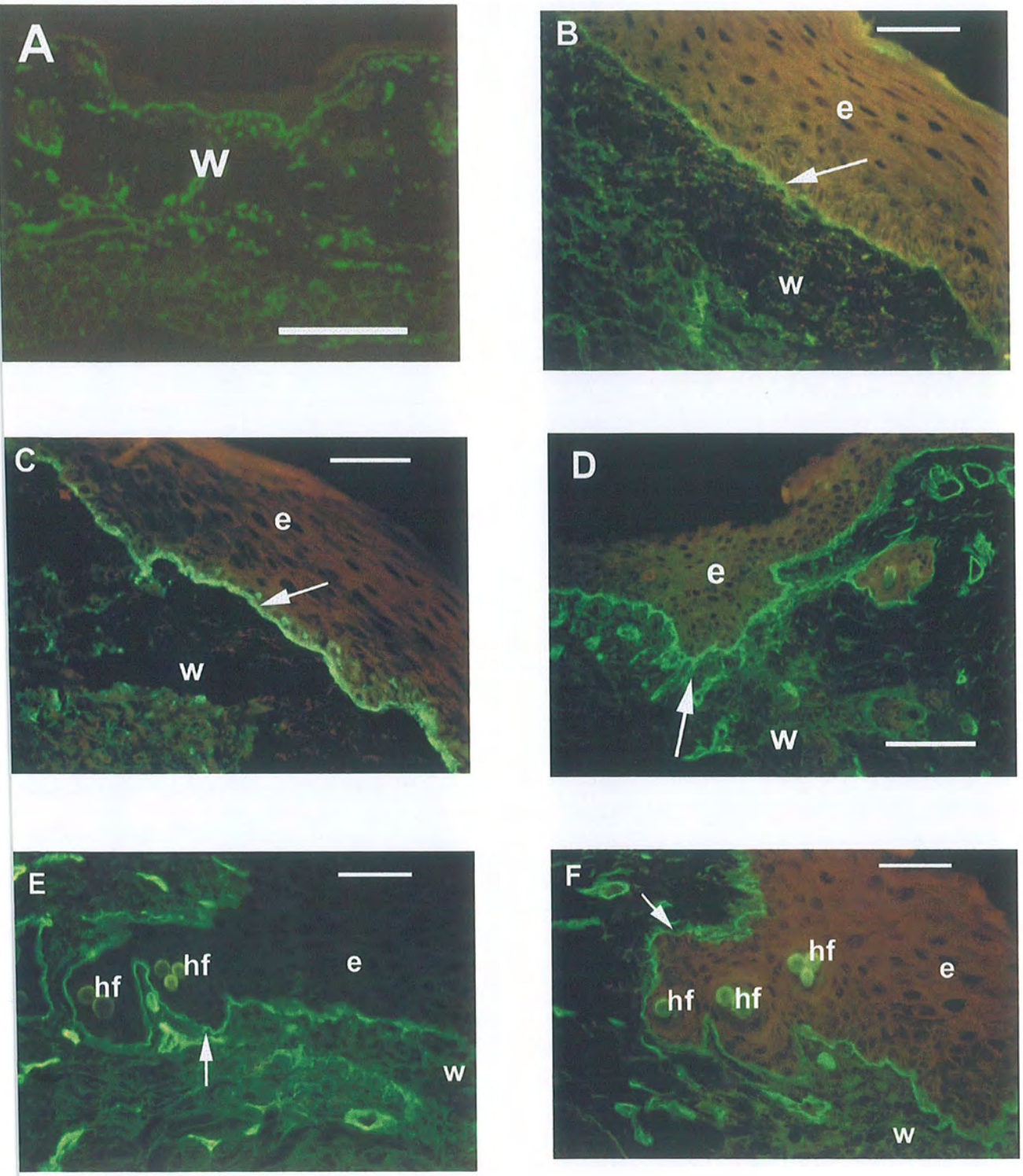
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Figure 28. Collagen IV And Laminin Day 5 Histological Sections.

A. High power section through a hair follicle (hf) deep in the wound that has lost the collagen IV staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).

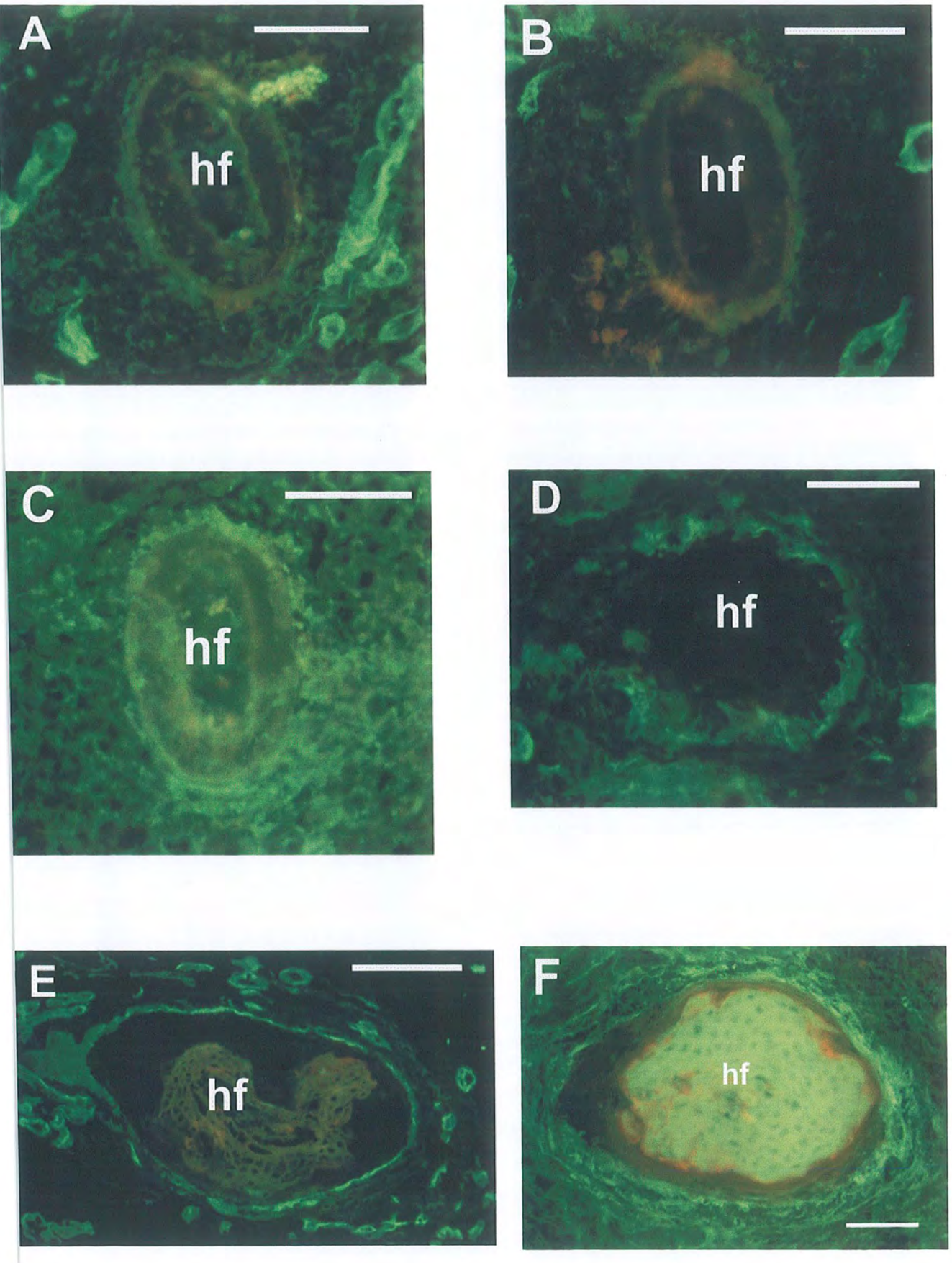
B. The same follicle (hf) as in **A.** showing the loss of laminin staining.
Laminin indirect immunofluorescence staining. (Scale bar = 50μ).

C. The same follicle (hf) as in **A.** and **B.** showing some α SMA positive marking.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

D. High power section through a hair follicle (hf) deep in the wound with positive Collagen IV staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).

E. The same follicle (hf) as in **D.** Note that it has retained the laminin basement membrane staining.
Laminin indirect immunofluorescence staining. (Scale bar = 50μ).

F. The same follicle (hf) as in **D.** and **E.** Note that it has retained the α SMA staining which has a radiating concentric pattern.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).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possibly indicate a heavier early deposition of laminin possibly associated with firm anchoring of the newly formed epithelium.Hair follicles at the edge of the wound generally expressed intact collagen IV (Figure 29 D) and laminin (Figure 29 C) basement membrane staining,however in some instances it was noted to be somewhat ragged or irregular.Hair follicles or hair follicle fragments in the wound again expressed a variegated pattern of staining.Some follicles lost the laminin staining (Figure 29 E) whilst they retained the collagen IV staining (Figure 29 F).Other follicles retained both the collagen IV and the laminin staining (Figure 29 E,F).
Even follicles very deep in the wound,at the level of the panniculus carnosus,retained positive basement membrane staining (Figure 29 G).

Day 8 Collagen IV And Laminin Staining.

Day 8 sections showed a well formed hypertrophied wound epithelium with a basement membrane that stained strongly for both collagen IV (Figure 30 A) and laminin(Figure 30 B). The basement membrane staining at this stage in general appeared smoother and more regular compared to earlier wounds (Figure 30 C).However in some areas the basement membrane was still irregular especially at the wound edge (Figure 30 D).The hair follicles or follicle remnants in the newly formed epithelium showed no basement membrane staining (Figure 30 B). Hair follicles at the wound edge retained both the collagen IV as well as the laminin basement membrane staining (Figure 30 A).The pattern of staining was sometimes irregular (Figure 30 A and D) however this could have been due to the angle and direction in which the follicles were cut in the cryostat.Hair follicles or follicle fragments in the wound seemed to behave in a similar fashion to the patterns observed in wounds of earlier timescales.Some follicle elements lost the collagen IV basement membrane staining (Figure 30 E).Other follicle fragments showed non-uniform laminin staining (Figure 30 F).This loss in collagen IV basement membrane staining can be contrasted with the expression in a follicle (Figure 30 G) away from the wound in the same histological section shown in Figure 30 E.

Figure 29. Collagen IV And Laminin Day 7 Histological Sections.

A. Low power section showing the laminin staining of a day 7 wound (w).
Laminin indirect immunofluorecence staining. (Scale bar = 400μ).

B. Collagen IV staining of a low power section through a day 7 wound (w).
Collagen IV indirect immunofluorescence staining. (Scale bar = 400μ).

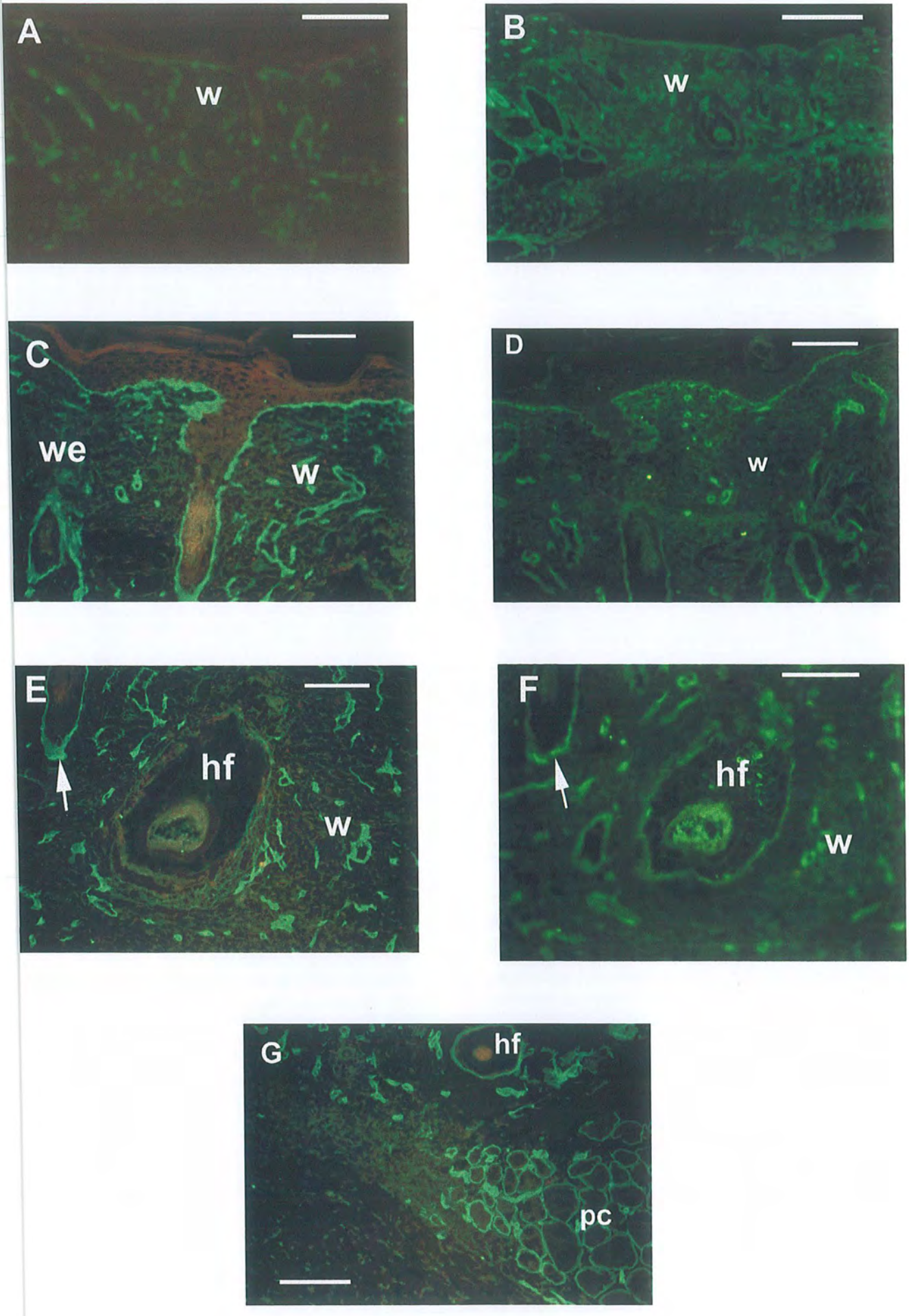
C. Section showing the laminin wound (w) epithelial basement membrane staining in continuity with the hair follicle basement membrane staining at the wound edge (we).
Laminin indirect immunofluorecence staining. (Scale bar = 100μ).

D. Section showing the wound (w) epithelium and hair folllicle (at edge of wound) basement membrane Collagen IV staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

E. Histological section through a hair follicle (hf) in the wound (w) that has lost the laminin basement membrane staining.
Laminin indirect immunofluorecence staining. (Scale bar = 100μ).

F. The same follicle (hf) within the wound (w) as in **E.** Note that it has retained the collagen IV staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

G. Section showing a hair follicle (hf) at the level of the panniculus carnosus (pc) that has retained the laminin basement membrane staining.
Laminin indirect immunofluorecence staining. (Scale bar = 100μ).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G
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pc

Figure 30. Collagen IV And Laminin Day 8 Histological Sections.

A. Section showing the Collagen IV wound (w) basement membrane staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

B. Section showing the laminin basement membrane (bm) staining of the wound (w) epithelium.Note a hair follicle (hf) element possibly regenerating within the wound epithelium (we).
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

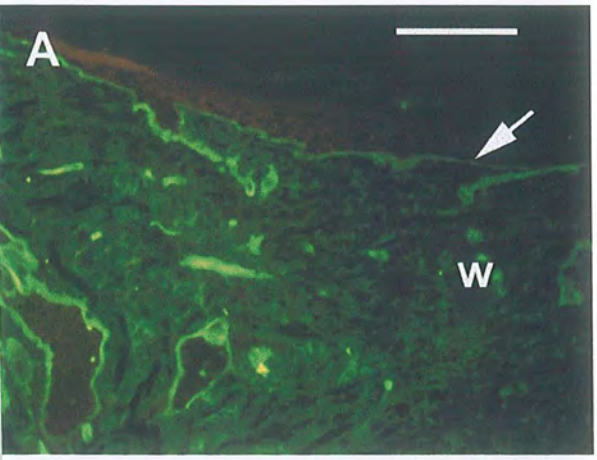
C. Section showing the laminin wound epithelium basement membrane (bm) staining.Note regularity.
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

D. Section showing irregular wound epithelium and hair follicle (hf) basement membrane laminin staining.
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

E. Histological section illustrating hair follicles (hf) within the wound tissue that have lost the collagen IV basement membrane staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

F. Section through hair follicles and follicle fragments in the wound tissue with non-uniform laminin basement membrane staining.
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

G. Section illustrating the collagen IV hair follicle basement membrane and papilla staining in a follicle away from the wound.
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).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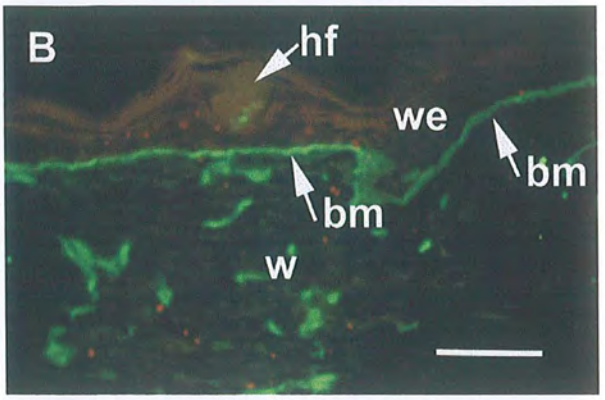
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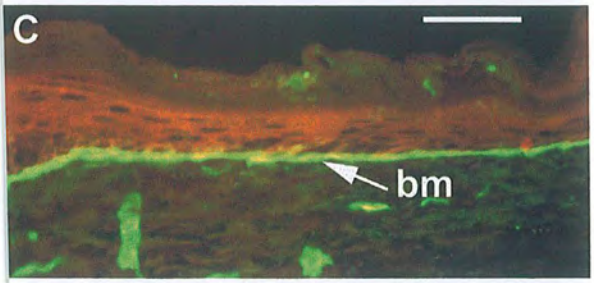
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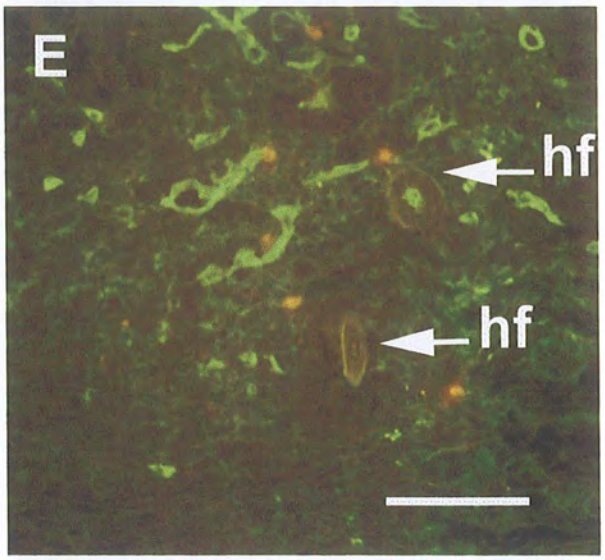
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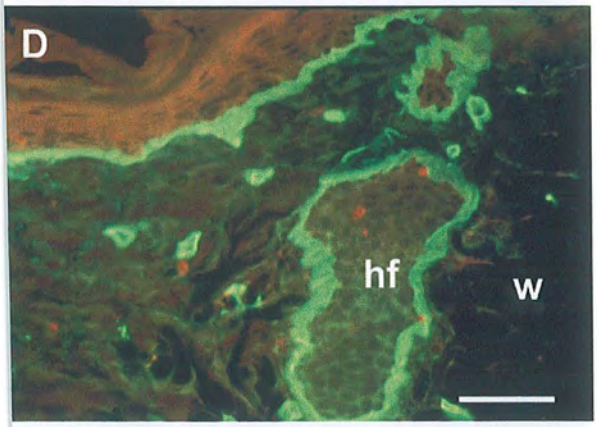
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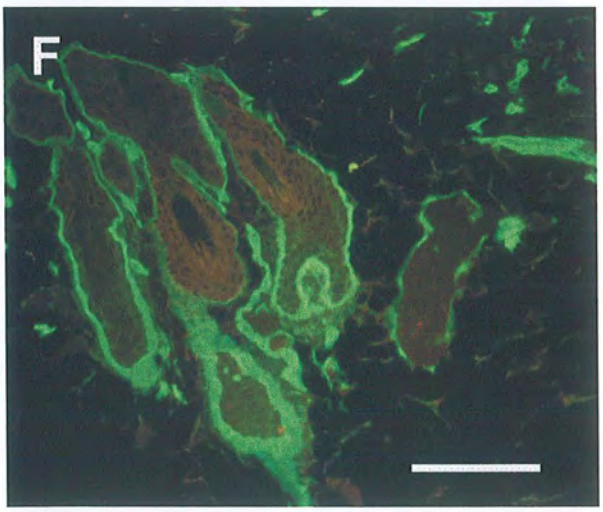
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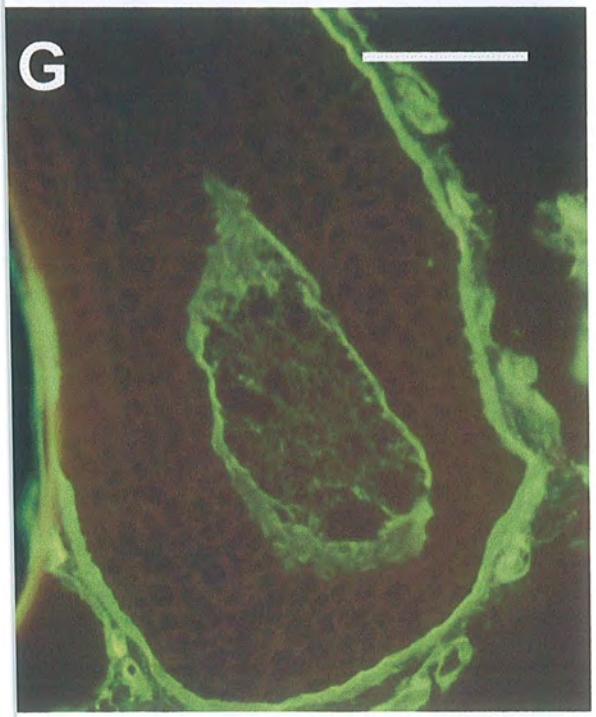

G

A general point of interest was the fact that at this stage of wound healing there were fewer hair follicles or follicle fragments within the wound substance as compared to wounds of earlier timescales.This observation was also noted during the study of the *Weigerts' Haematoxylin* stained histological sections.

During the course of recording the above observations no difference in staining pattern was identified anagen and telogen histological sections.

Day 10 Collagen IV And Laminin Staining.

At day 10 the wound epithelium and the wound tissue had become so well organised that it became difficult to differentiate the wound area from adjacent normal skin under UV light microscopy.The constant feature that regularly helped to identify the wound site was the definite irregularity in the structure of the wound epithelium as compared to the epithelium of normal skin.The basement membrane stained positively for both collagen IV (Figure 31 A) and laminin.

Hair follicles in the vicinity of the wound expressed strong positive basement membrane staining for both collagen IV (Figure 31 B) and laminin (Figure 31 C).The staining was irregular (Figure 31 B and C) possibly due to the angle and orientation in which the follicles were sectioned.However some follicles only expressed weak staining (Figure 31 C).Some follicles or follicle elements in the wound showed non-uniform positive staining (Figure 31 D) whilst other follicles deep in the wound showed strong positive marking (Figure 31 E).

Throughout the examination of the day 10 sections no difference in staining patterns could be identified between anagen and telogen skin tissue blocks.

Day 15 Collagen IV And Laminin Staining.

The day 15 wound was even better healed and organised than the day 10 wound and the same observations used to differentiate the wound area in the day 10 sections were used at this stage.The wound epithelium at day 15 became very regular,though it was still hypertrophied.Wound epithelial

Figure 31. Collagen IV And Laminin Day 10 Histological Sections.

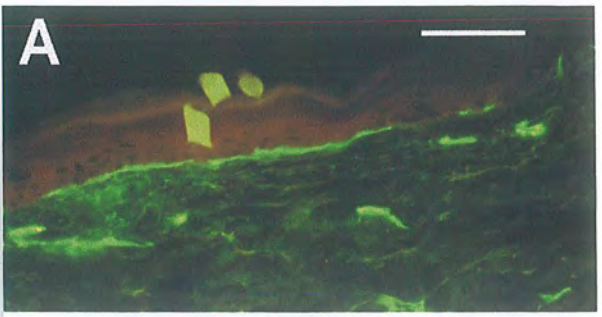
A. Section showing collagen IV staining of the wound epithelium basement membrane.
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).

B. Section showing collagen IV staining of the hair follicles in the vicinity of the wound.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

C. Section illustrating laminin staining of the hair follicles in the vicinity of the wound.
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

D. High power section showing hair follicles in the wound with partial loss of positive staining.
Laminin indirect immunofluorescence staining. (Scale bar = 50μ).

E. High power view of a hair follicle papilla in wound showing bright positive staining.
Laminin indirect immunofluorescence staining. (Scale bar = 50μ).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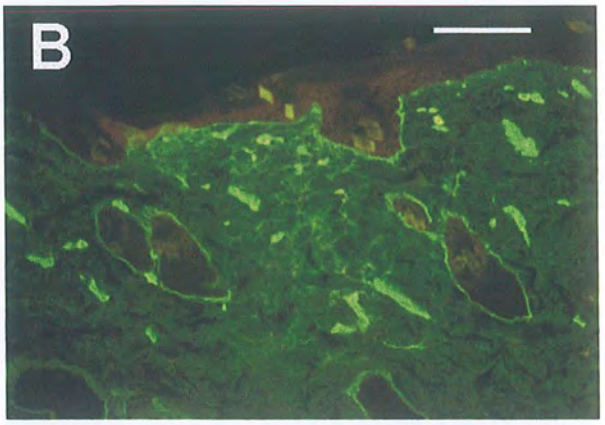
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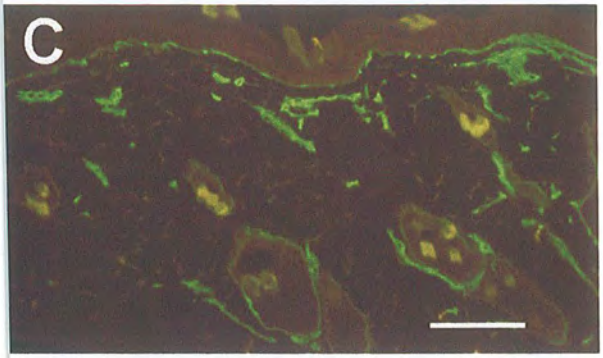
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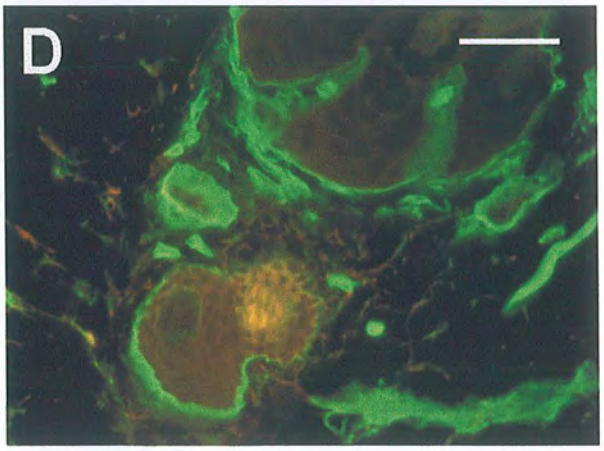
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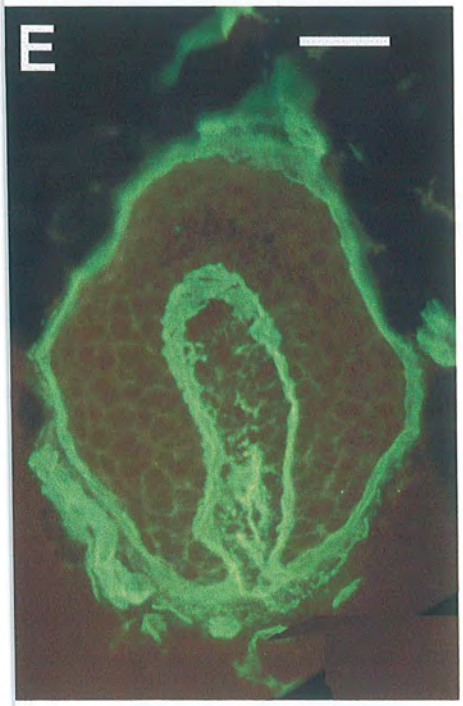

E

basement membrane staining was also more regular and showed strong expression for both the collagen IV (Figure 32 A),and the laminin,(Figure 32 B) antibodies.In some areas the basement membrane staining showed some irregularity especially in the vicinity of hair follicles at the wound edge (Figure 32 C).In general it was noted that the wound area was relativley devoid of hair follicles or hair follicle fragments (Figure 32 D).The hair follicles at the edge of the wound expressed positive collagen IV (Figure 32 D) and laminin (Figure 32 E) staining.

Figure 32. Collagen IV And Laminin Day 15 Histological Sections.

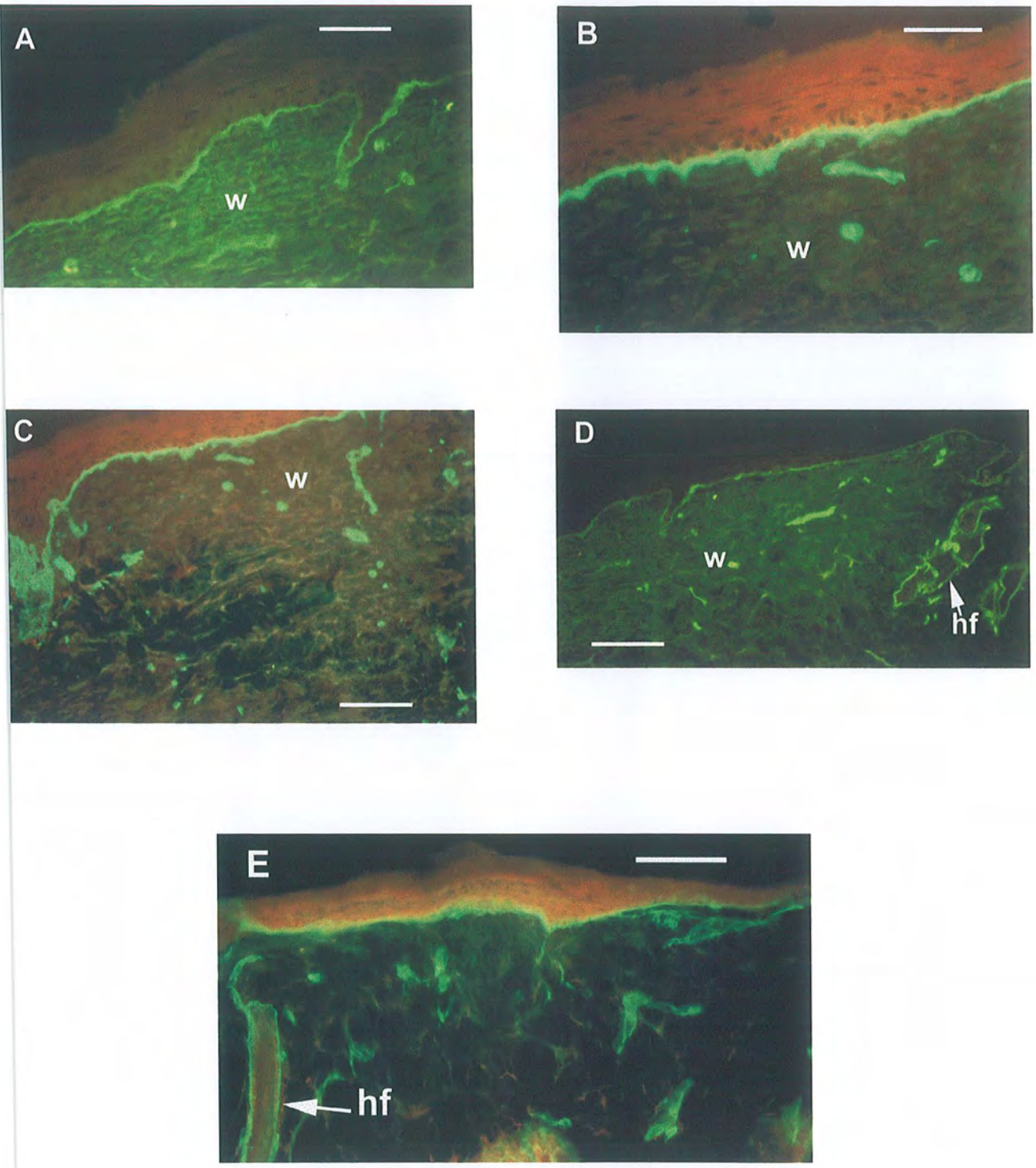
A. High power section showing collagen IV wound (w) epithelium basement membrane staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 50μ).

B. High power section showing laminin wound (w) epithelium basement membrane staining.
Laminin indirect immunofluorescence staining. (Scale bar = 50μ).

C. Section showing irregular wound (w) epithelium and hair follicle basement membrane staining.
Laminin indirect immunofluorescence staining. (Scale bar = 100μ).

D. Section showing that at day 15 the wound (w) area had very few hair follicles/fragments.Hair follicles (hf) at the edge of the wound expressed positive basement staining.
Collagen IV indirect immunofluorescence staining. (Scale bar = 100μ).

E. High power section showing a hair follicle at the edge of the wound that expressed positive basement staining.
Laminin indirect immunofluorescence staining. (Scale bar = 50μ).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4.6 Oservations On Alpha Smooth Muscle Actin (α SMA) Stained Sections.

4.6.1 Ultraviolet Light Microscopy.

Introduction.

α SMA staining of normal hair follicles using indirect immunofluorescence produces characteristic patterns depending on the stage of the hair follicle cycle.Hair follicles in the anagen phase exhibit the strongest pattern of staining: the marking appears as a fine spiral pattern of strong marking encircling the lower parts of the follicle (Figure 33 A. and B.) and disappears in the upper parts of the follicle.It is worth pointing out that the staining in normal follicles is very regular and limited to a single cell layer (Figure 33 A).Hair follicles in the telogen phase show very little or no staining at all in both the lower and the upper parts of the follicle.

Summary.

α SMA staining of histological sections of wounds of different stages showed a general increase in staining in the wound substance and perimeter around day 5.This was followed by a gradual decrease in brightness of the staining.Hair follicles in the wound tissue showed bright radiating concentric staining at day 5.This was most prominent in anagen follicles deep in the wound.Hair follicles at the wound edge showed radiating irregular staining on the side in contact with the wound whilst normal regular staining was noted on the side in contact with the dermis of the wound edge.Beyond day 5 these staining characteristics decreased so that by day 15,hair follicles in the wound demonstrated normal staining features.

Day 2 Alpha Smooth Muscle Actin Staining.

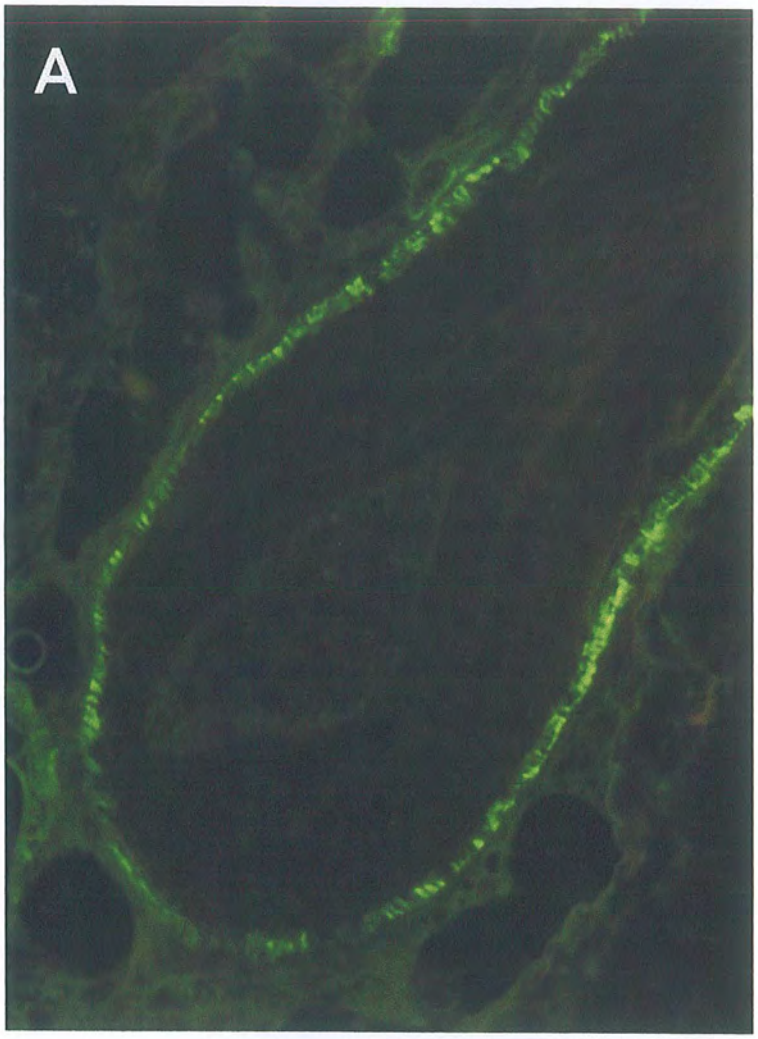
On inspection at low magnification the wound showed strong non-specific fluorescent marking in the most superficial parts as the wound scab stained brightly for α SMA (Figure 34 A).In general only diffuse streaky staining was noted in the rest of the wound.There was however positive staining around blood vessels in the base of the wound (Figure 34 B).

Enhanced α SMA staining was sometimes observed at the junction between the dermis of the wound edge and the wound infiltrate (Figure 34 C).However this was not a constant finding and in

Figure 33. Representative Photographs Showing The Normal Pattern Of αSMA Staining Of Anagen Hair Follicles.

A. Representative histological section showing the α SMA staining pattern around the bulb of a normal angen hair follicle.

B. A high power longitudinal section through an anagen hair follicle to show the α SMA staining arrangement around the hair shaft.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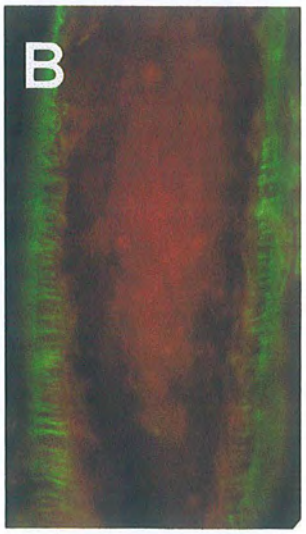

B

Figure 34. αSMA Stained Day 2 Histological Sections.

A. Section showing the strong non-specific α SMA marking of the day 2 wound scab.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

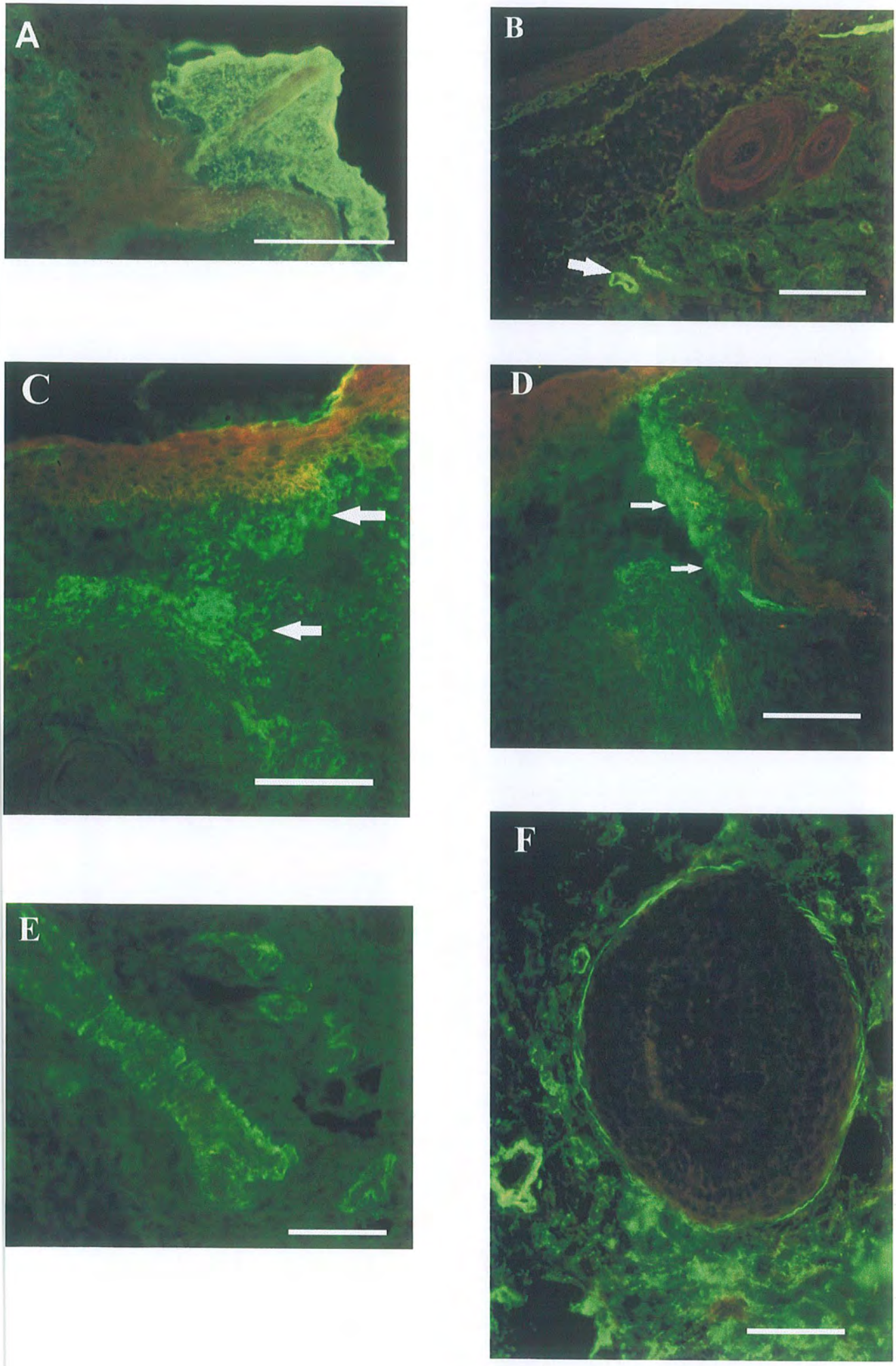
B. Section showing the day 2 wound and wound edge.Note the positive staining around blood vessels (arrow) at base of wound.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

C. Section showing positive αSMA marking at the junction between dermis edge of the wound and the wound tissue.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

D. Histological section showing positive staining (arrows) on the side of the follicle in contact with the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

E. Section showing the regular α SMA staining of a hair follicle in close proximity to the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

F. Tranverse section through a follicle close to wound.Note the surrounding speckled staining.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).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other wounds this phenomenon was not evident (Figure 34 B).Another interesting finding was an increased α SMA staining on the wound side of some hair follicles at the edge of the wound when compared to the other side in contact with the dermis of the wound edge (Figure 34 D).In some cases this was in continuation with the increased α SMA front at the junction between the dermis of the wound edge and the wound infiltrate.Hair follicles in the upper part of the dermis at the wound edge or in proximity to the wound showed no α SMA staining (Figure 34 B).Anagen follicles in the deeper dermis at the edge of the wound or in the proximity of the wound,in general showed strong and regular α SMA staining (Figure 34 E).Speckles of positive staining surrounding the bases of these positive follicles and increased positive staining in their vicinity was sometimes also noted (Figure 34 F).Another interesting observation was the fact that deep in the wound hair follicles positive for α SMA were noted adjacent to other follicles that were negative for α SMA.

Day 3 Alpha Smooth Muscle Actin Staining.

At day 3 the wound tissue was noted to be less fragile than at day 2 and it was therefore easier to obtain intact histological sections.Similar to the observations at day 2,a front of positive α SMA staining at the junction between the wound and the healthy tissue of the wound edge was observed at this stage.There was only moderately positive fine speckled α SMA staining in the substance of the wound itself when viewed at low power (Figure 35 A).The wound tissue was arranged in a rather homogenous network with speckles of positive staining (Figure 35 B).The wound scab,superficial to the newly formed epithelium showed strong non-specific staining (Figure 35 B). In some instances the positive α SMA staining in the wound was arranged in a homogenous pattern (Figure 35 C).Some hair follicles in the deep parts of the wound lost most of the positive α SMA staining and had only speckles of positive staining around them (Figure 35 D).

Some hair follicles at the edge of the deeper parts of the wound continued to grow up into the wound towards the surface taking some α SMA trails with them (Figure 35 E).Follicles adjacent to the wound edge in the upper or superficial parts of the dermis showed lack of α SMA staining (Figure 35 F).Blood vessels in the deeper parts of the wound stained positive for α SMA (Figure 35 G).

Figure 35. αSMA Stained Day 3 Histological Sections.

A. Section showing a day 3 wound.Note the fine speckled moderately positive staining.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

B. Section showing the homogenous arrangement of the wound tissue.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

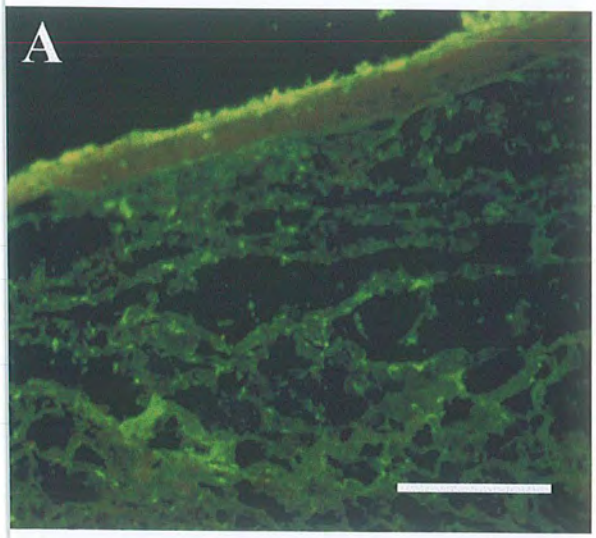
C. High power section showing the homogenous arrangement of positive α SMA staining in the wound tissue network.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

D. Section showing a hair follicle in the deep part of the wound.Note the loss of α SMA;only speckles of positive marking are present.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

E. Section showing trails of α SMA staining around a follicle in the wound that is possibly either repairing or regenerating.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

F. Section showing follicles at the edge of the wound in the upper parts of the dermis.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

G. Section showing blood vessels (arrows) in the deep parts of the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).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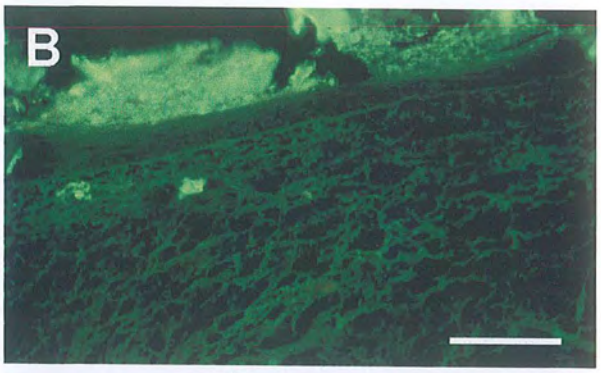
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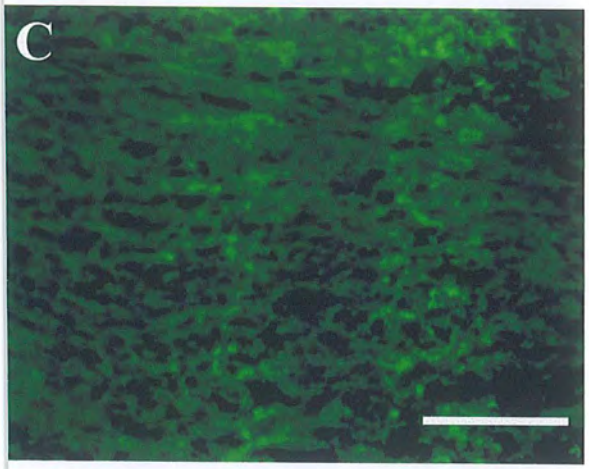
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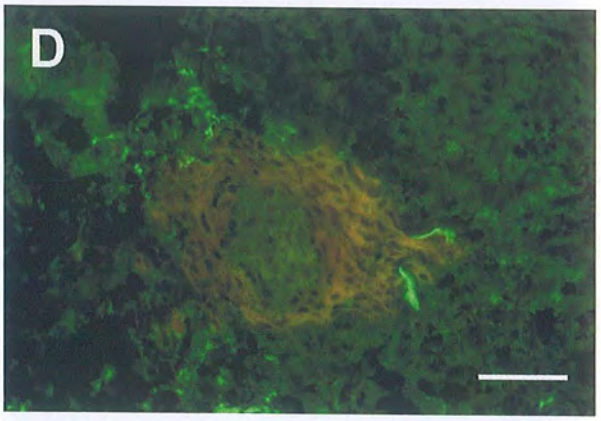
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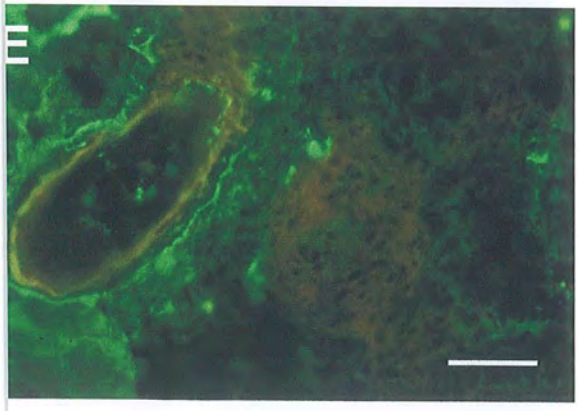
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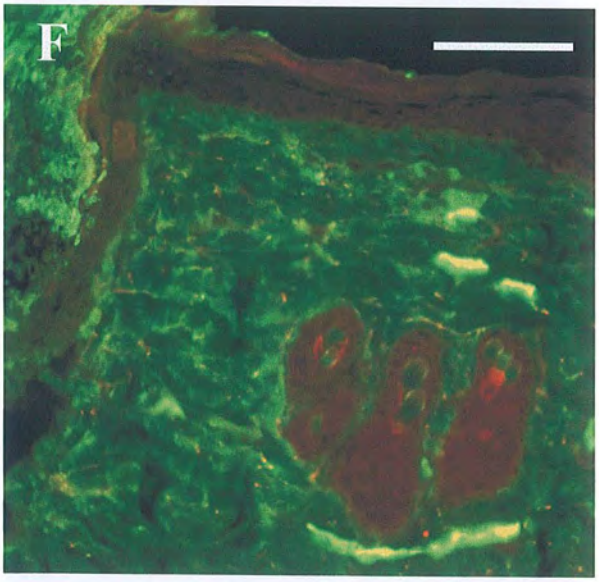
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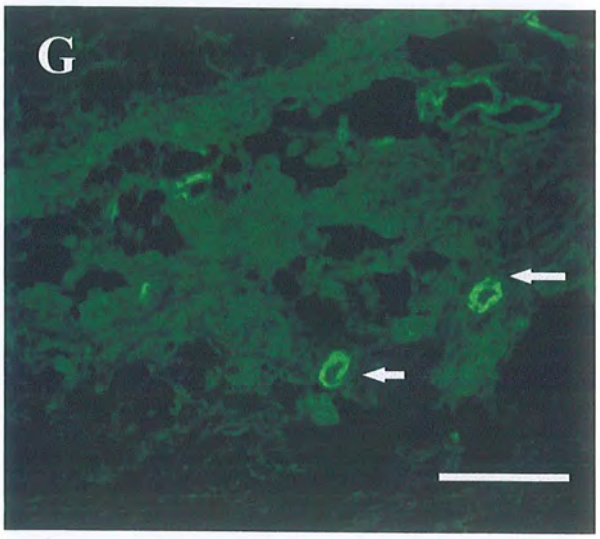
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Day 5 Alpha Smooth Muscle Actin Staining.

At day 5 a front of positive α SMA staining was noted at the junction between the normal dermis and the wound tissue (Figure 36 A and B).Some α SMA positive marking,was also present in the central region of the wound (Figure 36 C).Relative to day 2 and day 3,stronger and denser positive staining was seen in the deeper parts of the wound.The strongest positive labelling was observed at the level of the panniculus carnosus,where the marking appeared in the form of parallel transverse streaks traversing the width of the wound (Figure 36 D).Strong positive staining was also noted around the newly formed blood vessels.

Some hair follicles at the edge of the wound showed regular alpha smooth muscle actin staining (Figure 36 E).Others,right at the edge of the wound,had considerably more positive α SMA labelling on the side of the follicle in contact with the wound than on the side of the follicle in contact with the dermis of the wound edge.This staining was of a diffuse and irregular radiating pattern.Hair follicles away from the wound had normal regular spiral shaped α SMA positive staining.Hair follicles or follicle fragments within the wound tissue itself showed one of a few different patterns of staining.A frequently repeated observation in transversely sectioned hair follicles deep in anagen wounds was the positive staining that was strong,and radiated out from the follicles in a concentric pattern (Figure 36 F and G).In most cases this labelling was more or less uniform,and appeared thicker when compared to follicles at the wound edge or away from the wound.However in some follicles deep in the wound the staining was speckled and irregular (Figure 37 A and B).In longitudinal section some follicle elements deep in the anagen wounds were noted to have positive staining along their growth extensions.Some follicles demonstrated an element of radiating labelling (Figure 37 C and D) consistent with the observations on transverse sections.

In summary,at day 5 four patterns/grades of staining were observed around hair follicle elements in the wound:

- strong,thick and circular staining with concentric rings on transverse section.

Figure 36. αSMA Stained Day 5 Histological Sections.

A. Low power view of the day 5 wound.Note increased α SMA staining at junction of dermis with wound (arrow).
α SMA indirect immunofluorescence staining. (Scale bar = 400μ).

B. Section showing a front of α SMA staining at at the junction between the dermis at the wound edge and the wound tissue.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

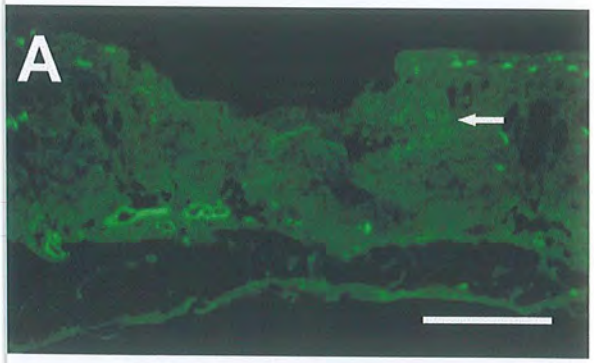
C. Section showing positive α SMA marking in the central part of the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

D. Section showing positive staining at the level of the panniculus carnosus.Note the parallel transverse streaks and the staining around the newly formed blood vessels.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

E. Section through a hair follicle at the edge of the wound (w) with regular α SMA staining (arrows).
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

F. High power tranverse section through an anagen hair follicle deep in the wound.Note the strong concentric and radiating positive α SMA staining around the follicle.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

G. High power section showing concentric positive staining around anagen hair follicle deep in the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).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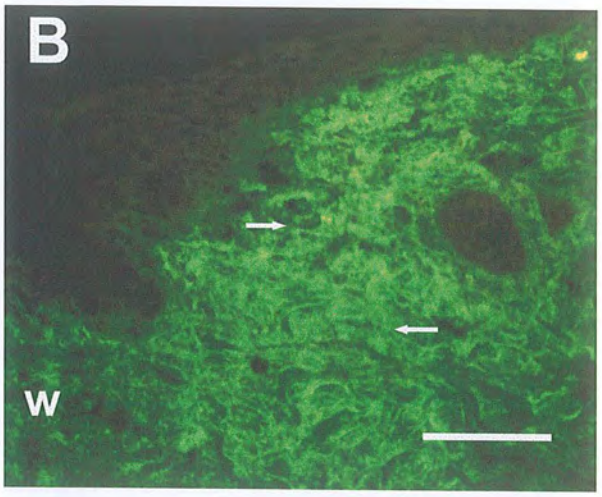
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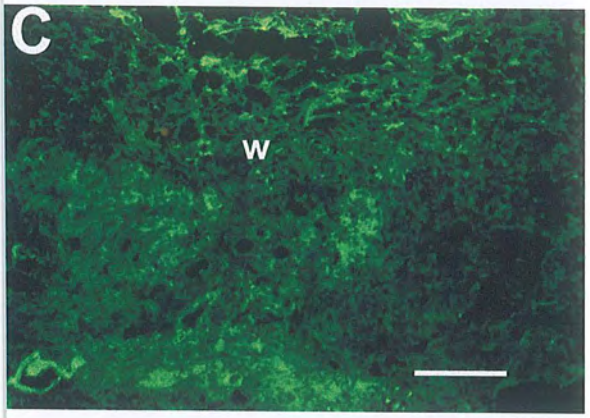
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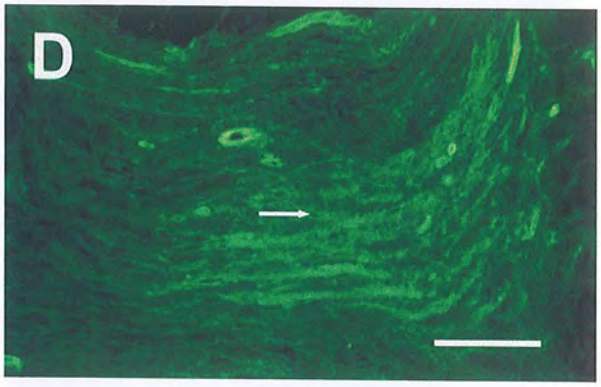
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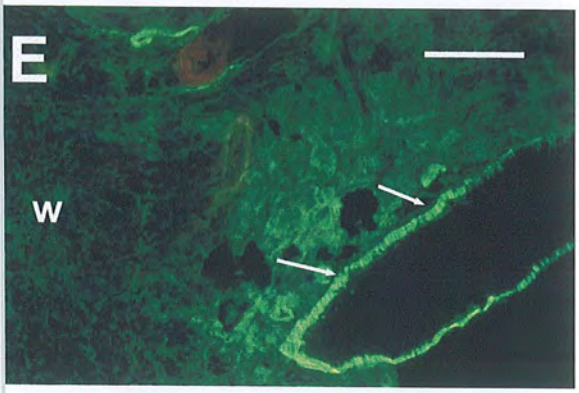
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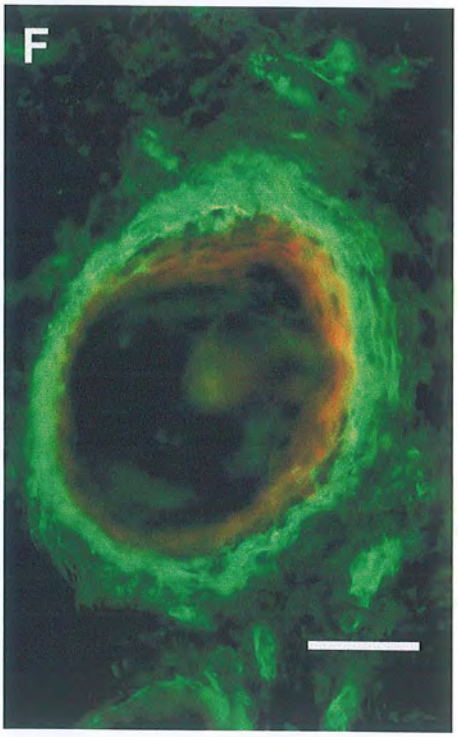
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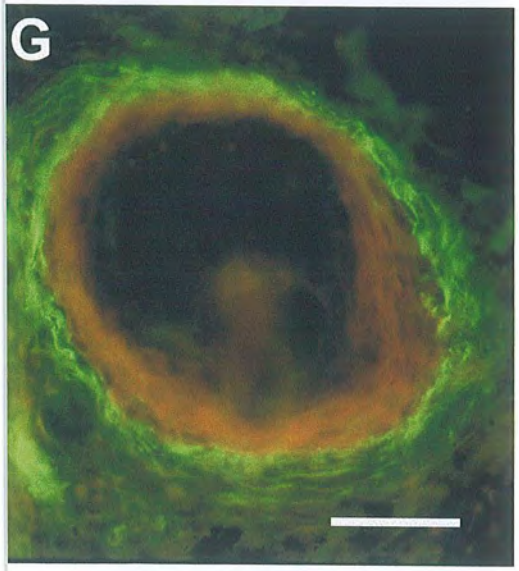

G

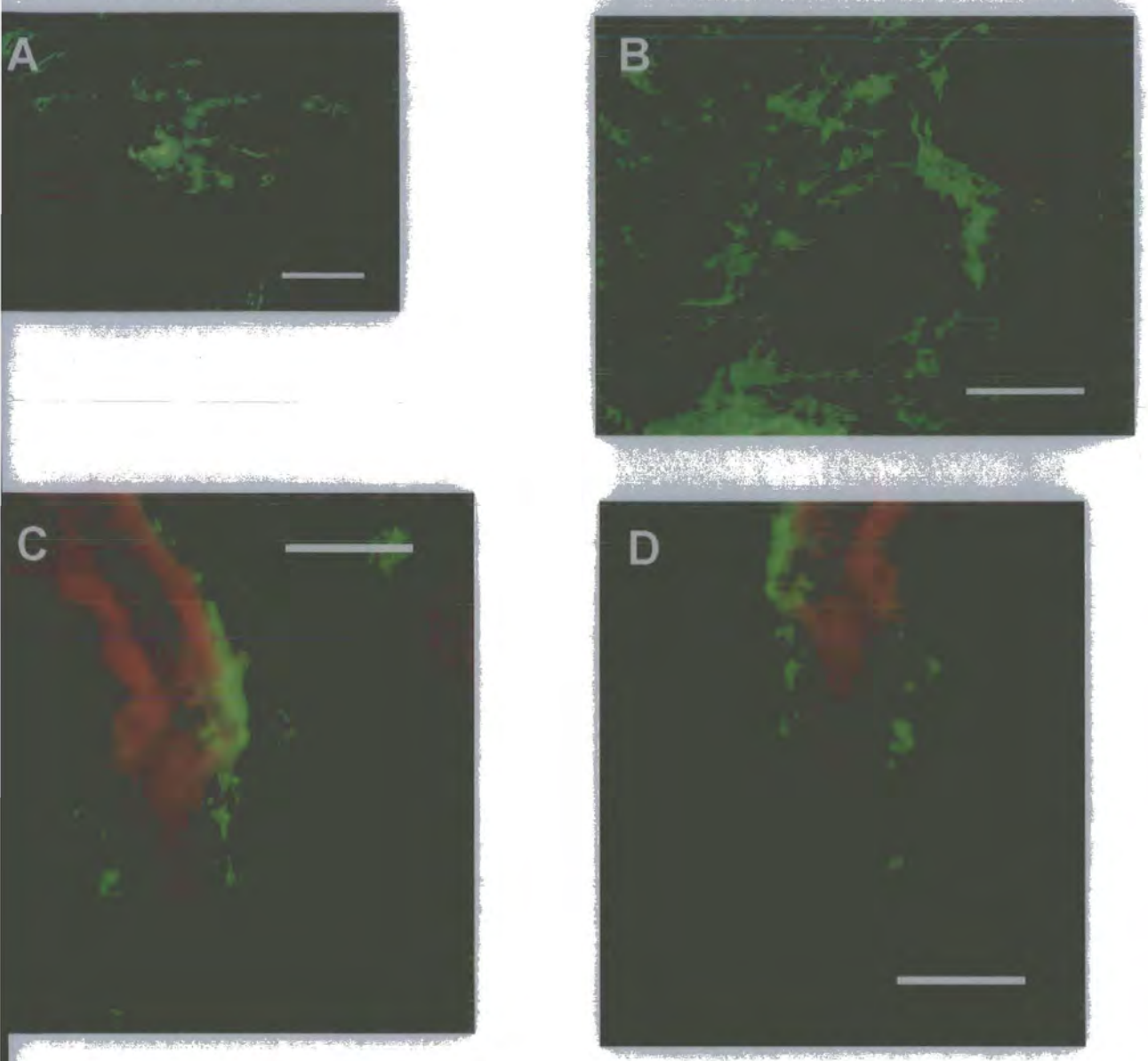
Figure 37. αSMA Stained Day 5 Histological Sections.

A. Section showing speckled,irregular and concentric staining around follicle deep in the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

B. Section showing irregular speckled staining around a hair follicle deep in the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

C. Longitudinal section through a hair follicle in the wound.Note the speckled radiating labelling.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

D. Section showing a hair follicle in longitudinal section in the wound with radiating irregular staining.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).


A
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D

- less thick,circular concentric staining.
- speckled staining.
- no staining at all.

The strong circular/spiral concentric pattern was observed around anagen follicles situated deep in the wound.This staining correlates in intensity with the strong positive marking observed in the lower parts of normal anagen follicles and is most likely to be derived from the dermal sheath cells of these parts of anagen follicles deep in the wound.The less strong circular concentric staining may correlate with the staining expressed by more upper parts of anagen follicles in the wound.

Day 7 Alpha Smooth Muscle Actin Staining.

At day 7,the scab over the wound still fluoresced strongly (Figure 38 A).The pattern of orientation of the positive staining in the wound seemed to be affected locally by the presence of hair follicles.In the vicinity of certain hair follicles in the wound the positive labelling had apparently assumed a fir-tree pattern radiating from the base and the sides of the lower part of the hair follicles (Figure 38 B and C).The radiating fir-tree pattern was coming out of follicular remnants that had no definite positive staining around them.In general there was an increased α SMA expression around intact hair follicles situated within the wound tissue itself especially in the deeper parts of the wound.When follicles were viewed in transverse section a definite irregular pattern of α SMA expression was seen in the vicinity of the follicles but with no concentric patterns (as for day 5),Figure 38 D.Follicles at the edge of the wound displayed no α SMA marking in the superficial part of the dermis but showed regular positive staining in the deeper parts of the dermis (Figure 38 E).This may have been due to the fact that anagen follicles normally express very little or no staining in the upper parts of the follicle but express strong positive markings in the lower parts of the follicle.However occasionally follicles at the edge of the wound did show irregular positive staining (Figure 38 F).

Day 8 Alpha Smooth Muscle Actin.

At day 8 the wound tissue looked very homogenous with diffuse positive alpha smooth muscle actin staining (Figure 39 A).Prominent staining was visible associated with new blood vessels within the wound (Figure 39 B).The wound fibroblasts and collagen network was very regularly aligned in a

Figure 38. αSMA Stained Day 7 Histological Sections.

A. Low power section showing the general appearance of the day 7 wound.Note the non-specific staining in the remnants of the wound scab.
α SMA indirect immunofluorescence staining. (Scale bar = 400μ).

B. Section showing hair follicles in wound.Note the fir tree pattern of radiating positive marking (arrow) around the lower part of the follicle.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

C. High power view of the follicle shown in **B.** highlighting the fir tree pattern of positive staining.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

D. Section showing irregular α SMA expression around an anagen follicle deep in the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

E. Section through a follicle at the edge of the deeper parts of the wound showing fairly regular positive marking.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

F. Section showing a follicle at the edge of the wound with irregular positive staining.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

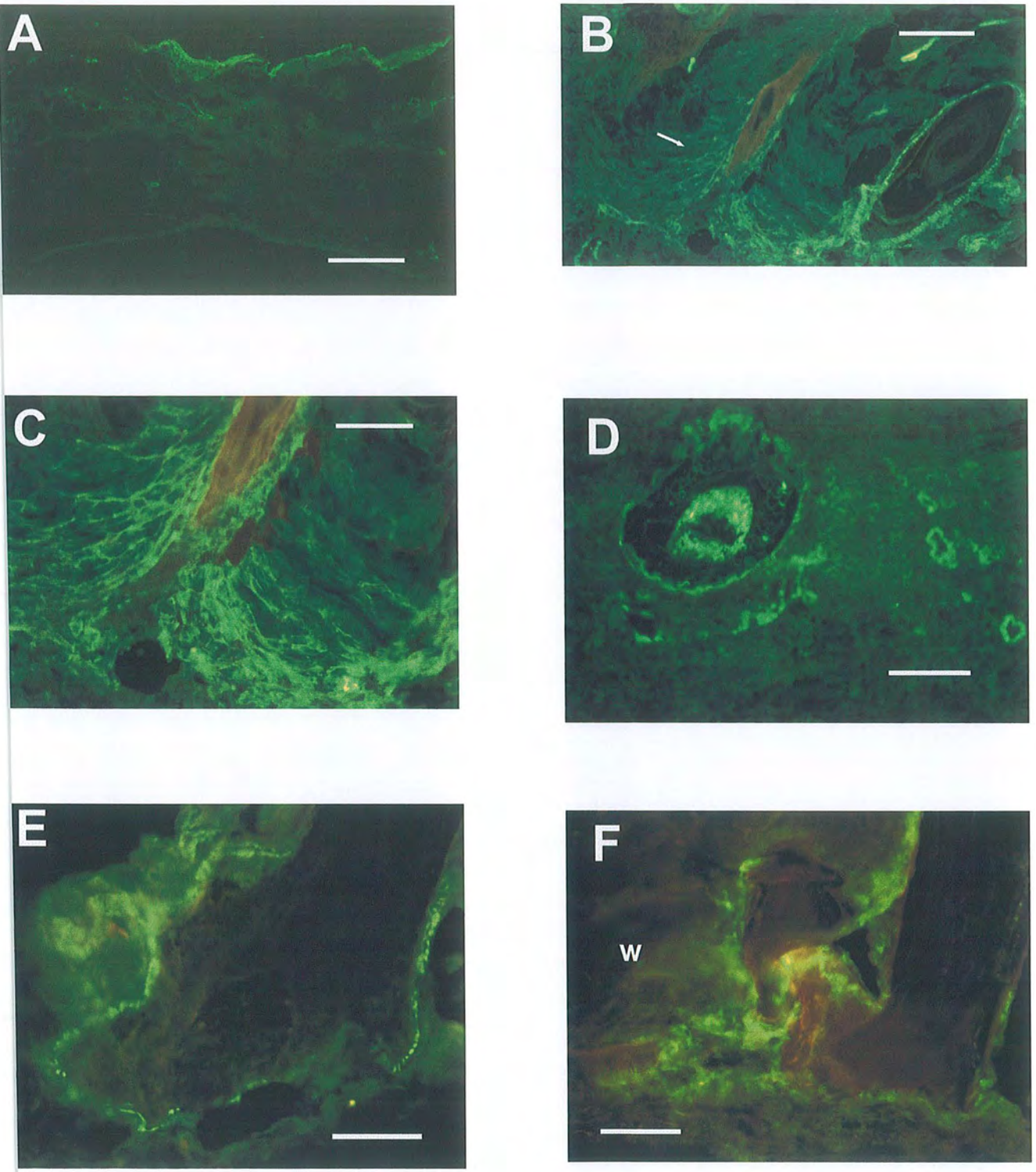
A
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Figure 39. αSMA Stained Day 8 Histological Sections.

A. Section showing a day 8 wound.Note the homogenous wound tissue arrangement and the diffuse positive α SMA marking.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

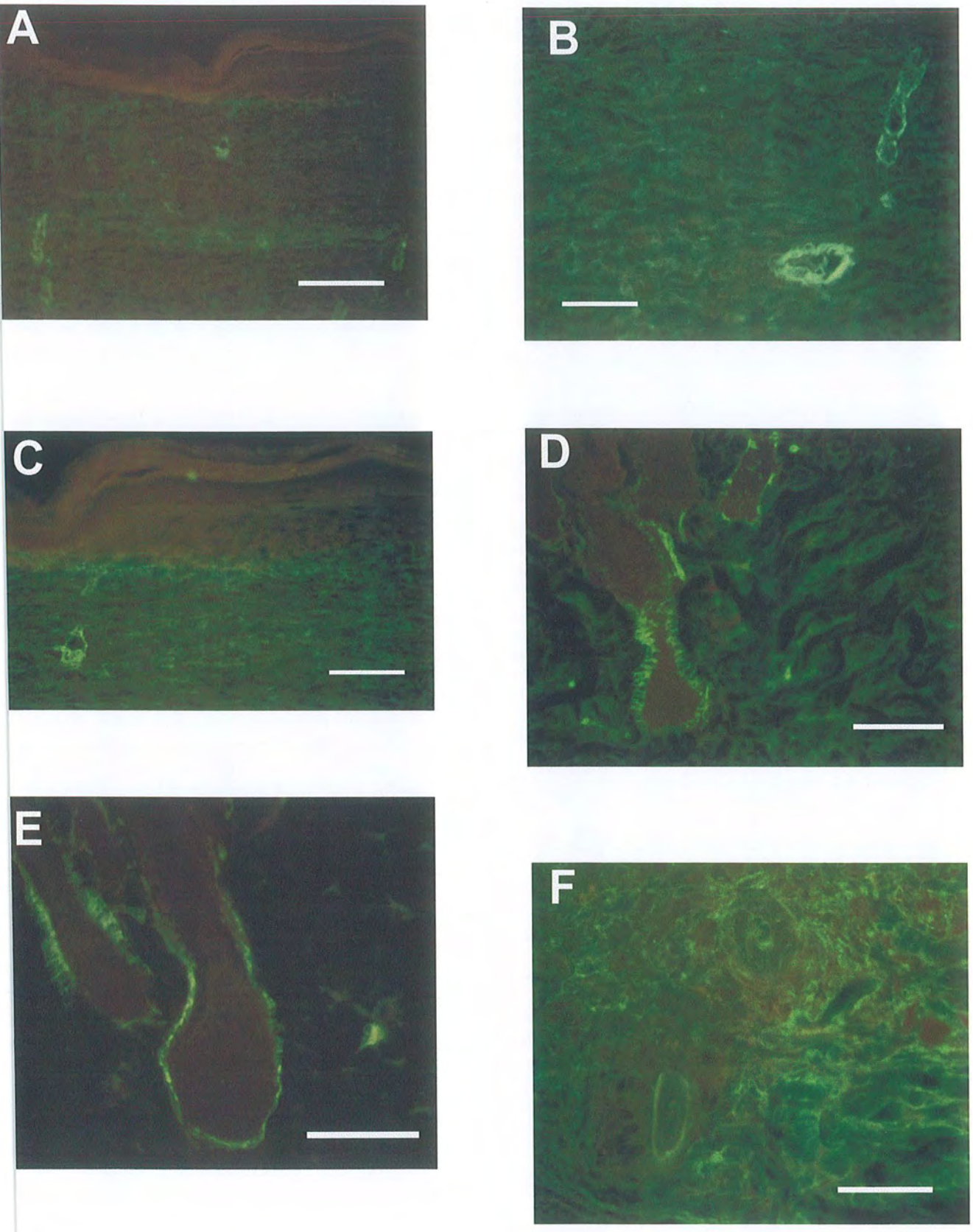
B. High power section showing the day 8 wound tissue.Note the positive staining around the newly formed blood vessels.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

C. High power section showing the uniform horizontal alignment of the wound fibroblast and collagen network.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

D. Section through a hair follicle in the peripheral part of wound showing regular positive staining.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

E. Section showing a hair follicle at edge of the wound.Note the regular α SMA marking.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

F. Section showing a hair follicle deep in the wound.Cells in the immediate vicinity of the follicle showed no specific pattern of staining.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).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horizontal fashion (Figure 39 C).Inspection of the deeper parts of the wound revealed a very similar uniform pattern,however the diffuse positive staining appeared to be somewhat stronger around the positively staining blood vessels (Figure 39 B).Hair follicles adjacent to or in the wound edge showed rather regular positive alpha smooth muscle actin staining (Figure 39 D and E).
Cells in the immediate vicinity of the follicle elements inside the wound showed some weak positive marking but this was similar to that expressed by cells further away from the follicle elements and showed no specific patterns (Figure 39 F).

Day 10 Alpha Smooth Muscle Actin.

At day 10 the wound looked very homogenous however the epithelium was still somewhat irregular.Diffuse irregular small streaks of positive staining were noted in the wound substance with no particular orientation pattern.Follicles in the vicinity of the wound showed streaks of positive staining associated with the lower parts of the follicles deep in the dermis (Figure 40 A).In general follicles deep in the dermis adjacent to the wound expressed regular positive markings (Figure 40 B).

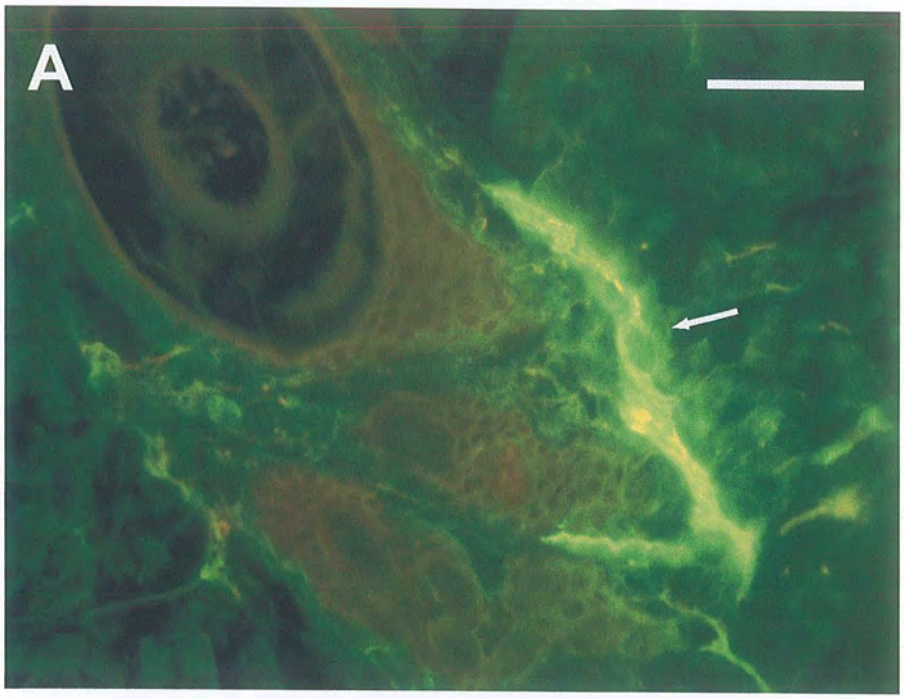
Day 15 Alpha Smooth Muscle Actin Staining.

At day15 the healed wound had a very homogenous texture with some irregular scattered streaks of positive α SMA marking as well as diffuse irregular areas of finer textured less bright positive staining (Figure 41 A).Positive staining in the form of streaks was also observed in relation to hair follicles (Figure 41 B).In some sections there was scattered marking in the form of streaks in the vicinity of possibly repairing or regenerating hair follicle fragments which were themselves surrounded by weak positive α SMA staining (Figure 41 C).Hair follicles in the vicinity of the wound or at the edge of the wound showed a regular α SMA staining pattern with no indication for radiating (Figure 41 D).Repairing or regenerating follicles in the wound were noted to have positive marking in the deeper parts of the wound (Figure 41 E).Some follicles in the wound or adjacent to the wound showed no positive staining at all.

Figure 40. αSMA Stained Day 10 Histological Sections.

A. Section showing streaks of positive staining (arrow) associated with the lower parts of the hair follicles deep in the dermis in the vicinity of the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

B. Section showing regular positive α SMA marking around follicles adjacent to the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).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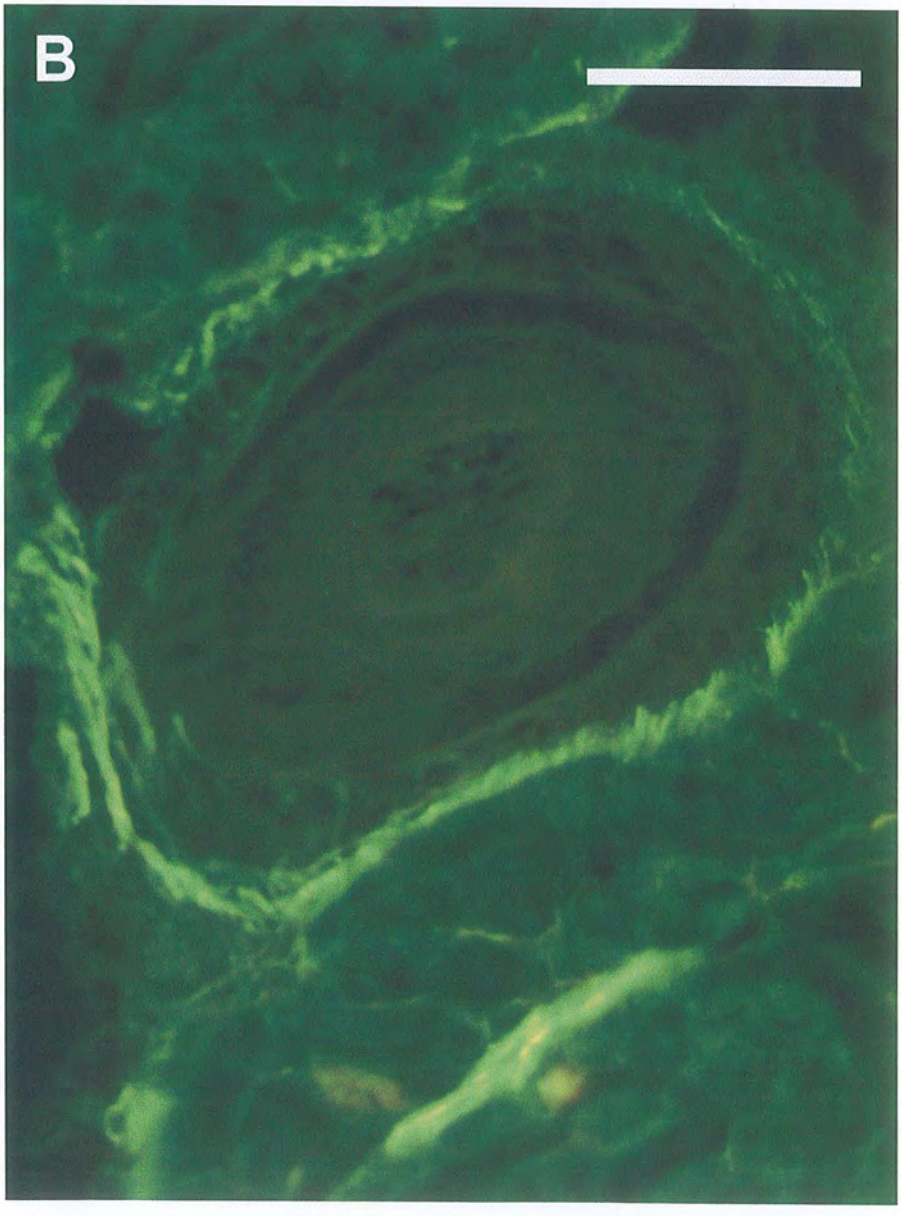

B

Figure 41. αSMA Stained Day 15 Histological Sections.

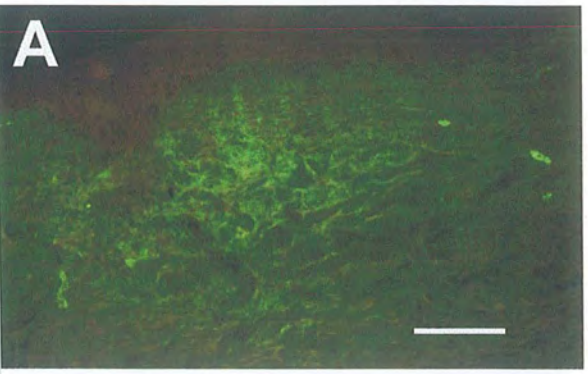
A. Section showing the day 15 wound tissue.Note the homogenous texture of the wound tissue and the fine diffuse positive α SMA marking with some scattered streaks of stronger staining.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

B. Section showing streaks of positive staining around and close to hair follicles/follicle elements in the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).

C. Section showing weak positive marking around and in the vicinity of possibly repairing hair follicle fragments in the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

D. High power section showing regular positive α SMA staining around follicles at the wound edge and in the vicinity of the wound.
α SMA indirect immunofluorescence staining. (Scale bar = 50μ).

E. Longitudinal section through a hair follicle within the wound.Note the positive marking around the base of the hair follicle (arrow).
α SMA indirect immunofluorescence staining. (Scale bar = 100μ).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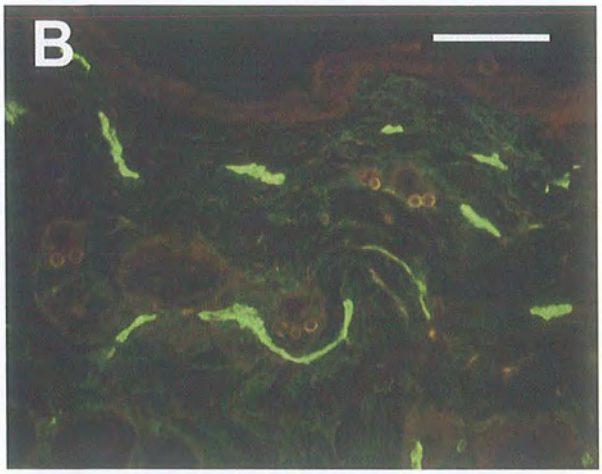
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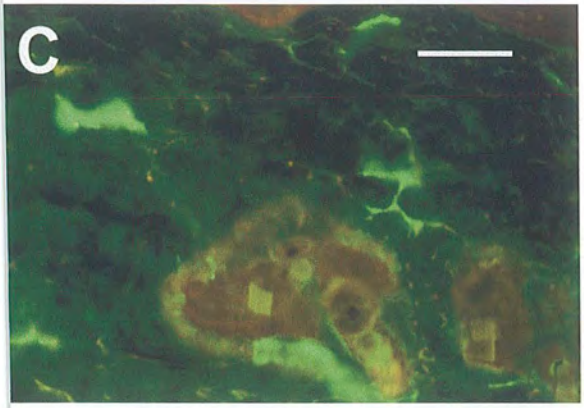
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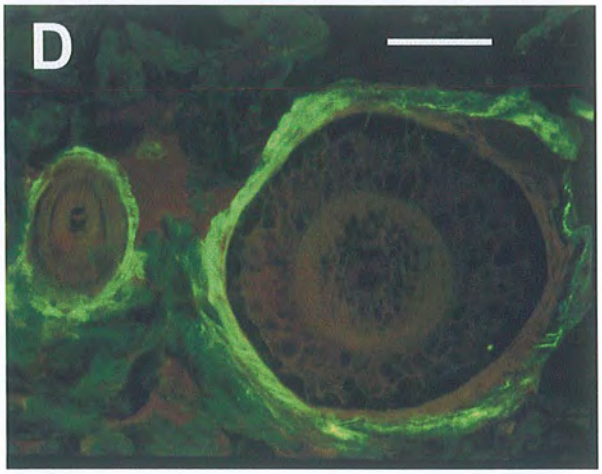
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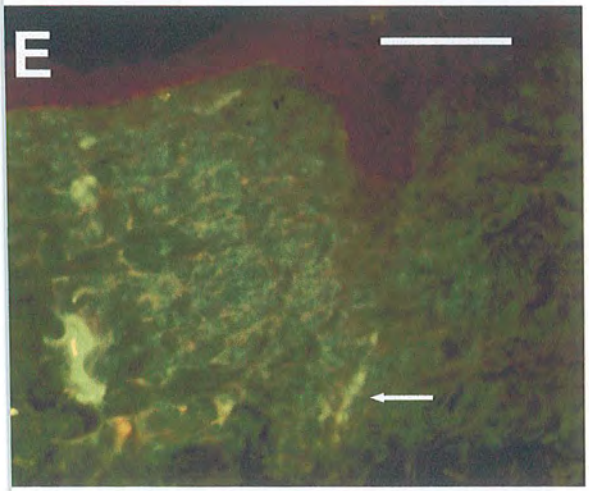
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4.6.2 Confocal Microscopy.

Histological sections stained using indirect immunofluorescence for alpha smooth muscle actin were also examined using confocal laser microscopy.In general the confocal microscopy examination confirmed in further detail the observations noted on light microscopy examination.In order to concentrate on the timing when the α SMA changes were observed;day 3,day 5 and day 7 sections only were studied under the confocal microscope.

Day 3 Alpha Smooth Actin Confocal Microscopy Observations.

Confocal microscopy examination of the day 3 α SMA labelled histological sections revealed some more detail of the staining characteristics of the hair follicles in the wound and in the vicinity of the wound.Hair follicles in the wound crater itself showed very little positive staining (Figure 42 A).Follicles right at the edge of the wound showed more positive marking,however this was still weak (Figure 42 B).Examination of hair follicles close to the wound showed stronger α SMA expression.The positive marking appeared as bright speckles of staining radiating out of the follicles (Figure 42 C).Similar observations were noted in follicles slightly further away from the wound (Figure 42 D).Follicles situated away from the wound showed a normal pattern of staining.

Day 5 Alpha Smooth Actin Confocal Microscopy Observations.

At day 5 the wound tissue itself contained more speckles of positive staining than the adjacent dermis (Figure 43 A).Hair follicles or follicle fragments in the wound demonstrated a varied pattern of staining.Some hair follicles in the depths of the wound lost most of the α SMA (Figure 43 B and C) whilst other neighbouring follicles in the wound showed bright positive marking with a concentric radiating pattern (Figure 43 D and E).Other follicles within the wound substance stained only partially for α SMA.

Day 7 Alpha Smooth Actin Confocal Microscopy Observations.

Examination of the day 7 alpha smooth muscle actin stained sections showed a varied pattern of staining of the hair follicles in and around the wound.Some follicles at the base of the wound and at

Figure 42. Confocal microscopy:Day 3 αSMA Stained Sections.

A. Section showing a hair follicle in a day 3 wound.Note the absence of positive staining.
α SMA indirect immunofluorescence staining. (Scale bar = 64μ).

B. Section showing a hair follicle at the wound edge in a deeper part of the dermis.Note the positive α SMA marking.
α SMA indirect immunofluorescence staining. (Scale bar = 64μ).

C. Section showing a hair follicle in the vicinity of the wound with stronger positive staining.
α SMA indirect immunofluorescence staining. (Scale bar = 64μ).

D. Section showing a hair follicle slightly further away from the wound expressing speckled positive marking.
α SMA indirect immunofluorescence staining. (Scale bar = 64μ).

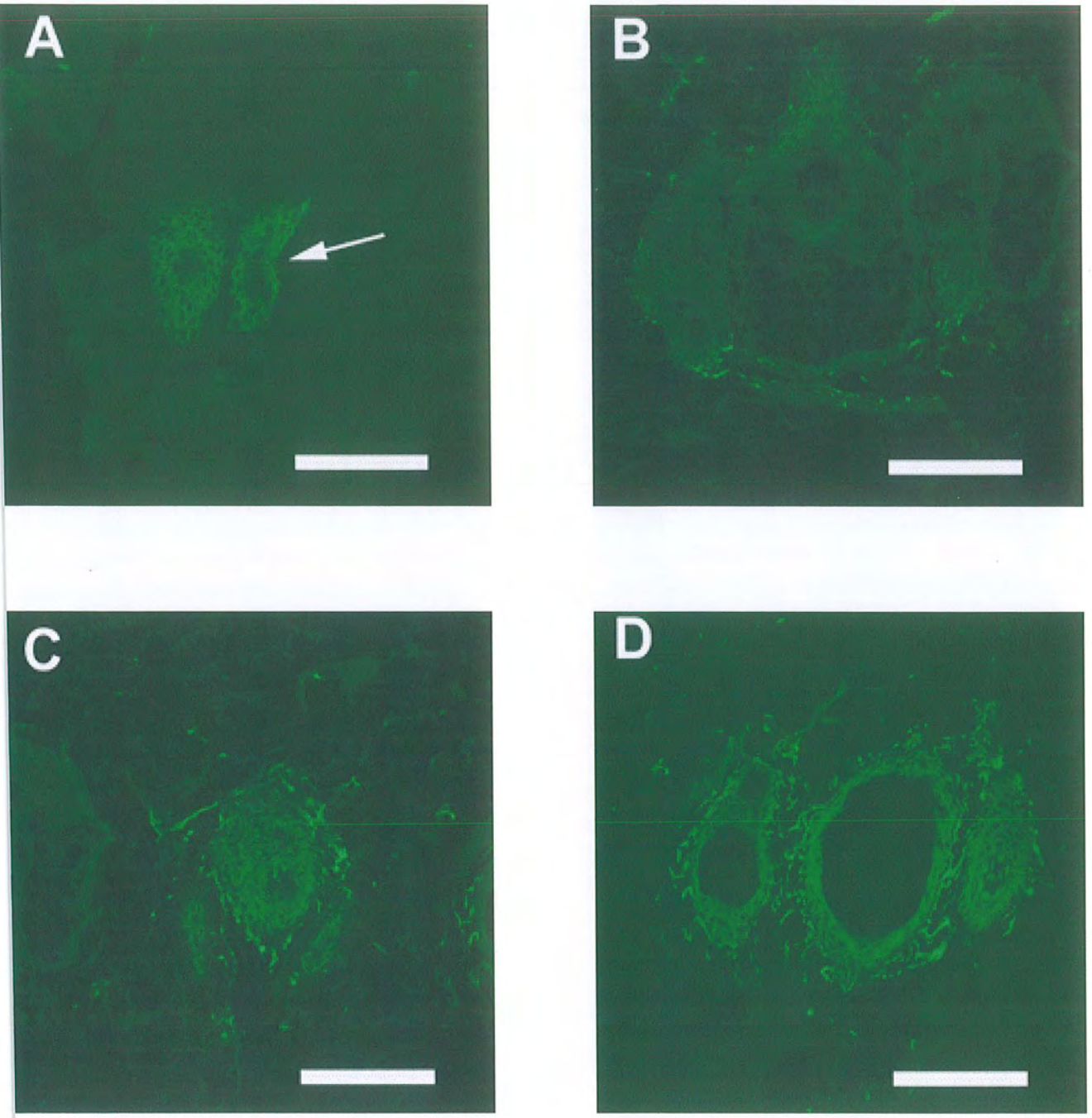
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Figure 43. Confocal microscopy:Day 5 αSMA Stained Sections.

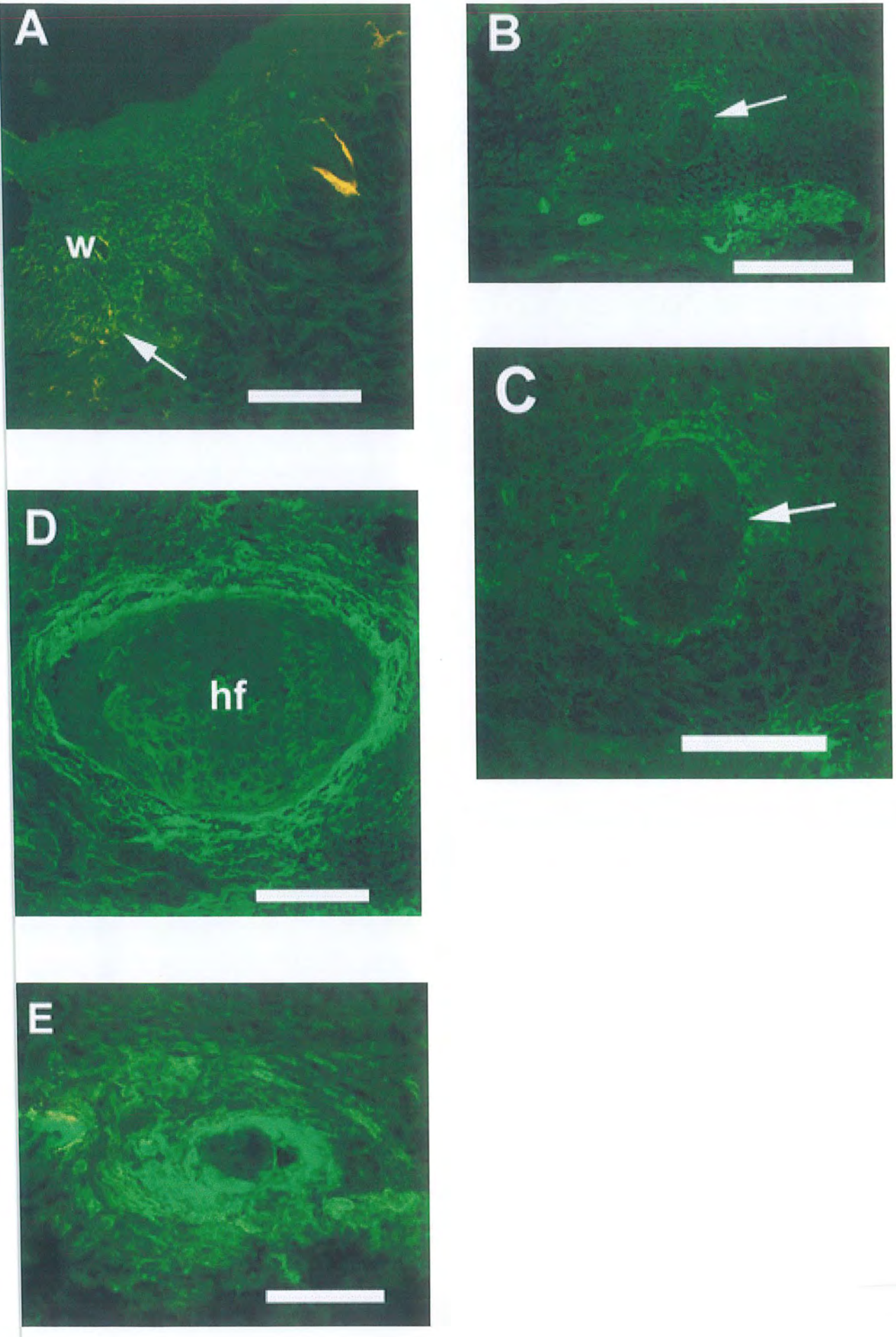
A. Section showing a day 5 wound (w).Note the diffuse speckled positive staining (arrow) in the wound compared to the nearby dermis.
α SMA indirect immunofluorescence staining. (Scale bar = 128μ).

B. Section showing a hair follicle (arrow) in the deep parts of the wound that has lost most of the positive marking.
α SMA indirect immunofluorescence staining. (Scale bar = 128μ).

C. High power section of the same follicle as in **B.** to highlight the weak positive staining.
α SMA indirect immunofluorescence staining. (Scale bar = 64μ).

D. High power section through a hair follicle (hf) in the wound with expressing concentric strong positive α SMA marking.
α SMA indirect immunofluorescence staining. (Scale bar = 64μ).

E. High power section showing a hair follicle in the wound with strong concentric/spiral radiating positive α SMA marking.
α SMA indirect immunofluorescence staining. (Scale bar = 64μ).


A
w
B
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hf
E

the edge of the wound expressed no positive staining.No follicles with radiating patterns of staining were noted in the wound.

Conclusions.

Confocal microscopy observations correlated well with the UV light microscopy findings.On several occasions it helped to eliminate background staining and clarified the true positive staining allowing more confident interpretation of the observations.In particular it reaffirmed the day 5 UV light microscopy findings of radiating concentric patterns of positive marking around hair follicles in the wound by providing images of higher magnification and definition and eliminating the effect of background staining.

4.7 Summary And Analysis Of Results.

(A summary of the results is shown in Tables 4. to 10.)

Day 2 (Table 4.).

At day 2 the wound area appeared to be mostly occupied by red blood cells of the original wound haematoma and a dense infiltrate of mononuclear cells.Very few follicle fragments or hair follicles could be seen in the wound cavity.The skin epithelium and the outer root sheath of the hair follicles at the edge of the wound were hypertrophied with an increased number of layers of cells and increased mitotic figures.The outer root sheath cell hypertrophy was in continuity with the hypertrophied adjacent skin epithelium.Cells from the epithelium at the edges had advanced steadily to cover the peripheral parts of the wound defect.The dermal sheath of the hair follicles at the edge of the wound was regular and normal in appearance.Indirect immunofluorescence staining for collagen IV and laminin showed a weak positive marking for both the antigens and was noted underneath the skin epithelium at the edge of the wound and in the middle of the wound indicating early basement membrane regeneration at the wound edge and at the middle of the wound.
There was an increase in alpha smooth muscle actin staining at the edge of the wound compared to other areas,however there was very little positive staining in the substance of the wound itself.

Table 4. Summary Of Results.

Day 2 wounds.

	Weigerts' Haematoxylin	**Collagen IV and Laminin**	**α SMA**
Wound	Wound haematoma. Mononuclear cell infiltrate.	Positive marking around blood vessels.	Some increased marking at junction between wound and adjacent dermis.
Wound Epithelium	Wound generally fully epithelialised. Hypertrophy.Increased mitotic figures.	BM staining (low intensity) at edge of wound and at centre of wound.	
Hair Follicles:			
• **Follicles in wound**	Normal DS. Surrounded by mononuclear cell infiltrate.	Either retained or lost BM staining.	Some follicle elements showed positive marking and some showed no positive marking.
• **Follicles at edge or close to of wound**	Hypertrophy of ORS. Surrounded by mononuclear cell infiltrate.	Normal undistorted BM staining.	Strong regular staining in anagen follicles situated in the deeper tissues.
• **Follicles away from wound**	No change in morphology.	Normal BM staining.	Normal staining patterns.

	Anagen Wounds		**Telogen Wounds**		**Difference in wound diameter (*p* value)**
Wound Diameters (mm)	**Wound 1.**	**Wound 2.**	**Wound 3.**	**Wound 4.**	
Rat 1.	2.1	1.7	2.1	2.2	**>0.05**
Rat 2.	1.8	1.7	2.2	2.0	
Rat 3.	1.7	1.75	2.0	2.1	
Macroscopic Observations	Red wound scab. Steep wound edges.		Red wound scab. Steep wound edges.		

Day 3 (Table 5.).

By day 3 the epithelial covering of the wound had advanced further to cover the entire surface of the wound.The skin epithelium at the wound edge was even more hypertrophied than at day 2.The outer root sheath of hair follicles at the edge of the wound was also more hypertrophied and composed of multiple layers of cells with mitotic figures increasing in number towards the edge.

The dermal sheath of hair follicles adjacent to the wound was hypertrophic and made up of multiple layers of pink staining cells (Weigerts' Haematoxylin).The dermal sheath cells of these follicles appeared to radiate and blend with the adjacent connective tissue.Hair follicles directly in contact with the wound edge also showed a radiating dermal sheath pattern which blended into the wound infiltrate itself and was surrounded by an dense mononuclear infiltrate.

The laminin marking had advanced from the edges of the wound towards the central part of the wound to a greater extent than the collagen IV,possibly indicating a more rapid lay down of laminin compared to collagen IV.

There was only moderate positive alpha smooth muscle actin staining in the wound itself however there was an area of increased staining at the perimeter of the wound suggesting myofibroblast activity.Some follicles in the deepest parts of the wound lost the positive staining whilst others showed speckles of positive alpha smooth muscle actin staining.The fact that the dermal sheath hypertrophied in the vicinity of the wound indicates that it is possibly involved in or contributes to the wound healing process.The fact that the dermal sheath tends to radiate out and blend into the adjacent tissues including wound tissue suggests a possible migration of dermal sheath cells into the vicinity of the wound and into the wound itself.The alpha smooth muscle actin speckles of positive staining around some follicles deep in the wound suggests dermal sheath cell activation and migration out of the deeper parts of the follicle into the wound and its vicinity.

Table 5. Summary Of Results.

Day 3 wounds.

	Weigerts' Haematoxylin	Collagen IV and Laminin	α SMA
Wound	Predominantly mononuclear infiltrate. Fewer red blood cells.	Homogenous tissue. Positive marking around blood vessels.	Homogenous tissue with speckles of positive staining. Positive marking at junction between wound and adjacent dermis. Positive marking around blood vessels.
Wound Epithelium	Wound fully epithelialised. Hypertrophied at wound edge.	Advancement of BM staining from edges to centre of wound. BM staining also observed at central part of wound.	
Hair Follicles:			
• **Follicles in wound**	Early signs of blending of DS with wound cellular network.	Some follicle fragments retained BM marking and some partially or completely lost the BM expression.	Some follicles in the deep parts of the wound lost the positive marking. Only surrounding speckles of positive staining.
• **Follicles at edge or close to of wound**	Hypertrophied ORS. Hypertrophied and blended DS. Increased mononuclear cell infiltrate around these follicles.	In general,strong regular BM staining. Occasionally irregular.	Growth extensions with trails of positive staining. No positive marking in follicles in upper parts of dermis.
• **Follicles away from wound**	Normal DS. No surrounding mononuclear cell infiltrate.	Normal BM staining.	Normal marking.

	Anagen Wounds		Telogen Wounds		Difference in wound diameter (*p* value)
Wound Diameters (mm)	**Wound 1.**	**Wound 2.**	**Wound 3.**	**Wound 4.**	
Rat 4.	2.0	2.0	2.0	2.0	
Rat 5.	2.0	2.2	1.8	1.8	**>0.05**
Rat 6.	1.8	2.1	1.8	1.9	
Macroscopic Observations	Darker red scab. Sloping wound edges.		Darker red scab. Sloping wound edges.		

Day 5 (Table 6.).

The wound epithelium was very irregular (wavy) and hypertrophic.It was relatively thicker (more hypertrophied) at the wound edges.Hair follicles at the wound edge as well as follicles close to the wound still had a hypertrophied outer root sheath.Follicles or follicle fragments in the substance of the wound also showed hypertrophy of the outer root sheath on transverse section.On longitudinal section,these follicles elements demonstrated a hypertrophied outer root sheath growing towards the surface to join with the surface epithelium.Hair follicle reparative processes were also noted.The outer root sheath of injured follicles or follicle remnants in the wound grew towards the surface or towards the epithelial components of the other hair follicles to attempt to repair the sleeve for the new hair fibre growth.

The dermal sheath of hair follicles or follicle fragments in the wound had intricately blended and integrated with the cellular network of the wound and the dermal sheath could no longer be identified as a separate structure.The dermal sheath cells had possibly migrated out into the wound so that the wound fibroblasts were now in contact with the glassy membrane of the hair follicle or alternatively the dermal sheath cells underwent a change in phenotype to fibroblasts so that they were now unidentifiable from the surrounding fibroblast network.The wound fibroblasts aligned themselves in a concentric fashion around the hair follicles in the wound.These concentrically aligned fibroblasts may have been normal wound fibroblasts or they may have been dermal sheath cells which have undergone a phenotypic change to wound healing fibroblasts.The dermal sheath of follicles at the wound edge or close to the wound was also noted to be hypertrophied and radiating into and blending with the adjacent tissues.This increase in number of cells and the tendency to migrate out is a further indication of the activity going on in the dermal sheath at this stage.An observation which more directly shows the effect of the wound stimulus on the hair follicle dermal sheath is the fact that in hair follicles precisely at the edge of the wound the dermal sheath was indistinguishable from the wound fibroblasts on the side of the wound whereas it was hypertrophied and had a radiating pattern on the side away from the wound.The dermal sheath cells on the side of

the wound may have either migrated away or have transformed into fibroblasts.Hair follicles examined in longitudinal section in the wound were noted to be surrounded by a fir-tree pattern of fibroblast arrangement.The presence of hair follicles in the wound may have influenced the alignment of the fibroblasts around them or the phenotypic fibroblasts in the concentric/fir-tree pattern are derived from the dermal sheath of the follicle they surround,hence the orientation pattern.

Laminin and collagen IV basement membrane antigen expression was seen along the entire span of the wound epithelium and the staining was now stronger in intensity than in earlier wounds.

Alpha smooth muscle actin staining at day 5 showed a stronger positive staining in the periphery of the wound as compared to earlier wounds.The wound substance itself showed some definite positive staining indicating the presence of myofibroblasts in the wound centre.The deeper parts of the wound showed stronger positive staining.The strongest staining was at the level of the panniculus carnosus.This observation correlates with the fact that the most positive staining part of the hair follicle dermal sheath is situated in the deeper layers of the skin. If the dermal sheath was contributing to the wound myofibroblasts population then the deeper skin layers would be expected to have a higher concentration of positive staining cells during wound healing.The increased staining at the level of the panniculus carnosus was orientated in parallel streaks aligned transversely across the wound.This orientation pattern is partly in correlation with the *organizational theory* of wound contraction (Ehrlich et al 1986 and Ehrlich1988). Follicles at the edge of the wound showed increased positive alpha smooth muscle actin staining on the side in contact with the wound.This staining was irregular and had a radiating pattern blending into the wound.Anagen follicles in the wound itself showed concentric radiating patterns of alpha smooth muscle actin on transverse section. Longitudinal sections revealed radiating labelling around the bases of the follicles and positive staining along the growth extensions of the follicles.These observations suggest that the dermal sheath cells of the hair follicles may have migrated out in a concentric fashion into the wound.The strong positive staining of these possibly migrating dermal

sheath cells indicates a high level of intracellular alpha smooth muscle actin isoform,making them phenotypically very similar to myofibroblasts and highly suggestive of their possible phenotypic change to myofibroblasts.

Table 6. Summary Of Results.

Day 5 wounds.

	Weigerts' Haematoxylin	Collagen IV and Laminin	α SMA
Wound	Well organised tissue architecture composed of fibroblasts and mononuclear cells.	Positive staining around blood vessels.	Positive marking at junction between wound and adjacent dermis. Positive staining in central part of wound. Positive staining around new blood vessels.
Wound Epithelium	Complete epithelialisation. Irregular and hypertrophic epithelium.	BM expression along entire length of wound epithelium.	
Hair Follicles:			
• **Follicles in wound**	DS blended and integrated with surrounding wound tissue. Concentric arrangement of wound fibroblasts or DS cells around hair follicles.	Some follicles lost both collagen IV and laminin staining (retained some α SMA marking). Some follicles retained both the collagen IV and laminin staining (concentric radiating α SMA pattern).	Thick concentric pattern of strong positive marking. Speckled concentric staining. Loss of positive marking. Positive staining along growth extensions.
• **Follicles at or close to edge of wound**	Hypertrophied ORS. Hypertrophied DS. DS blended with wound tissue on side of follicles directly in contact with wound.	In general,normal BM staining.	Some follicles had increased marking on side in contact with wound.
• **Follicles away from wound**	No DS hypertrophy.	Normal BM staining.	Normal spiral α SMA staining.

	Anagen Wounds		Telogen Wounds		Difference in wound diameter (*p* value)
Wound Diameters (mm)	**Wound 1.**	**Wound 2.**	**Wound 3.**	**Wound 4.**	
Rat 7.	2.0	2.0	2.0	2.0	**>0.05**
Rat 8.	2.1	1.9	2.0	2.0	
Rat 9.	1.75	1.9	1.9	2.0	
Macroscopic Observations	Shiny new epithelium at wound edge. Wound less deep,smoother concavity.		Shiny new epithelium at wound edge. Wound less deep,smoother concavity.		

Days 7 (Table 7.) and Day 8 (Table 8.).

At day 7 and day 8,the histological sections showed even more advanced epithelial and hair follicle reparative processes.The wound surface epithelium remained hypertrophic but was now smoother and more regular.The outer root sheath of the follicles close to the wound and at the edge of the wound was hypertrophied with multiple layers of cells.Mitotic figures were noted in the outer root sheath of follicles at the edge of the wound.Regenerating or repairing hair follicles were observed both in the substance of the wound tissue itself and in the substance of the newly formed hypertrophied epithelium. Injured hair follicles in the wound were noted to grow extensions directed either towards the surface epithelium or laterally to join neighbouring epithelial components of other repairing injured hair follicles.These growing branches showed trails of positive marking in α SMA stained sections indicating dermal sheath growth and regeneration along the new extensions.

At day 7 the wound fibroblast arrangement in concentric and fir-tree patterns around the hair follicles was still very conspicuous.The dermal sheath of follicles in the wound blended with the adjacent fibroblast network and was no longer identifiable as a separate layer of cells.In follicles exactly at the edge of the wound the dermal sheath was identifiable on the side of the follicle in contact with normal dermis but not identifiable on the side in contact with the wound.

Examination of the day 8 sections continued to reveal the blending of the dermal sheath of the hair follicles in the wound and at the edge of the wound with the adjacent wound tissue.The dermal sheath was still indistinguishable from the surrounding wound fibroblasts.

At day 7 and day 8 the epithelial basement membrane showed complete and bright collagen IV and laminin staining.Follicles in the wound either retained or lost the collagen IV and laminin basement membrane staining.An interesting point noted on day 8 was the fact that hair follicle elements included in the newly regenerated epithelium showed no positive marking for laminin and collagen IV.

In day 7 sections alpha smooth muscle actin staining in the wound showed fir-tree patterns of labelling around hair follicles in longitudinal section and irregular positive increased staining with no definite concentric patterns in transverse section.In day 8 wounds alpha smooth muscle actin revealed a diffuse pattern of weak staining with a horizontal alignment of the collagen-fibroblast network.There was no particular increased positive labelling in the wound apart from diffuse irregular streaks of positive staining.

Table 7. Summary Of Results.

Day 7 wounds.

	Weigerts' Haematoxylin	Collagen IV and Laminin	α SMA
Wound	Hour glass shape (wound contraction). Less mononuclear cells. Higher concentration of fibroblasts.	Positive staining around blood vessels.	Some positive marking in wound tissue.
Wound Epithelium	Hypertrophic. Smoother and more regular.	Complete and bright BM staining.	
Hair Follicles:			
• **Follicles in wound**	Concentric fibroblast pattern. Fir-tree fibroblast pattern. Blended DS.	Some follicles retained both laminin and collagen IV staining. Some follicles lost either laminin or collagen IV staining.	Increased expression around intact follicles but no definite concentric patterns. Fir-tree pattern of positive marking.
• **Follicles at or close to edge of wound**	Hypertrophied ORS. Mononuclear cell infiltrate around bulbs. DS blended with fibroblast network on side in contact with wound.	In general showed regular BM marking. Occasionally somewhat ragged or irregular.	Regular positive staining. Occasionally irregular staining.
• **Follicles away from wound**	No change in morphology.	Normal BM staining.	Normal staining.

	Anagen Wounds		Telogen Wounds		Difference in wound diameter (*p* value)
Wound Diameters (mm)	**Wound 1.**	**Wound 2.**	**Wound 3.**	**Wound 4.**	
Rat 10.	1.0	0.9	1.6	1.7	
Rat 11.	1.3	1.1	1.9	1.8	**< 0.05**
Rat 12.	1.0	0.9	1.2	1.4	
Macroscopic Observations	Further decrease in scab size and increase in peripheral new epithelium. Shallower wound depth. Hair follicle regrowth started at edges.		Further decrease in scab size and increase in peripheral new epithelium. Shallower wound depth.		

Table 8. Summary Of Results.

Day 8 wounds.

	Weigerts' Haematoxylin	Collagen IV and Laminin	α SMA
Wound	Fibroblast network with little mononuclear infiltrate.	Positive staining around blood vessels.	Diffuse positive marking. Regular arrangement of wound fibroblasts. Positive staining around blood vessels.
Wound Epithelium	Hypertrophied. More regular.	Strong BM staining. Smoother and more regular BM staining.	
Hair Follicles:			
• **Follicles in wound**	DS blended with fibroblast network.	Some follicles lost collagen IV staining. Some showed non-uniform laminin staining.	Some positive marking in cells in vicinity of follicle fragments.
• **Follicles at or close to edge of wound**	Hypertrophied ORS. DS blended with fibroblast network.	Positive BM staining,sometimes irregular.	Regular positive staining.
• **Follicles away from wound**	Normal morphology	Normal staining.	Normal marking.

	Anagen Wounds		Telogen Wounds		Difference in wound diameter (*p* value)
Wound Diameters (mm)	**Wound 1.**	**Wound 2.**	**Wound 3.**	**Wound 4.**	
Rat 13.	1.8	1.0	1.5	1.5	
Rat 14.	1.4	1.5	1.5	1.7	**< 0.05**
Rat 15.	0.8	0.7	1.8	1.5	
Macroscopic Observations	Further decrease in size of wound scab and increase in area of new epithelium. Wound depth very shallow.		Further decrease in size of wound scab and increase in area of new epithelium. Wound depth very shallow.		

Day 10 (Table 9.).

By day 10 the wound epithelium was still hypertrophied however it was even more smooth and regular.The outer root sheath of follicles in the wound and adjacent to the wound was still hypertrophied.An interesting observation at this stage was the fact that the outer root sheath of hair follicles at the edge of the wound had more layers of cells on the side of the wound than on the side in contact with the dermis at the wound edge.This was a repeated finding.The fact that there were consistently more layers of cells on the side of the outer root sheath in contact with the wound indicates that the wound may have a direct stimulus on outer root sheath cell hypertrophy.It also indicates that the wound stimulates to a greater extent the part of the outer root sheath on the side of the wound.This side contributes most of the cells to the new epithelium at the wound surface and ensures a good reservoir of epithelial cells for repair.Mitotic figures were very common on this side of the outer root sheath.Within the substance of the wound itself hair follicle regeneration was now more prominent with follicles at various stages of repair.The dermal sheath was no longer blended with the adjacent tissues and could be identified as a separate layer of cells.No concentric or fir-tree staining patterns of fibroblast arrangement could be identified.
As the wound matured the epithelial basement membrane staining became more regular and laminin and collagen IV antibodies were both well expressed.Alpha smooth muscle actin staining showed a homogenous fibroblast network with diffuse irregular streaks of positive staining.Follicles in the wound showed weak positive staining whilst follicles close to the wound had streaks of positive staining associated with the deeper parts of the follicles.

Table 9. Summary Of Results.

Day 10 wounds.

	Weigerts' Haematoxylin	Collagen IV and Laminin	α SMA
Wound	Very regular fibroblast arrangement. Mononuclear cells only observed at base of wound.	Positive marking around blood vessels.	Very homogenous fibroblast network. Diffuse irregular streaks of positive marking.
Wound Epithelium	Hypertrophied. Still irregular.	Positive BM staining.	
Hair Follicles:			
• **Follicles in wound**	DS not blended,identified as a separate layer of cells. No concentric or fir-tree pattern of fibroblast arrangement.	Non uniform or strong BM staining.	Weak positive marking.
• **Follicles at or close to edge of wound**	Hypertrophied ORS,especially on side in contact with wound.	Positive BM staining,sometimes irregular.	In general.regular staining. Streaks of positive marking associated with deep parts of follicles.
• **Follicles away from wound**	Normal histology.	Normal staining.	Regular staining.

	Anagen Wounds		Telogen Wounds		Difference in wound diameter (*p* value)
Wound Diameters (mm)	**Wound 1.**	**Wound 2.**	**Wound 3.**	**Wound 4.**	
Rat 16.	0.75	0.8	1.25	1.4	
Rat. 17.	1.0	1.1	1.5	1.6	**> 0.05**
Rat 18.	1.4	1.1	1.5	1.3	
Macroscopic Observations	No scab;entire surface covered with shiny new epithelium. Hair regrowth in periphery of wound, more pronounced in anagen wound.		No scab;entire surface covered with shiny new epithelium. Hair regrowth in periphery of wound, less pronounced in telogen wound.		

Day 15 (Table 10.).

The day 15 wound epithelium was even more regular however it was still hypertrophied.The outer root sheath of follicles close to the wound was still hypertrophied in continuity with the wound epithelium and the outer root sheath of follicles in the wound substance was also still hypertrophied with branching.Hair follicle regeneration was noted to be more prominent at this stage.Many newly regenerated hair follicles were noted to be budding from the substance of the hypertrophied wound epithelium with the developing follicles growing downwards into the wound tissue.These hair follicles possibly arose *de novo* as there were no adjacent follicle fragments or repairing follicles.They may have arisen from either hair follicle pleuripotent stem cells which may have been implanted into the wound tissue during the wounding process or from cells with hair follicle regenerating potential attracted into the area by the wound healing process.The dermal sheath of hair follicles in the wound substance was now identifiable as a distinct structure and was no longer blended into the adjacent wound fibroblast network.The fibroblast network around hair follicles in the wound was no longer arranged in a fir-tree or concentric pattern. The wound epithelium basement membrane expressed strong collagen IV and laminin staining. Some follicles in the wound showed irregular basement membrane staining whilst other follicles showed loss of basement membrane staining.Alpha smooth muscle actin stained sections revealed a very homogenous wound appearance with only some scattered streaks of positive staining. There was some positive alpha smooth muscle actin staining around the deep parts of follicles in the wound.

Table 10. Summary Of Results.

Day 15 wounds.

	Weigerts' Haematoxylin	Collagen IV and Laminin	α SMA
Wound	Lower part of wound contracted further (inverted cone). Composed almost entirely of fibroblasts, very few mononuclear cells.	Positive marking around blood vessles.	Very homogenous texture. Scattered streaks of positive marking.
Wound Epithelium	Still hypertrophied. More regular. Areas of possible hair follicle formation.	Strong BM expression. More regular BM.	
Hair Follicles:			
• **Follicles in wound**	DS visible as a distinct cell layer (not blended). No specific fibroblast patterns. Hair follicle buds.	Few follicle fragments observed in wound. Some loss of BM staining. Some irregular BM staining.	Positive marking around deep parts of follicles. Streaks of positive marking.
• **Follicles at or close to edge of wound**	DS still not clearly distinct on side of follicle in contact with wound whilst well distinct on side in contact with dermis.	Regular BM staining.	Regular positive staining.
• **Follicles away from wound**	Normal morphology.	Normal BM staining.	Normal staining.

	Anagen Wounds		Telogen Wounds		Difference in wound diameter (*p* value)
Wound Diameters (mm)	**Wound 1.**	**Wound 2.**	**Wound 3.**	**Wound 4.**	
Rat 19,	0.9	0.9	1.2	1.5	
Rat 20.	0.9	0.8	1.1	1.0	**> 0.05**
Rat 21.	0.8	0.9	0.9	1.0	
Macroscopic Observations	Wound healed.No central depression. Further hair regrowth at periphery of wound.		Wound healed.No central depression. Hair regrowth less pronounced than in anagen wounds.		

Section 5. Discussion.

5.1 Hair Growth Patterns.

Haddow et al (1945) established the constantly moving wave-like patterns of hair growth in the rat. They injected 1ml of a 15% solution of chlorazol sky blue *FFS* into the femoral vein of the anaesthetized albino rat and were able to visualize the skin capillary circulation.The greatest density of capillaries was observed at the advancing edge of the growing hair and there was a sudden reduction in skin capillaries at the receding edge. They concluded that the moving wavelike pattern of hair regrowth was controlled by the constantly changing capillary blood supply to the skin. Anagen skin had a more well developed capillary blood supply compared to telogen skin especially at the front of the hair growth wave.Geary (1952) and Zawacki and Jones (1967) further mentioned the hair growth wave-form pattern and the latter emphasised the need for standardisation of the skin thickness prior to any skin wound healing experiment as this varied with the hair cycle. Later, Moserova and Houskova (1989) further emphasised this point.

The observations of this present study,made during the process of standardising the skin for the study of the wound healing process in excised wounds,confirm the wave-like pattern of hair re-growth described above. However on many occasions the hair growth pattern was very irregular and patchy and no distinct wave-like pattern could be identified. Furthermore it was noted that areas of skin with no hair growth at all were a very rare occurrence and it was therefore very difficult to standardise areas of skin as pure telogen.

Geary (1952) had pointed out that even animals from the same litter vary in skin thickness in any particular region at any point in time.In this present experiment,it was observed that animals from the same litter had almost identical patterns of hair growth however the phases of the cycle did vary in particular regions confirming the observations made by Geary (1952).

From this present experiment it can be concluded that skin hair shaving using clippers is a satisfactory way of determining which parts of the skin are in anagen and which parts are in telogen however it is not as specific as proposed by Zawacki and Jones (1967) and Moserova and Houskova (1989).Areas of overlap between late telogen skin and early anagen are very common and reduce the accuracy of the standardisation.

5.2 Wound Contraction.

The macroscopic appearance of the healing excisional wounds at the various stages corresponded with the histological findings.Up to day 3 the wound tissue appeared to be still mainly composed of haematoma.The newly formed epithelium was not visible as it was covered by the wound scab.However on the fifth day it was noted that the epithelium was reforming as a shiny white layer at the edges as the wound scab was shed from the perimeter of the wounds.As more epithelium and fibroblast plus collagen fibres scaffolding were laid down the wound crater appeared shallower and covered with the shiny new epithelium.The wound was gradually filled with more and more repair tissue which gradually completely replaced the original haematoma,the wound diameter decreased progressively and the wound crater was completely obliterated.

The results of this study indicate that the wound diameter of circular excised wounds in general decreases progressively over the first fifteen days after wounding. Furthermore,anagen wounds contract significantly more than telogen wounds after the fifth day post-wounding.In particular the difference in diameter between anagen and telogen wounds was noted to be statistically significant on days 7 and 8 with *p values* being well below 0.05. These results therefore clearly indicate that anagen skin contracts in a different way to telogen skin.

Upon literature review very little could be found relating to the difference in wound healing between anagen and telogen skin.Zawacki and Jones (1967) acknowledged the importance of hair growth in the study of burn wound models in hairy animals and stressed that the skin in hairy animals has to first be standardised before starting any experiment.They point out that as the thickness of the skin varies with the hair cycle so does the depth of the skin adnexae and they feared that this can confound any difference in healing of burn wound models of different depths unless the skin was standardised.However no comments were made regarding any difference in wound healing between anagen and telogen skin.They were merely concerned about the different skin thickness between anagen and telogen skin and the differences that this

might cause in the burn wound model.

Moserova and Houskova (1989) mention that skin defects in areas where the dermis is thicker and where the skin appendages are deeper in the skin heal faster other areas, however they provided no scientific proof for their clinical observations.They also pointed out that such skin properties were more favourable for wound epithelialization than at other areas.A difference in wound healing between anagen and telogen skin was therefore specifically noted and further stressed the need for standardisation of the skin before any form of wound healing experiment.

An important clinical observation (in the human) made by Berkowitz (1981) emphasising the point that split thickness skin grafts should never be harvested from bald areas of the scalp as they take very long to heal compared to hairy scalp clearly demonstrates the extreme end of the spectrum of differences in wound healing between skin with actively growing hair follicles and bald skin.

Analysis,interpretation and extrapolation of the data from the present experiment allows a partly hypothetical explanation of the pathophysiological mechanisms responsible for the difference in wound healing between anagen skin and telogen skin:

Anagen skin has a greater concentration of myofibroblasts than telogen skin during wound healing and contracts differently.

The histological and immunohistochemical differences observed during anagen and telogen skin wound healing show that the dermal sheath cells are highly likely to be responsible for this difference.Keeping in mind the close phenotypic similarities amongst dermal sheath cells, follicular fibroblasts and wound fibroblasts and considering the observed dermal sheath radiating patterns the most appealing explanation is that dermal sheath cells from around anagen follicles migrate out into the wound and function as myofibroblasts, hence the increased number of myofibroblasts in anagen skin.

Other potential explanations for the difference in wound contraction between anagen and telogen skin include:

1. Anagen skin is thicker than telogen skin and hence the raw surface of the sides of the excised wounds wound be larger in anagen skin thereby allowing more migration of fibroblasts into the wound with the consequent synthesis of more collagen leading to a thicker collagen network with greater remodelling and contraction capacity (Pull Theory),(Majno et al,1971 and Lawrence,1998).

2.Anagen skin has a higher blood flow than telogen skin and hence a higher delivery of oxygen and nutrients to the healing wounds leading to faster healing and contraction. Haddow et al (1945) investigated the differences in capillary density over different areas of skin with different hair growth rate in the albino rat.During the initial part of their study they noted that areas with actively growing hair had taken up an injected dye (9-phenyl-5:6-benzo-iso-alloxazine) whilst areas with no actively growing hair did not take up the dye.Further investigation revealed that the orange dye was being taken up by actively growing anagen follicles.They therefore performed a further experiment whereby the skin capillary system was visualised using an intravenous injection of chlorazol sky blue FFS.The greatest density of capillaries was confirmed to correspond to the areas of actively growing anagen hair in particular at the advancing edge of hair growth.They also noted a sharp decrease in capillary density at the receding edge.It was therefore concluded that anagen skin with actively growing hair has a higher blood supply than telogen skin.This increase in blood supply was noted to be in a constantly moving pattern leading to the rhythmic alterations in hair growth cycle.These points were further highlighted by Geary (1952) and later by Zawacki and Jones (1967).

The rich capillary network around the actively growing anagen follicles may also facilitate the recruitment of myofibroblasts and fibroblasts into the wound area allowing faster wound healing compared to telogen skin.

Wound contraction has been noted by various authors to be affected by the shape of the

wound and to occur in various phases. Billingham and Russell (1956) concluded that wound contraction occurred in a logarithmic process in which circular wounds contracted slower than straight-sided wounds. However, Kennedy and Cliff (1979) showed that there was no apparent difference in the wound contraction curve between circular and square wounds and that the logarithmic rate of wound closure was the same in the two types of wound. They also noted that the wound contraction curves showed three distinct phases: an early closure phase,a stationary phase and a logarithmic closure phase. Further work by McGrath and Simon (1981) also showed that the wound contraction curve was made up of three phases which were named: the pre-exponential or plateau phase,the exponential phase and the post-exponential phase.

Pre-exponential or plateau phase :Immediately after wounding there was an increase in the wound surface area attributed to the retraction of the skin margins by the adjacent skin.
The plateau part of the curve then starts on the second day and continues to the sixth day with very little reduction in wound surface area.

Exponential phase :After the sixth day the exponential phase starts whereby the wound surface area decreases in size in a logarithmic fashion and continues to do so to beyond thirty days post wounding.

Post-exponential phase :After approximately forty days after wounding no significant change in wound size was noted.

The findings of the above study correlate well with the findings of my experiment.In my experiment the mean wound diameter was noted to have increased slightly on day 2 in telogen wounds whilst there was a decrease in diameter in anagen wounds, Figure 7. This increase in diameter in telogen wounds on day 2 may be attributed to the elasticity of the adjacent skin retracting the wound edges of the relatively thin telogen skin. The decrease in diameter in anagen wounds may reflect the faster degree of wound healing whereby they enter the plateau

phase earlier.The mean diameter of anagen wounds however increased on day 3 possibly due to the stronger retraction forces in the thicker anagen skin overcoming the early contractile forces.It may also be due to the lower elasticity of the thicker anagen skin resisting the retractile forces in the initial period after wounding.

In general,up to day 5 very little change in wound diameter could be noted and this corresponds well with the pre-exponential or plateau phase described by McGrath and Simon (1981).However from day 7 onwards a significant decrease in wound diameter was noted with the anagen skin contracting significantly more than the telogen skin.This in general corresponds with the exponential phase described by McGrath and Simon (1981).From these findings it can be concluded that my experimental findings correlate well with the literature and contribute further by showing that anagen skin wounds contract significantly more than telogen wounds.

5.3 Wound Epithelial Regeneration And Hair Follicle Repair And Regeneration.

Several authors have mentioned that skin appendages contribute to the healing of skin partial thickness wounds (Miller et al,1998 and Martin 1997) especially burns (Holmes et al,1983), however apart from the work by Ordman and Gillman (1966) very little is described in the literature explaining the mechanisms by which it actually takes place.

The observations on epithelial and hair follicle regeneration made during the present experiment in general correlate with the observations previously made by Ordman and Gillman (1966),however they contribute further valuable data.The outer root sheath hypertrophy of follicles at the edge of the wound clearly demonstrates the epithelial cell reservoir function of the hair follicles for epithelial regeneration.The differential hypertrophy observed in the outer root sheath of follicles in contact with the wound further demonstrates the possible direct positive influence of the wound on the outer root sheath cell multiplication.A further contribution is the observation of the *de novo* regenerating follicle bulbs in the wound substance and within the new epithelium.

Eisen et al (1955) first showed that the outer root sheath of the hair follicle contributes to

the regeneration of the skin epithelium after wounding. This has also been pointed out by Martin (1997).The regenerative powers of the outer root sheath cells as a reservoir of epidermal keratinocytes have been further investigated by Cotsarelis et al (1990) and Rochat et al (1994). Cotsarelis et al (1990) attributed 'stem cell' or pleuripotent properties to the outer root sheath cells of the bulge region of the hair follicle. They also indicate that these cells are ultrastructurally relatively undifferentiated but could be stimulated to proliferate. All these findings correlate with the observations of the present experiment as the outer root sheath clearly hypertrophied in response to skin wounding and contributed to both hair follicle repair as well as to epithelial regeneration. The presence of pleuripotent stem cells in the bulge region of the hair follicle may provide an explanation for or at least an indication of the origin of the regenerating follicles and follicle buds arising *de novo* both in the substance of the wound epithelium as well as in the wound itself. Rochat et al (1994) showed that the hair follicle contains keratinocytes with stem cell properties,specifically with extensive proliferative properties allowing regeneration of large amounts of epithelium.They also attributed these cells to the bulge area of the outer root sheath.

Ferraris et al (1997) have recently shown that cultured epidermal stem cells retain their embryonic pleuripotentiality during wound healing and are capable of producing hair follicle bulbs and pilosebaceous units.This work provides a further possible explanation for the origin of the *de novo* regenerating follicles and the small hair bulb buds observed in the wound epithelium and in the wound tissue in the present experiment.

Williams et al (1994) showed that *in vitro* transection of the hair follicle above the level of the pilosebaceous unit led only to growth of the hair shaft with no growth of the outer root sheath whilst transection below this level led to growth of both structures.This correlates with the findings of this experiment as damaged follicles deep in the dermis showed outer root sheath hypertrophy and branches growing from damaged follicles deep in the wound showed both

outer root sheath and hair fibre growth.However follicles at the edge or close to the edge of the wound even if undamaged still developed outer root sheath cell hypertrophy.This indicates that the outer root sheath responds to stimuli from the wound itself even though the hair follicle itself was not damaged.It may therefore be concluded that hair follicles will respond with outer root hypertrophy when the follicle is wounded and when the follicle is close to a wound.

The observations of the present experiment clearly indicate very active hair follicle repair and regeneration activity in rat skin during the observed 15 day period after wounding. At day 15 the wound scar was still macroscopically bald possibly because the regenerating follicles had not matured enough to produce visible hair fibre growth. During preliminary experiments it was found that beyond the 15 day period the wound scar became progressively smaller and smaller with time and at the end (day 20-25) the healing was so good that it was impossible to identify the previous wound area as there was no bald patch left;all the hair regrowth or contraction was complete.However in the human scar tissue in the scalp is permanently bald,with hair follicles being practically absent in the scar area.It would be very interesting to know whether in human skin hair follicle repair and regeneration after wounding occurs in a similar way as in the rat.If that is so the key question would be what then happens to the repairing/regenerating follicles entrapped in the scar tissue as the scar tissue is ultimately bald. Scar tissue is by nature dense and very tough in texture and it may well be that this physical framework prevents further hair follicle regeneration.It may well also be that the scar tissue resorbes the repairing follicles possibly by an immune mediated mechanism. However interestingly enough a case has been reported (Buckland et al,1986) whereby hair regrew in the scar in a previously bald scalp (male pattern baldness) after the area sustained a burn.The wound healing response to the burn trauma might have triggered epithelial stem cells to regenerate hair follicles!.

5.4 Epidermal And Hair Follicle Basement Membrane Repair And Regeneration After Wounding.

Fine (1994) showed that part of the epithelial basement membrane laminin is produced by the epithelial cells.Furthermore Fleischmajer et al (1998) showed that 20% of the basement membrane laminin is produced by the epithelium whilst 80% is produced by the dermis.The observation of positive laminin marking in the basement membrane area of the middle parts of the wound independent from the marking at the edges of the wound and in the absence of dermis in the early stages of wound healing (day 2 and 3) confirms the capacity of the epithelial cells to synthesise laminin in the absence of dermis.

Fleischmajer et al (1998) showed that,*in vitro,* fibroblasts were a more major source of collagen IV than keratinocytes.In the present study the wound epithelial collagen IV staining became stronger in intensity at day 5 which corresponds to the time when the wound was well infiltrated by fibroblasts.These findings therefore support the *in vitro* findings of Fleischmajer et al (1998).

The present experimental findings also indicate a faster rate of epithelial basement membrane laminin deposition compared to the collagen IV deposition.This may a reflection of the stronger anchoring power of laminin through its anchoring fibrils making it an early necessity for epithelial stability in the early phases of wound healing.

Hair follicles at the edge of the wound and close to the wound in general retained their collagen IV and laminin basement membrane staining in its entirity,however on some occasions the staining was irregular at the edge of the wound as a reflection of the trauma sustained by the follicles during the wounding process.Hair follicles or hair follicle fragments in the wound substance itself in general either completely or partly lost their basement membrane expression. Specifically at day 5 some follicles in the wound itself lost both the collagen IV and the laminin staining. On comparative alpha smooth muscle actin stained sections these same follicles were noted to have also lost their alpha

smooth muscle actin staining.These follicles were deemed to be follicles that had sustained irrecoverable damage from the wounding process and were in the process of being resorbed.Other follicles in the same wounds were noted to have retained the positive marking for the two basement membrane antigens and expressed a radiating pattern of positive alpha smooth muscle actin staining.These follicles were considered to be viable and in the process of recovery/regeneration.At day 7 some follicles in the wound lost the laminin staining whilst retaining their collagen IV staining.This possibly reflects a situation in which the follicles were either being resorbed or were in an early stage of repair.

Jahoda et al (1992) established that hair follicle regeneration after traumatic amputation is accompanied by hair follicle basement membrane repair.The observations in this present study support this and indicate that various degrees of injury to the hair follicles lead to different consequences depending on the degree of initial damage.Whereas the trauma to the hair follicles in the study by Jahoda et al (1992) was controlled and standardised,the hair follicle trauma observed in this present experiment was of a variable severity, hence the varied degrees of regeneration, resorption and final outcome.

5.5 Hair Follicle Dermal Sheath Cell Behaviour During The Healing Of Excisional Wounds.

As discussed in the introduction of this thesis (Section 1), dermal sheath cells and myofibroblasts share many common features including their mesodermal origin,their phenotypic similarity to dermal and follicular fibroblasts and the fact that both stain positive for the same specific type of alpha smooth muscle actin (Skalli and Gabbiani,1987, Jahoda and Oliver,1981, Messenger, 1984, Horne,1987, Messenger et al,1991 and Almond-Roesler et al,1997).Furthermore both types of cells possess contractile properties and like other mesodermal cells may have the potential of cell migration especially when one considers their cytoskeletal framework.

These similarities provide enough ground to postulate that dermal sheath cells and myofibroblasts may under the specific influence of a wound stimulus undergo a phenotypic change from one cell type to the other and perhaps vice versa,depending on the reparative

needs.In view of this,the results of this experiment are interpreted with this basic idea in mind, so as to highlight all the observational findings that support or disprove this hypothesis.

Rat fibroblasts cultured from different organs (dermis,skeletal muscle, subcutaneous tissues and lung) exhibit varying proportions of cells that are alpha smooth muscle actin positive and cells that are alpha smooth muscle actin negative (Dugina et al,1998). However on comparison of the cell shape,cytoskeleton and focal contact organisation of cells which were alpha smooth muscle actin positive and cells that were alpha smooth muscle actin negative from various organs with their own category,no significant difference in morphology can be found. Contrary to this, on comparing alpha smooth muscle actin positive cells with alpha smooth muscle actin negative cells significant differences were noted between the two groups independent of the tissue of origin: alpha smooth actin positive cells were larger in size,had larger numbers of cellular extensions at the edges,showed more focal adhesions to the substratum and had a more pronounced intracellular fibronectin network than alpha smooth muscle actin negative cells (Dugina et al,1998).Thus alpha smooth muscle actin positive fibroblasts and alpha smooth muscle actin negative fibroblasts exhibit definite important phenotypic differences whereas there was no morphological phenotypic differences between fibroblasts of different tissues. These observations support the hypothesis, previously out forward by Jahoda et al (1991) and Reynlods et al (1993), suggesting that since the dermal sheath cells are phenotypically very similar to dermal fibroblasts, they can behave as fibroblasts and undergo the phenotypic change to myofibroblasts under the influence of the wound stimulus. They then migrate out from around the deeper parts of the anagen follicles as brightly staining alpha smooth actin positive fibroblasts / myofibroblasts, hence the concentric and fir-tree patterns of the alpha smooth muscle actin staining observed around follicles in the depth of the wound.

Furthermore Reynolds and Jahoda (1991) and Reynolds et al (1993) have shown that hair follicle pleuripotent cells or stem cells are situated in the lower end bulb region of the follicle.The fact that germinative epidermal cells are situated deep in the dermis provides a possible explanation for for the great cellular activity and possible phenotypic changes occurring deep in the wound and the adjacent dermis.It also helps to explain the origin of the regenerating hair bulbs/buds arising *de novo* in the wound tissue.Germinative epidermal cells from the lower end bulb might have been

released from hair follicles during the wounding procedure using the punch biopsy or possibly might have migrated out from neighbouring follicles as a response to the wound healing process.

The actual presence of actin filaments in hair follicle dermal sheath cells is highly suggestive of their potential for motility and migration as an actin filament framework is essential for cell motility (Schafor et al,1998). Furthermore, it is well established that actin expression is a dynamic process in which actin synthesis and disassembly is closely auto-regulated by intracellular mechanisms (Lyubimova et al,1997). These findings support the view that dermal sheath cells, under the effect of a stimulus as a result of the wound injury, may have their actin assembly mechanisms unregulated leading to increased actin synthesis, hence the cells then gain the motility power and migrate into the wound where they may either become myofibroblasts and contribute to wound contraction. This mechanism may provide an explanation as to why wounds in anagen skin contracted more than wounds in telegen skin: anagen skin wounds have a higher number of hair follicles with bulbs deep in the dermis and hence a higher succession of dermal sheath cells which assume migratory powers and behave as myofibroblasts leading to increased wound contraction. Fibroblasts cells spread area is dynamically regulated by a balance between cell protrusion and cell retraction. The lack of protrusion remains relatively constant and it is the changes in the rate of reaction that produce changes in the area of spread of the fibroblasts. (Dunn and Zicha,1995). The constantly dynamic processes of protrusion and retraction may be accurately controlled by the cytoskeleton, and fibroblast motility may be a result of increase in all retraction leading to a complementary increase in protrusion and hence cellular vectorial motility. Similar regulatory mechanisms may be operating in the dermal sheath cells of the anagen hair follicles and the wound stimulus may disturb the balance leading to an increase in cell retraction with a resulting increase in protrusion and hence cellular migration into the adjacent dermal connective tissue and wound tissue. Fibroblast motility has mow been under investigations for many years. Armstrong and Armstrong (1978), looked at the migratory potential of skin fibroblasts and found that fibroblasts moved in

solid tissue only 36% of the distance they transverse in monolayer cell culture indicating that and motility in tissues is much less than the motility in tissue culture.

Dunn (1980) summarised the locomotory machinery of fibroblasts by suggesting that on a substratum,e.g. extracellular matrix,the cells first need to adhere to the substratum, then move the bulk of the cell towards the adhered area and eventually protrude new cellular protrusions to make new adhesions. In this process he emphasised the need of releasing the adhesions at the rear of the cell and retracting the rear end of the cell forwards into the main cell bulk. This was basically a description of lamellopodial cellular locomotion and is a co-ordination between protrusion and retraction. Butcher (1998) reviewed the locomotory mechanisms of fibroblasts and concluded that there were two possible models: The cytoskeletal model and the membrane flow model. The cytoskeletal model presumed that the cells feet one present over the plasma membrane and are attached to the intracellular actin framework and the cell movement is achieved by anchoring the feet and applying a vectorial force generated by actin myosin interaction. The membrane flow model presumes that there exists a continual flow of the plasma membrane from the front part of the cell towards the rear on both the superior and inferior surfaces of the cell, and further explains that the plasma membrane flow is related to the endocytosis/exocytosis cycle of the cell. The presence of alpha smooth muscle actin in dermal sheath cells and radiating patterns of staining seen around anagen hair follicles deep in the wound suggest that the dermal sheath cell motility is possibly by a cytoskeletal mechanism involving the abundant intracellular alpha smooth muscle actin framework of these cells. Couchman and Ross (1985) provided further support for the involvement of the actin cytoskeleton and membrane recycling mechanism in the fibroblast protrusive activity leading to motility.

Ronnov-Jessen and Peterson (1996) during *in vitro* studies on fibroblast function showed that the presence of alpha smooth muscle actin in fibroblasts led to slower migration activity as compared to fibroblasts that were negative for alpha smooth muscle actin.They concluded that alpha smooth muscle actin was a signal for retardation of fibroblast migration.This effect may be beneficial in

wound healing as the alpha smooth muscle actin positive fibroblasts (myofibroblasts) would be less motile and stay in the wound area where they are required for wound contraction.However in the present experiment the dermal sheath cells of the hair follicles in the wound even though they were alpha smooth muscle actin positive they were still capable of migrating out in a concentric spiral fashion into the adjacent wound tissue.The fact that they were alpha smooth muscle actin positive did not seem to make them immobile or inhibit their migratory potential.In this context the present observations on motility of alpha smooth muscle actin positive dermal sheath cells or fibroblasts around anagen hair follicles in the wound do not correlate with the *in vitro* findings of Ronnov-Jessen and Peterson (1996).

Dunlevy and Couchman (1993) stated that fibroblast motility was a major component in the biological process of wound healing and embryogenesis. Their work pointed out that conditioning of the medium readily controlled fibroblast migration by influencing the presence or absence of focal adhesions. By manipulating the presence or absence of focal adhesions they were able to directly manipulate and control fibroblast locomotion. By extrapolating the principles of these findings to the phenotypically similar hair follicle dermal sheath cells, it may be that the wound fluid nuclei affects the focal adhesions of the dermal sheath cells in such a way as to reduce the number of cells containing focal adhesions of the and allowing them to become motile and migrate into wound tissues to help in the reparative process.

Migrating mesodermal cells in culture have been shown to organise themselves in an orderly 'halo' around tissue explants (Li et al 1997). The individual cells were noted to be elongated and parallel to each other and at a right angles to the tissue explant.The cytoskeletal structure of the individual cells was aligned in a similar fashion. This orientation was derived to be due to soluble factor : a cell orientating factor-possibly a protein affecting the cell to cell interactions and promoting the alignment pattern of the migratory mesodermal cells.

Other theories attempting to explain cellular alignment have been put forward by authors including: contact guidance by the aligned fibrils (Rovasio et al,1983); contact inhibition of cell movement

(Abercombie and Heaysman,1966 and Bard and Hay,1975); lateral adhesions (Elsdale and Bard, 1972) and initiation of movement as a result of contact stimulation (Thomas and Yamada,1992). My observations of concentric centrifugal radiation of alpha smooth muscle actin positive dermal sheath cells from around anagen hair follicles deep in the wound substance suggests a comparable picture of mesodermal cell migration,the hair follicle representing the centre of the pattern. However the alignment of the migrating dermal sheath cells was parallel to the hair follicle in contrast with the right angle orientation of the mesodermal cells in relation to the tissues explant. Nonetheless,the pictures of migrating dermal sheath cells in a concentric centrifugal pattern in general compare well with the previously described patterns of mesodermal cell movement.One or more of the mechanisms of cell alignment described above may be responsible for operating the characteristic pattern.

5.6 More Scope For The Scalp To Be Used As A Donor Site For Split Thickness Skin Graft Harvesting.

The findings of this study clearly indicate the enormous healing potential of hairy skin as a result of the involvement of the various different hair follicle elements.Harvesting split thickness skin stimulates the remaining skin epithelial cells and hair follicle outer root sheath cells in the donor area to divide and regenerate the new epithelium.In hair dense skin,like the scalp,the contribution of the outer root sheath cells greatly enhances the healing process and clearly explains the rapid healing of scalp skin after split thickness skin graft harvesting. In summary this study contributes further direct histological evidence favouring the scalp as a donor site.

5.7 The Potential For Incorporating Living Hair Follicle Dermal Sheath Cells In Skin Equivalents.

The ideal skin equivalent would need to effectively replace both the epidermis and the dermis of normal skin. Epidermal keratinocytes obtained from small skin biopsies can now be serially cultured *in vitro* to produce sheaths of keratinocytes that can be used to provide the outer cover to large wounds such as burns and giant hairy naevi (Coulumb et al,1998, Rheinwald et al,1975, and Parenteau et al,1991).

Limat et al (1994) studied organotypic cultures of human hair follicles outer root sheath cells and moved the formation of a stratified epithelium generally resembling the interfollicular epidermis. Further work by the same group (Limat et al,1996) showed that an epidermal equivalent derived from culture of autologous outer root sheath cells can be used to graft successfully chronic leg ulcers. Therefore both interfollicular keratinocytes and outer root sheath cells can be used to establish *in vitro* cell culture sheaths or epidermis equivalents.

Grafting a full thickness wound with epidermis equivalent only however leads to unstable grafts and it is well recognised that a dermal equivalent is also required (Coulumb et al,1998) to achieve a stable skin replacement.

Furthermore in the absence of dermal replacement full thickness wounds grafted only with keratinocytes develop hypergranulation, eventually lead to increase wound contraction leading to contracture and hypertrophic scarring. A certain amount of dermis or its equivalent is required to prevent this (Bell et al,1998). To achieve skin cover using a dermal and an epidermal equivalent a two stage cover procedure is required. At the first operation the dermal equivalent is grafted onto the wound bed and allowed to take.When the dermal graft is vascularised, epidermal cover is carried out using either split thickness graft or cultured sheets of keratinocytes. Dermal grafts incorporating living fibroblasts have further advantages besides those mentioned above. Independent of the epidermal grafting technique, a living dermal equivalent was found to reduce pain in the area, provide good homeostasis and improve the physical strength and cosmetic appearance of the graft (Coulumb et al,1998).

Living fibroblasts incorporated in the dermal equivalent clearly lead to a skin replacement that more resembles normal skin especially in its suppleness and mechanical strength (Coulumb et al,1998).

A further important point is the fact that living fibroblasts from different donor tissue sources when incorporated into the dermal component of living skin equivalents determine epidermal differentiation and influence the regeneration time period of the epidermis (Konstantinova et al, 1998). Fibroblasts cultured from eyelid skin biopsies seem to produce the more favourable results.

Keeping in mind the similarities between skin fibroblasts and hair follicle dermal sheath cells discussed in the introduction of this thesis and the radiating pattern of alpha smooth muscle actin staining around hair follicles in wounds suggesting dermal sheath cell migration into the wound, the most logical step forward would be to incorporate hair follicle dermal sheath cells into the dermal

component (collagen matrix) of skin equivalents and asses the outcome. This dermal sheath cell seeding of a collagen matrix has been used in our laboratory in the PVG rat model by one of my colleagues, Dr A Gharzi (PhD Thesis,Durham University) with promising results. However further work in this field is required. It is therefore envisaged that in the future a complete living skin equivalent (dermal plus epidermal) can be derived *in vitro* from the epidermal and dermal components of the hair follicle. This further reflects the great regeneration and differentiating potential of the hair follicle.

5.8 Further Work.

Di I Labelling Of Dermal Sheath Cells To Directly Follow Their Behaviour In The Healing Wound.

Dermal sheath cells harvested by microdissection from the vibrissa follicles of the PVG rat would need to be incubated in vivo with Di I to obtain a red fluorescent cytoplasmic labelling.Standard excisional wounds would then need to be applied to the homogenous PVG rat and the labelled dermal sheath cells implanted into the wounds at various timings during the process of wound healing and their fate objectively followed using immunofluorescence histology techniques.In this way the behaviour of dermal sheath cells in the healing wound would be followed more directly especially with respect to any phenotypic change to myofibroblasts.

Measurement Of BrdU Uptake By Dermal Sheath Cells Of Anagen Hair Follicles Within A Wound During The Various Stages Of Wound Healing.

Specific evidence related to the observed dermal sheath hypertrophy during the process of wound healing can be obtained by measuring the BrdU uptake of dermal sheath cells in anagen follicles within the wound.This would provide direct evidence of the degree and site of cell division that occurs in the hair follicle dermal sheath during the process of skin wound healing.

Gene Probe Labelling Of Hair Follicle Dermal Sheath Cells To Follow Their Behaviour In The Healing Wound.

This would involve genetic labelling of vibrissa follicles dermal sheath cells to objectively follow their behaviour during the process of wound healing.This would involve histological and gel electrophoresis techniques.

Skin Equivalent Containing Dermal Sheath Cells.

Labelled hair follicle dermal sheath cells cultured in vivo after several passes would be incorporated into a collagen gel sheath and the living 'skin equivalent' would then be applied to excised wound and the activities of the dermal sheath cells closely followed up especially with regards to myofibroblast production,collagen production,wound contraction and rate of wound healing as

compared to control collagens containing either living fibroblasts or no living cells at all.The advantages of dermal sheath cells in wound healing wound then be demonstrated in vivo.

Investigate For Any Peptides Or Growth Factors Produced By Dermal Sheath Cells Or To Which Dermal Sheath Cells Respond During The Wound Healing Process.

This would be the next logical step to explain how dermal sheath cells actually perform their beneficial functions in the wound healing process and interact with the neighbouring fibroblasts and myofibroblasts.

Section 6.

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Section 7.

Appendix 1. Weigert's Haematoxylin Staining Schedule.

Staining Step	Proceedure	Time Duration
1.	Wash in Histoclear	3 minutes
2.	Wash in Histoclear	3 minutes
3.	Wash in absolute alcohol	3 minutes
4.	Wash in absolute alcohol	3 minutes
5.	Wash in 95% alcohol	2 minutes
6.	Wash in 70% alcohol	2 minutes
7.	Wash in distilled water	2 minutes
8.	Stain in Alcian Blue	20 minutes
9.	Rinse in distilled water	1 minute
10.	Stain in Weigerts Haematoxylin	30 minutes
11.	Rinse in 50% alcohol	1 minute
12.	Differentiate nuclei in 1% acid alcohol *	Just a dip
13.	Rinse in 70% alcohol	1 minute
14.	Wash in distilled water	1 minute
15.	Stain in Ponceau S	20 seconds
16.	Differentiate in 90% alcohol	10 seconds
17.	Dehydrate in absolute alcohol	1 minute
18.	Wash in absolute alcohol	2 minutes
19.	Wash in Histoclear	2 minutes
20.	Wash in Histoclear	2 minutes

*1% acid alcohol was prepared by adding 1ml concentrated hydrochloric acid to 100ml of 70% alcohol.

Appendix 2. Proceedure For Making And Storing Mowiol.

The Mowiol was made by mixing 2.4gm of Mowiol 4-88 (Hoechst) with 6gm of glycerol and adding 6ml of deionized distilled water,allowing the mixture to stand overnight at 37°C.12ml of 0.2M Tris (pH 8.5) was then added and the solution heated to 50°C for 10 minutes with occassional mixing.The solution was then clarified by centrifugation at 5000g for 15 minutes in an ultracentrifuge and the supernatant was recovered.DABCO (1,4-diazobicyclo-[2.2.2]-octane) was then added to 2.5% to the supernatant to prevent fading.The solution was then divided into aliqouts and stored by freezing at -20°C.

Appendix 3. Weigerts' Haematoxylin,Alcian Blue And Curtis Ponceau S.

Weigerts' Haematoxylin:Solutions A and B.

Solution A was made by adding 10g. of Haematoxylin to 1000ml. of absolute alcohol and allowed to stir on an electric stirrer for one hour.Solution B was prepared by adding 40ml of 30% ferric chloride and 10ml. of concentrated hydrochloric acid to 950ml. of distilled water and again allowed to stir for one hour.

Alcian Blue.

The Alcian Blue solution was prepared by adding 10g. of Alcian Blue and 30ml of glacial acetic acid to 1000ml of distilled water followed by stirring.

Curtis Ponceau S.

The Curtis Ponceau S solution was prepared adding 12ml of glacial acetic acid and 900ml of a saturated aqueous solution of picric acid to 1000ml of 1% aqueous solution of Ponceau S.

Appendix 4. Phosphate Buffered Saline.

Phosphate buffered saline (PBS) solution was prepared by adding 40g. of sodium chloride, 5.75g. of disodium hydrogen phosphate and 1g. of potassium dihydrogen phosphate to 5 litres of distilled water.The solution was placed on a stirrer for one hour after which the pH was checked using a pH meter and adjusted if required to 7.35-7.4.The PBS solution was then filtered using fine filter paper.