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# Scar-free healing: from embryonic mechanisms to adult therapeutic intervention

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In man and domestic animals, scarring in the skin after trauma, surgery, burn or sports injury is a major medical problem, often resulting in adverse aesthetics, loss of function, restriction of tissue movement and/or growth and adverse psychological effects. Current treatments are empirical, unreliable and unpredictable: there are no prescription drugs for the prevention or treatment of dermal scarring. Skin wounds on early mammalian embryos heal perfectly with no scars whereas wounds to adult mammals scar. We investigated the cellular and molecular differences between scar-free healing in embryonic wounds and scar-forming healing in adult wounds. Important differences include the inflammatory response, which in embryonic wounds consists of lower numbers of less differentiated inflammatory cells. This, together with high levels of morphogenetic molecules involved in skin growth and morphogenesis, means that the growth factor profile in a healing embryonic wound is very different from that in an adult wound. Thus, embryonic wounds that heal without a scar have low levels of TGFβ1 and TGFβ2, low levels of platelet-derived growth factor and high levels of TGFβ3. We have experimentally manipulated healing adult wounds in mice, rats and pigs to mimic the scar-free embryonic profile, e.g. neutralizing PDGF, neutralizing TGFβ1 and TGFβ2 or adding exogenous TGFβ3. These experiments result in scar-free wound healing in the adult. Such experiments have allowed the identification of therapeutic targets to which we have developed novel pharmaceutical molecules, which markedly improve or completely prevent scarring during adult wound healing in experimental animals. Some of these new drugs have successfully completed safety and other studies, such that they have entered human clinical trials with approval from the appropriate regulatory authorities. Initial trials involve application of the drug or placebo in a double-blind randomized design, to experimental incision or punch biopsy wounds under the arms of human volunteers. Based on encouraging results from such human volunteer studies, the lead drugs have now entered human patient-based trials e.g. in skin graft donor sites. We consider the evolutionary context of wound healing, scarring and regeneration. We hypothesize that evolutionary pressures have been exerted on intermediate sized, widespread, dirty wounds with considerable tissue damage e.g. bites, bruises and contusions. Modern wounds (e.g. resulting from trauma or surgery) caused by sharp objects and healing in a clean or sterile environment with close tissue apposition are new occurrences, not previously encountered in nature and to which the evolutionary selected wound healing responses are somewhat inappropriate. We also demonstrate that both repair with scarring and regeneration can occur within the same animal, including man, and indeed within the same tissue, thereby suggesting that they share similar mechanisms and regulators. Consequently, by subtly altering the ratio of growth factors present during adult wound healing, we can induce adult wounds to heal perfectly with no scars, with accelerated healing and with no adverse effects, e.g. on wound strength or wound infection rates. This means that scarring may no longer be an inevitable consequence of modern injury or surgery and that a completely new pharmaceutical approach to the prevention of human scarring is now possible. Scarring after injury occurs in many tissues in addition to the skin. Thus scar-improving drugs could have widespread benefits and prevent complications in several tissues, e.g. prevention of blindness after scarring due to eye injury, facilitation of neuronal reconnections in the central and peripheral nervous system by the elimination of glial scarring, restitution of normal gut and reproductive function by preventing strictures and adhesions after injury to the gastrointestinal or reproductive systems, and restoration of locomotor function by preventing scarring in tendons and ligaments.

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## 1. CLINICAL PROBLEMS OF SCARRING

Scarring occurs after trauma, injury or surgery to any tissue or organ in the body. Such scars are a consequence of a repair mechanism that replaces the missing normal tissue with an extracellular matrix consisting predominantly of fibronectin and collagen types I and III, as such scarring represents a failure of tissue regeneration. In man and animals, scarring causes major medical problems: e.g. in the eye, where scarring can cause hazy vision or blindness, in the peripheral and central nervous systems where scarring prevents neuronal reconnections and hence restoration of neuronal function, in the gastrointestinal and reproductive organs, where strictures and adhesions caused by scarring can give rise to serious or life-threatening conditions such as infertility or failure of bowel function, and in ligaments and tendons where scarring restricts movement, decreases strength and prevents normal function. Not surprisingly the skin represents the most frequently injured tissue, and dermal scarring after injury, trauma, surgery, etc. results in adverse medical consequences including loss of function, restriction of movement (particularly because of contractures over joints), restriction of growth, poor aesthetics and adverse psychological effects (particularly in children, the most common group to sustain burn injuries). Our work has focused predominantly on skin wounds. This review will therefore concentrate on scarring in the skin after injury or surgery, but results are applicable to other organs and tissues as well as to chronic fibrotic disorders such as glomerulonephritis, pulmonary fibrosis, etc., which share many of the cellular and molecular mechanisms common to scarring.

A scar in the skin may be defined as 'a macroscopic disturbance of the normal structure and function of the skin architecture, resulting from the end product of a healed wound' (Ferguson *et al.* 1996, p. 854). The severity of skin scarring in man and laboratory animals can be measured macroscopically (clinically) by using a variety of criteria such as scar volume, pliability, contour, colour, etc. and clinically and histologically by using a visual analogue scale where '0' represents normal skin and '10' a very poor scar (Beausang *et al.* 1998).

There are currently no mechanistic-based therapies, e.g. pharmaceutical drugs, to prevent or improve scarring (Bayat *et al.* 2003). Current therapies, e.g. pressure garments, silicone dressings, hydrocortisone injections, etc. are empirical, unpredictable and largely ineffective (Bayat *et al.* 2003).

## 2. SCAR-FREE EMBRYONIC WOUND HEALING

Skin wounds on early mammalian embryos heal perfectly with no signs of scarring and complete restitution of the normal skin architecture (Whitby & Ferguson 1991*a*). We have conducted systematic experiments making defined skin wounds on mouse and sheep embryos at varying gestational ages (Whitby & Ferguson 1991*a*,*b*; Whitby *et al.* 1991). These *in vivo* surgical experiments clearly demonstrate that in all mammalian species investigated so far (mice, rats, rabbits, sheep, pigs, marsupials, monkeys), skin wounds made during the first one-third to one-half of gestation heal perfectly with no scars (for a review see McCallion & Ferguson 1996). In mice, the latest time when a small incisional skin wound heals with no discernible scar is embryonic day 16 (time of birth normally embryonic day 20 or 21). The transition from scar-free embryonic wound healing to scar-forming adult wound healing is a gradual one and is characterized by the abnormal organization of the neo-dermis, predominantly the abnormal deposition of small parallel bundles of extracellular matrix (consisting largely of collagen types I and III and fibronectin) to form the scar as opposed to the deposition of large bundles of extracellular matrix in a normal basketweave orientation in the normal skin and in the neo-dermis of an embryonic (pre-embryonic day 16) wound. Scarring is therefore a morphogenetic problem, i.e. a failure of the regeneration of the normal skin structure as opposed to a biochemical problem, e.g. abnormal composition of the scar tissue.

## 3. MECHANISMS OF MAMMALIAN EMBRYONIC SCAR-FREE HEALING

There have been numerous investigations of the differences between embryonic wounds (pre-embryonic day 16 in the mouse) that heal without a scar, late foetal wounds (post-embryonic day 16 in the mouse) and adult wounds which heal with a scar (for a review see McCallion & Ferguson 1996). There are a large number of differences between the healing of embryonic and adult wounds. Many of these differences are epiphenomenon, i.e. not causative of the scar-free healing phenotype because, of course, embryos are still developing and do not have the same stable phenotype as the adult. Consequently many obvious differences between embryonic and adult wounds have been shown to be irrelevant to scar formation or the lack of it. Thus, for example, mammalian embryos develop surrounded by the sterile aqueous environment of the amniotic fluid, whereas adult wounds are exposed to air and numerous potential contaminating agents, e.g. bacteria, viruses, foreign bodies, etc. A particularly elegant demonstration of the irrelevance of the sterile, fluid, embryonic environment to scar-free healing was an ontological investigation of wound healing and scarring in the pouch young of the marsupial *Monodelphis domestica* (Armstrong & Ferguson 1995, 1997). Marsupial embryos are born at an early stage of immunological development, but at an advanced stage of skin development (the epidermal layer is well formed and highly keratinized to prevent dehydration of the pouch young on the mother's nipple). Furthermore these early pouch young are regularly contaminated with maternal urine and faeces: in stark contrast to their eutherian counterparts in sterile amniotic fluid! Nonetheless, despite these striking differences skin wounds on early pouch young of *M. domestica* heal perfectly with no scars, demonstrating the irrelevance of the external embryonic environment to scar-free healing (Armstrong & Ferguson 1995, 1997). Equally, adult sheep skin grafted to a sheep embryo and subsequently wounded heals with a scar (Longaker *et al.* 1994).

Many other differences between embryonic scar-free healing wounds and adult scar-forming wounds have been shown, e.g. increased levels of hyaluronic acid, more primitive fibroblasts, or absence of a fibrin clot in embryonic wounds (for a review see McCallion &

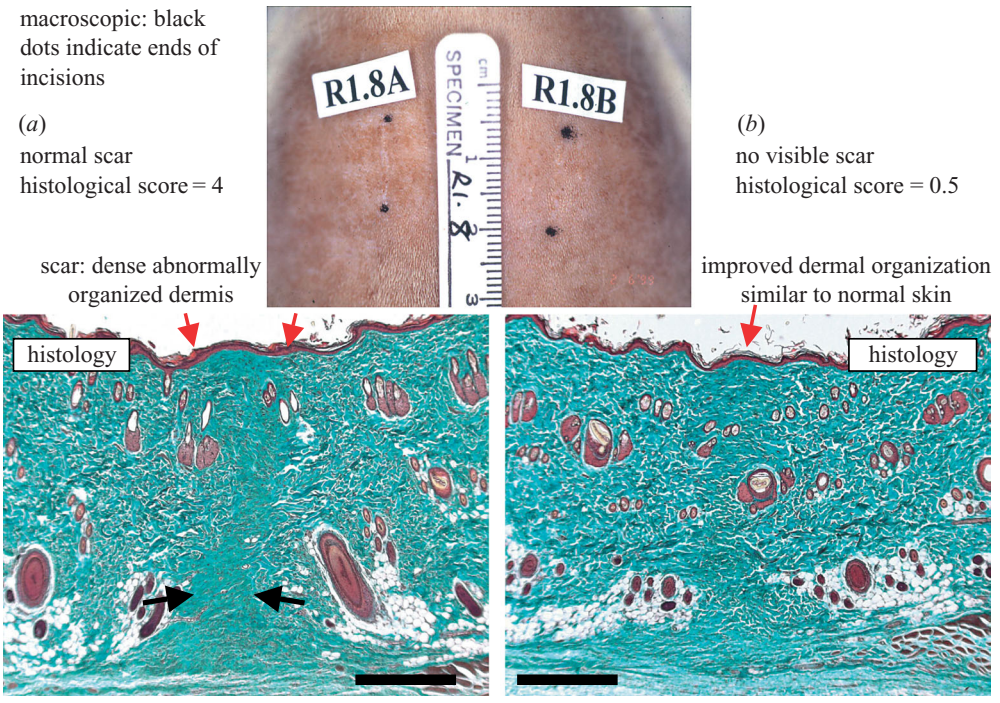


Figure 1. (*a*) A placebo-treated wound (R1.8A) compared with (*b*) absent scarring in a TGFβ3-treated (R1.8B) 1 cm rat incisional wound, 84 days post-wounding. Scale bar, 250 μm.

Table 1. Summary of TGFβ isoform differences in scar-free embryonic wound healing and scar-forming adult wound healing.

| TGFβ isoform | embryonic scar-free healing | adult healing with a scar |
|---|---|---|
| TGFβ1 | low/absent | high (platelets and inflammatory cells) |
| TGFβ2 | low/absent | high |
| TGFβ3 | high (keratinocytes and fibroblasts) | low |

Ferguson 1996). The key question is which of these numerous variables are involved in embryonic scar-free healing and adult scar-forming healing. To demonstrate such causality, several criteria, in addition to mere presence or absence in embryonic and adult wounds, must be fulfilled (Ferguson *et al.* 1996). As a first step, manipulation of the variable should induce an improvement or absence of scarring in the adult (which normally heals with a scar) and a manipulation in the reverse direction should induce scarring in the early embryonic wound (which normally heals without a scar). When such stringent criteria are employed, only a few of the myriad of cellular and molecular differences between embryonic and adult healing remain as potential mechanisms and therapeutic targets involved in skin scarring (McCallion & Ferguson 1996; Ferguson *et al.* 1996).

Thus, for example, embryonic wounds elicit a very different inflammatory response compared with adult wounds (Cowin *et al.* 1998). In the embryo the immune system is developing and the response to injury of these primitive immune cells is different from that in the adult. As a consequence, embryonic wounds have far fewer inflammatory cells (markedly reduced numbers of neutrophils, lymphocytes, monocytes and macrophages), the inflammatory cells present are less differentiated (e.g. fewer activated macrophages) and the length of time that inflammatory cells are present is markedly reduced compared with adult wounds (Cowin *et al.* 1998). In addition, the embryo is rapidly developing and growing with a considerable expansion of skin volume. Consequently, normal embryonic skin and embryonic wounds contain high levels of morphogenetic factors involved in skin growth, remodelling and morphogenesis. As a consequence of these two principal variables (altered inflammatory response and skin morphogenesis) the growth factor profile at a healing embryonic wound is very different qualitatively (i.e. the types of growth factor present), quantitatively (i.e. the amounts of such growth factors present) and temporally (i.e. the length of time the growth factors are present) compared with an adult wound (Whitby & Ferguson 1991*b*; O'Kane & Ferguson 1997; Shah *et al.* 2000; Cowin *et al.* 2001). Thus, for example, there are major differences in the TGFβ isoforms present in embryonic and adult wounds (table 1). Embryonic wounds express very high levels of TGFβ3, a skin morphogenetic factor predominantly synthesized by keratinocytes and fibroblasts and very low levels of TGFβ1 and TGFβ2. By contrast, adult wounds contain predominantly TGFβ1 (and TGFβ2), which is derived initially from degranulating platelets and subsequently from inflammatory cells such as monocytes and macrophages. Likewise, adult wounds contain large quantities of PDGF, which is virtually absent in embryonic wounds (owing to the lack of platelet degranulation), whereas embryonic wounds contain higher levels of endogenous FGFs involved in skin morphogenesis (Whitby & Ferguson 1991*b*).

## 4. EXPERIMENTAL MANIPULATION OF ADULT WOUND HEALING

Using information on the cellular and molecular differences between scar-free embryonic wound healing and scar-forming adult wound healing, we have experimentally manipulated the healing wounds of adult mice, rats and pigs (see McCallion & Ferguson 1996; O'Kane & Ferguson 1997; Shah *et al*. 2000 for reviews). These manipulations have been conducted using pharmacological or genetic approaches in transgenic animals. Thus, for example, application of neutralizing antibodies to TGFβ1 and or TGFβ2 (preferably both) to healing adult rodent wounds results in markedly improved scarring (Shah *et al.* 1992, 1994, 1995). Equally, prevention of activation of TGFβ1 and TGFβ2 at the wound site by competitive inhibition with mannose 6 phosphate also results in markedly improved healing and scarring, whereas wounds in mannose 6 phosphate receptor/insulin-like growth factor II knockout mice heal with markedly improved scarring (our unpublished observations). Interestingly, pan-neutralization of all three TGFβ isoforms (TGFβ1, TGFβ2 and TGFβ3) does not improve scarring, suggesting that neutralization of TGFβ3 may be detrimental (Shah *et al.* 1994, 1995). By contrast, exogenous addition of TGFβ3 to healing adult wounds (to elevate levels similar to those seen in scar-free embryonic wounds) results in markedly improved or absent scarring during adult wound healing (Shah *et al.* 1995). To further investigate the role of TGFβ3 in scarring we wounded homozygous null, heterozygous and homozygous positive (wild-type) embryos in crosses between heterozygote transgenic mice null for TGFβ3 (Proetzel *et al.* 1995). Interestingly, wounds on mouse embryos homozygous null at the TGFβ3 locus show delayed healing and scarring compared with their wild-type litter mates, homozygous positive at the TGFβ3 alleles, which heal rapidly with no scarring (Qiu *et al.* 2004). The abnormal, scar-forming, embryonic healing in the TGFβ3 null mice is suggestive of a defect in cell migration. Fibroblasts recovered from TGFβ3 null skin migrate significantly slower in a collagen filter assay compared with their wild-type counterparts. This deficit in fibroblast cell migration can be rescued by exogenous addition of TGFβ3, but not TGFβ1 or TGFβ2 (Qiu *et al.* 2004), suggesting a TGFβ3 isoform-specific effect on cell migration. By contrast, the effects of TGFβ1, TGFβ2 and TGFβ3 on fibroblast cell proliferation are similar. These data clearly indicate that among the three TGFβ isoforms there are both promiscuous (cell proliferation) and isoform-specific (cell migration) effects. Stimulation of fibroblast cell migration into a healing wound results in a more normal basketweave organization of the fibroblasts and the extracellular matrix molecules that they deposit, thus restituting the normal dermal architecture and producing a marked improvement or absence of scarring (figure 1).

Such experimental approaches indicate that TGFβ3 may be a particularly important molecule for healing with improved scarring (table 2).

## 5. THERAPEUTIC MANIPULATION OF SCARRING DURING ADULT HUMAN WOUND HEALING

On the basis of this research we have developed novel pharmaceutical agents to improve or prevent scarring in man. A central therapeutic approach is to alter the ratio of TGFβ isoforms in healing adult human wounds: elevating the levels of TGFβ3 and/or reducing the levels of TGFβ1 and TGFβ2. Such putative human pharmaceutical agents have successfully completed phase 1 safety and toleration studies under the auspices of a biotechnology company, Renovo Limited (see www.renovo.com). Efficacy studies have been conducted initially in human volunteers. With appropriate regulatory permissions these volunteers have received experimental 1 cm incisions or 3 mm punch biopsy excisions under their arms; the experimental drug has been applied to one wound with placebo or standard care to the other, thus allowing the comparison of wound healing and scarring within an individual subject as well as between subjects. These human volunteer studies have progressed well and demonstrated efficacy comparable to that seen in animals, thereby facilitating the initiation of patient-based clinical trials, e.g. in skin graft donor sites. A full length publication describing the results of these ongoing human clinical trials will be published separately when the trials have been fully analysed and the blind broken.

Although initial human clinical trials have been conducted in the skin, these scar-improving pharmaceuticals may find widespread use, e.g. in the eye, abdominal and reproductive organs, nerves, ligaments, etc. as well as in systemic fibrotic disorders such as glomerulonephritis or pulmonary fibrosis, all of which share conserved molecular and cellular mechanisms with dermal scarring.

## 6. TIMING OF INTERVENTION

We have investigated the time during healing when agents such as TGFβ3 or neutralizing antibodies to TGFβ1 and TGFβ2 should be applied to elicit their scar-improving effects. Surprisingly we have shown that early application, at the time of or shortly after (within 48 hours) wounding, produces the best results (Shah *et al.* 1994, 1995). However, scars are the final endpoint of a healing wound. In rodents, scars are not normally stable and mature until some 80 days post-wounding. Likewise in man, scars are not normally mature and stable until at least six months post injury. Scarring has therefore been thought of as a late event in wound healing, predominantly involving extracellular matrix remodelling (Ferguson & Leigh 1998; Cherry *et al.* 2001). Why then should a therapeutic agent to improve scarring need to be administered early, i.e. around the time of surgery or injury? There are many possible explanations. The initial trigger in a healing wound involves the release of active molecules, predominantly TGFβ1 and PDGF from degranulating platelets at the wound site within seconds of wounding. This initial response is triggered not by gene transcription or translation but rather by release of considerable quantities of stored active molecules. These initial triggers rapidly induce several overlapping and redundant cytokine and cellular cascades. Thus, for example, TGFβ1 is autoinductive binding at its own promoter and upregulating its own synthesis. Low levels of active TGFβ1 stimulate the recruitment of immature monocytes to the wound site, which in turn secrete TGFβ1 (Niesler & Ferguson 2001). Interestingly, high levels of TGFβ1 inhibit the recruitment of mature

Table 2. Summary of the evidence for the centrol role of TGFβ3 in scarring.

| TGFβ3 |
|---|
| present at high levels in developing skin and in embryonic wounds that heal with no scar |
| present at low levels in adult skin and wounds that scar |
| induced late in adult wound healing when levels of TGFβ1 start to fall |
| neutralization of TGFβ3 in adult wounds makes the scar worse |
| addition of TGFβ3 to adult wounds reduces or eliminates scarring |
| genetic deletion of TGFβ3 causes scarring following embryonic wounding (litter mate +/+ embryos heal with no scar) |

monocytes, thus regulating the inflammatory response. Consequently, early intervention at the time of, or shortly after, wounding has two major consequences: (i) a small alteration in the early mediators, e.g. reduction of TGFβ1, can have a major long-term effect due to alteration and reduction of these autoinductive regulatory cascades; and (ii) early in the healing process there are only a small number of major signalling molecules. As wound healing rapidly progresses, additional cytokines and growth factors are induced or secreted and a large number of overlapping and interacting cytokine cascades are established. These cascades have been evolutionarily optimized to be both robust and redundant, meaning that they can withstand considerable pertubations in the quantities and types of molecule present. A result of this is that later (after 48 hours) therapeutic or experimental interactions are difficult and produce much less significant effects on scarring compared with earlier interactions when the overlapping multiply redundant cascades have yet to be established.

Furthermore, early application of master regulatory cytokines such as TGFβ3 can induce a different repertoire of receptors and responses on the target cells, resulting in a markedly different response to the various factors released during subsequent stages of healing and thus a markedly improved scar. Early application of therapeutic agents may also result in altered recruitment and persistence of cellular populations during subsequent healing phases, e.g. inflammatory (monocyte, macrophage, lymphocyte, etc.) or fibroblast populations (Shah *et al.* 1995). Furthermore, there is evidence from other inflammatory diseases, e.g. glomerulonephritis, uveoretinitis (Erwig *et al.* 1998, 2001; Robertson *et al.* 2002), that the cytokine that a macrophage first sees may alter or render it refractory to signalling from the same or different cytokines. This phenomenon has been described as macrophage programming (Erwig *et al.* 1998, 2001). Therefore early exposure of cells in a healing wound to particular growth factors may programme them to produce markedly different long-term outcomes. Such cellular programming may occur, for example, by the entrainment of various second messenger pathways (rather like neuronal gate control mechanisms in pain), by voiding particular second messenger responses because of lack of substrate molecules that have been used in the earlier signalling and/or by the induction of specific transcription factors, which facilitate the transcription of particular genes, whose products may effect the differentiation of that cell and perhaps its neighbours.

Furthermore, in the case of healing skin wounds treated at the time of injury with TGFβ3, the stimulation of fibroblast cell invasion into the fibrin clot has at least two beneficial effects. First, the pioneer cells open up pathways facilitating subsequent cellular invasion. Second, the highly motile fibroblasts deposit extracellular matrix in a basketweave orientation (owing to their haphazard migratory pathways within the fibrin clot) and this restitutes the normal dermal architecture, whereas non-treated wounds show an alignment of fibroblasts predominantly at the interface between the fibrin clot and the wound margins resulting in abnormally organized parallel bundles of scar tissue collagen. This early organization of the healing wound may be self reinforcing: rapid early restitution of the normal dermal architecture normalizes the force distribution on that area of the skin, which in turn facilitates normal dermal regeneration. By contrast, the early abnormal alignment of scar tissue extracellular matrix establishes, and/or perpetuates, abnormal tensile forces within the wound, which in turn reinforce the subsequent abnormal alignment of the scar tissue extracellular matrix thus causing the scar to persist and indeed become more prominent with time.

Thus timing of experimental or therapeutic intervention to produce adult scar-free healing is critical. In clinical practice, this timing is advantageous and not problematic. During surgical operations the time of wounding can be anticipated accurately and is precisely known: surgery is trauma by appointment! In the case of major traumatic injury, e.g. road traffic accidents, sporting injuries, domestic accidents, burns, violence, etc., patients are transported rapidly to the hospital, where any scar-improving drug could be appropriately administered, certainly within the 48 hour therapeutic period. Indeed, the observation that experimental or therapeutic agents appear necessary only in acute doses in the early phases of healing is a major clinical advantage obviating the necessity of developing long-term dosing strategies, ensuring patient compliance, etc. Scar-improving human pharmaceuticals will probably be given by the physician or surgeon acutely by direct intradermal injection into the margins of the wound (wound sites are normally anaesthetized with local anaesthetic to facilitate suturing, and/or the patient may be under a general anaesthetic thus rendering such simple methods of administration clinically acceptable).

Scientifically, this long time interval between experimental intervention and phenotypic effect appears common to many regenerating systems, e.g. lung regeneration induced by retinoic acid (Maden & Hind 2004), and neuronal restitution of the central nervous system after stem-cell application (McKay 2004), and indeed is a hallmark of axolotl limb regeneration where the initial phases of blastema formation, cellular de-differentiation and subsequent re-specification are essential to producing the missing part (Imokawa *et al.* 2004).

## 7. EVOLUTIONARY CONSIDERATIONS

Most people believe scarring to be an inevitable consequence of injury and by inference to be an evolutionarily optimized endpoint. This prevalent view is intellectually at odds with the results of our experimental manipulations and often raises questions about the potential disadvantages of having no scar. In our experiments there are no such disadvantages. Despite its morphological appearance, a skin scar is actually weaker than the normal skin. With our scar-improving and scar-preventing experimental and therapeutic regimes, we observe no decrease in the tensile strength of the wounds (they are actually stronger than normal scars as the extracellular matrix is more normally aligned) (Shah *et al.* 1992, 1994, 1995), the wounds heal as fast (often faster) as controls (Shah *et al.* 1994, 1995) and the incidence of wound infection is not increased (and indeed might be predicted to decrease owing to the faster healing). Such considerations throw into question the original premise that the scar is an evolutionarily optimized endpoint of the healing wound. We reject that premise and provide below an alternative evolutionary hypothesis for scarring and regeneration.

## 8. WOUND SIZE AND TYPE

We hypothesize that evolutionary selective forces on different wound-healing mechanisms (scarring or regeneration) were directed at a particular type of wound that is no longer a common wound type seen today. If an animal encounters a very major wound, such as the avulsion of a limb, then the healing of this large wound is likely to be subjected to minimal evolutionary selective pressures, as the animal will probably die shortly after wounding. In nature if a major wound is sustained in any mammal then the probable effect will either be: rapid death, e.g. from exsanguination; fairly rapid death, e.g. from septicaemia; or secondary death due to the general illness and morbidity that the wound induces, preventing the animal from functioning normally (for example, escaping from a predator). We therefore hypothesize that there have been few evolutionary selective forces on large mammalian injuries as quite simply the animal rapidly dies. Equally, we argue that there are only a few specific, selective forces on very small mammalian wounds, such as those that could be induced for example by a thorn on a plant. In such small wounds the overriding survival pressure will be to minimize wound infection and facilitate the expulsion of the foreign body. A consequence of that will be selective forces for a robust inflammatory response to kill invading micro-organisms and a robust inflammatory response combined with a fibrotic walling-off response to 'isolate' the foreign body and facilitate its expulsion, e.g. by liquefaction of the tissues between the walled-off capsule and the foreign body to form an abscess which then ruptures expelling the foreign body and liquefied contents and allowing the abscess cavity to heal. In such situations those evolutionary selective forces that are brought in to play on the healing response will be ones that select for a fibrotic walling-off effect, i.e. a scarring effect (abnormal organization of extracellular matrix).

We hypothesize that wounds of intermediate size have been the subject of the major evolutionary selective forces shaping the cellular and molecular mechanisms of wound healing. By intermediate wounds we mean wounds that are neither very small, e.g. thorn pricks or so large as to compromise the life of the animal. In evolutionary terms these intermediate wounds will be mostly bites, blows, contusions, degloving injuries, etc. caused by damage from other animals, blows from falling objects or from the animal falling or impaling itself on a foreign object. These intermediate wounds would typically involve widespread tissue damage, haematoma formation and bruising, variable degrees of tissue damage with varying amounts of normal tissue remaining in the damaged area, and they would frequently be dirty, i.e. with impaled foreign bodies such as dirt, wood splinters, bacteria, etc. We hypothesize that the evolutionary forces shaping healing in these wounds have been directed, as with the small wounds, to the prevention of infection and the walling off of foreign bodies (as described above) and additionally to the rapid restitution of missing tissue which needs to function quickly even at the expense of functioning optimally and certainly, as a consequence, functioning suboptimally long term. Effectively this is a selective pressure to rapidly form granulation tissue and to rapidly remodel that granulation tissue into some form of scar tissue, such that the injured body part, e.g. limb can function at least partly so as to preserve the life of the organism. Thus we argue that the major evolutionary forces shaping the cellular and molecular mechanisms underlying adult wound healing are those to wall off foreign bodies and infection and to rapidly replace missing tissue with rapidly, but only partly, functioning repair tissue. This is the phylogenetically optimized response to a widespread dirty wound with variable amounts of tissue damage such as would be encountered in a primitive bite, blow, etc.

These are not the types of wound commonly encountered today. In evolutionary history sharp injuries, such as those inflicted by mechanical equipment, glass, surgical instruments, swords or bullets are very rare. In evolutionary history sharp, clean injuries such as those encountered in surgery or after the surgical debridement of a traumatic injury, are extremely rare, whereas in evolutionary history, sharp, clean injuries with close approximation of the wound margins such as those that occur after surgical repair by suturing or glues are completely unheard of. It is therefore obvious that the most common type of wound in contemporary man and animals, i.e. a wound made by a sharp object under clean or sterile conditions (or having been cleaned by debridement) and with its margins approximated by sutures, glues or bandages is a completely new evolutionary condition, which has arisen only in the past 500 years or so and is *not* a wound type that has been optimized by the evolutionary forces shaping wound-healing mechanisms. In brief, we hypothesize that this, the most common wound injury seen in contemporary man or animals, heals by inappropriate and suboptimal cellular and molecular mechanisms that have been phylogenetically selected over a long time period for the healing of a different type of wound (bite, blow, contusion, etc.) with different degrees of tissue damage, wound infection, widespread impalement of foreign bodies and different wound morphology (no approximation of the wound margins). The result of this evolutionary mismatch is a scar that can be excessive and debilitating even after minor injury,

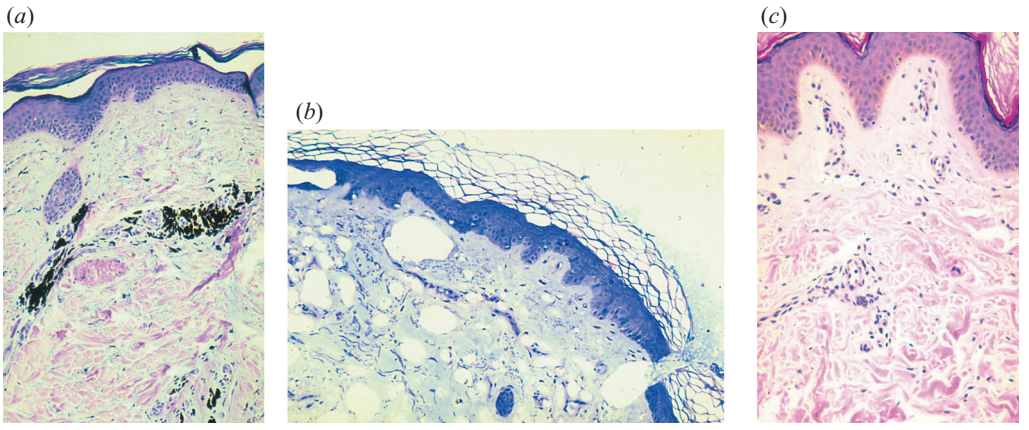


Figure 2. (*a*) Histological section of a human skin tattoo biopsy. Note the black pigment particles within fibroblasts adjacent to blood vessels and hair follicles. (*b*) Histological section of human skin biopsied within seconds of treatment of a blue/black tattoo with a dermatological Yag laser. Note the numerous small wounds caused by lysis of pigment-containing cells. (*c*) Histological section of human skin biopsied from a tattoo site treated 7 days earlier with a laser. Note the lack of inflammatory cells and the colonization of the small wounds with fibroblast cells which ultimately regenerate the dermis without scarring.

e.g. simple surgical incision under sterile conditions. We hypothesize that the normal response to a wound made with a sharp object under clean conditions and with apposition of its margins is inappropriate, indeed pathological, and that therapeutic manipulation of these normal but inappropriate healing mechanisms can result in an improved healing response of scar-free healing and/or tissue regeneration. Scarring, we submit, is essentially an evolutionary response to wall off foreign bodies and infection and to rapidly reconstitute semi-functional missing tissue. None of these considerations applies in modern wounds. We hypothesize that the normal healing response in these sharp, clean, margin approximated wounds is excessive, with inappropriate levels of inflammatory cells and mediators, inappropriate stimulation of granulation tissue and inappropriate fibrotic differentiation signals resulting in walling off and scarring.

We further hypothesize that the differences between this inappropriate scarring response and the appropriate regeneration (scar-free healing) response are subtle and involve similar molecular players, thereby rendering dramatic therapeutic intervention feasible and straightforward. What is the evidence for such a bold statement?

## 9. REGENERATION AND SCARRING OCCUR IN THE SAME ANIMAL AND EVEN IN THE SAME TISSUE

Although it has been well documented that certain adult amphibian parts can regenerate if removed, e.g. limbs, jaws, lens of the eye, etc. (see Imokawa *et al.* 2004), it is also true that other parts of the animal do not regenerate, e.g. retina of the eye and if an incisional wound is made on the flank of an axolotl it heals with a scar. Therefore in the same primitive amphibian, under certain circumstances, particular tissues undergo a regenerative response whereas others undergo a scarring response. Equally, in mammalian livers, if two-thirds of the liver mass is removed by partial hepatectomy, the remaining liver regenerates (Fausto 2000). By contrast, if a stab incisional wound is made into the same liver, it heals with a scar. Equally, in the MRL mouse a complete through-and-through circular punch wound in the ear regenerates (Rajnoch *et al.* 2003; Heber-Katz *et al.* 2004), whereas an incisional wound to the back skin heals with a scar identical to that in a control C57 mouse (M. W. J. Ferguson and A. Beare, unpublished data). Even in adult humans, there are tissues that are capable of complete regeneration: the gums and gingival (interdental) papillae between the teeth will completely regenerate without scarring if excised. This regenerative mechanism is the basis of the clinical dental specialty of periodontics. In human adults, the oral mucosa and gums scar little (if at all) after surgical incision, whereas an identical surgical incision e.g. 1 cm cut, will scar much worse if made on the skin covering the deltoid region and sternum of the chest than for example on the face, abdomen or legs. It is clear that, even in man, there are tissue-specific and regional variations in regeneration and the severity of scarring.

Such considerations suggest that the mechanisms underlying both scarring and regeneration must be compatible and use subtly, but not substantially, different signalling molecules or combinations of signalling molecules. These observations indicate that regeneration is not necessarily a specialized response restricted only to amphibians and lower animals and completely different from the repair we associate with common injuries in adult mammals. Such considerations suggest that the intellectual paradigm of regeneration being different and signalled by substantially different (? novel) molecules from scarring must at least be questioned.

## 10. REGENERATION AND SCARRING OCCUR EVEN WITHIN THE SAME AREA OF HUMAN ADULT SKIN

To further strengthen the above argument, it should be noted that under different circumstances a piece of adult human (or animal) skin can either scar or regenerate! Evidence for this is widespread but rarely considered or discussed. Thus, at a simplistic level, a very small injury to the skin, e.g. pin-prick, needle insertion, etc., heals

perfectly with no scar. By contrast, a surgical incision in the same piece of skin (even if it is only 1 cm long) heals with a scar. There are even situations in man where multiple minor injuries with the trapping of numerous foreign bodies regenerate perfectly with no scars, e.g. tattooing! When tattoos are placed in the skin, multiple pin-prick injuries, each depositing small pigment particles, are made in the skin to the depth of the dermis or even the subcutaneous fat. Despite these numerous small injuries and the incorporation of considerable amounts of foreign material, tattoos normally heal perfectly with no scars. The great thing about human tattoos is no sooner have people had the tattoo performed than they want it removed or altered: for example, individuals with 'I love Mary' or the like tattooed on their arm frequently want it removed when Mary is no longer in favour and someone else is! We have therefore studied the cellular mechanisms underlying tattoo placement and removal. After the initial minor injury to the skin, the small tattoo particles are rapidly phagocytosed within fibroblast cells located predominantly around hair follicles and blood vessels (figure 2*a*). Presumably these tattoo particles remain in these cells (or cells that engulf them) for the lifetime of the individual! If individuals with tattoos containing black or blue pigment are treated with a laser of the appropriate excitation frequency and immediately biopsied the results are shown in figure 2*b*. Here, the rapid absorption of the laser energy causes the cells containing the pigment particles to lyse, releasing their contents and creating numerous microwounds within the tissue. If the healing of these wounds is then followed with time (figure 2*c*) they regenerate perfectly with no scarring and with minimal early inflammatory response. In this way one can compare wounds involving the same volume of tissue damage in man (or animals) created either by a single incision (or excision) or by multiple small injuries, e.g. tattoo removal or small perforating injuries, such as those inflicted in tattoo placement. In the former case the wounds scar, in the latter they heal with perfect regeneration of normal skin structure and function.

Why do these small skin wounds heal with perfect regeneration even in man? From our studies we conclude that the mechanisms are similar to those involved in embryonic scar-free healing and in our experimental manipulations of animal and human wounds. Thus small skin wounds elicit minimal blood clotting and platelet degranulation and consequently show low levels of early TGFβ1. Small skin wounds also elicit a minimal inflammatory response, again resulting in low levels of TGFβ1 and TGFβ2 as well as other inflammatory mediators. Small skin wounds are exposed to higher levels of TGFβ3, which is synthesized by overlying keratinocytes and adjacent fibroblasts and which rapidly diffuses into the small wound compared with the distant cellular sources and poor diffusion present in a larger wound. Small skin wounds have minimal amounts of missing tissue, show minimal granulation tissue formation and presumably receive important cues and signals from the surrounding tissues, e.g. appropriate cytokine signals such as TGFβ3, appropriate extracellular matrix substrates (as opposed to fibrin), thus facilitating normal cell alignment and tensile forces. As in scar-free embryonic wound healing, the duration of the response phase of healing is short. By contrast, in an incisional or excisional wound, the wound space is initially filled with fibrin and numerous degranulating platelets which release large amounts of TGFβ1. There is poor diffusion of TGFβ3 from surrounding epithelial and fibroblast cells; there is prolonged and sustained inflammation with the release of TGFβ1 and other inflammatory mediators; there is recruitment of additional cells to the wound site whose alignment is often inappropriate; and there are altered tensile forces on the wound and the healing response is prolonged and evolutionarily inappropriately optimized to wall off foreign material or generate copious granulation tissue (i.e. a scarring response).

Even subtle alterations to the type of injury can result in a different outcome, presumably because of subtle alterations in the signalling mechanisms. Thus for example in human skin, simple needle injuries, e.g. for injection or made by a fine suture needle, will heal perfectly with no scar and complete tissue regeneration. By contrast, if that small needle is used to insert a suture, which is either made of inappropriate pro-inflammatory material, e.g. catgut, and/or is left in place too long, is tied too tight or is subject to movement, then the result is a small suture track scar, frequently seen after numerous surgical operations in man. Presumably, subtle changes induced by the circumstances, e.g. increased inflammation resulting in increased levels of TGFβ1, induce the small wound to heal with a scar.

These common but frequently neglected observations are in keeping with the molecular mechanisms underlying embryonic scar-free healing and experimental manipulation of adult wounds to heal without scarring. Collectively, these observations suggest a different intellectual paradigm for scarring and regeneration. This would suggest that the mechanisms underlying regeneration and scarring are similar, subtle and interchangeable. The paradigm would further suggest that they exist in many adult tissues and organs and that subtle alteration of the signalling environment of the healing wound can produce dramatically different outcomes. The most exciting conclusion from this is that improved scar-free healing (partial regeneration) or complete regeneration may be a more easily and rapidly attainable therapeutic outcome in man than has previously been thought.

## 11. MOLECULAR SIMILARITIES AND EVOLUTIONARY CONTEXT OF SCARRING AND REGENERATION

How could this new paradigm of scarring and regeneration operate at the molecular, cellular and phylogenetic levels? First, there is already evidence that identical growth factors in different combinations can produce dramatically different outcomes. The initial priming signal for liver regeneration is the simultaneous effects of interleukin 6 and TNF alpha (Yamada *et al.* 1997; Fausto 2000; Campbell *et al.* 2001). Interestingly, TNF alpha on its own elicits a pro-inflammatory scarring response such as one might see following a stab injury to the liver, which results in a scar. Thus one of the signals for liver regeneration is not a novel regenerative molecule, but rather the coincident signalling of a pro-inflammatory cytokine!

Equally, in the skin we demonstrate that there are major differences between the effects of on the one hand TGFβ1

(and or TGFβ2) and on the other hand TGFβ3 when applied early to a healing wound. TGFβ1 induces a markedly fibrous repair, characterized by a scar, whereas TGFβ3 induces normal skin morphogenesis and regeneration. Subtly different isoforms of the same growth factor produce dramatically different results. Such observations raise the question as to why these very similar molecules binding to the same receptor should have such different effects in the context of scarring and regeneration, when they have identical effects, for example, on cell proliferation.

It is now clear that most major signalling molecules consist of families with several biological variants (isoforms, splice variants, etc.). Often in many artificial assays *in vitro* or *in vivo*, these molecules behave identically. They are therefore often described as being functionally redundant. We believe it more important to consider the emergence and function of such variants in the context of protein evolution. In terms of protein evolution, the overriding initial requirement is to be compatible with normal function and life. Thus most isoforms of, e.g. a growth factor, are thought to arise by the initial duplication of a single ancestral gene. Initially the duplicated gene encodes the same molecule. Then with time, presumably mutations arise in that molecule which are compatible with the normal widespread cellular functions of the growth factor, but which may convey a new and advantageous characteristic on the mutated molecule in a specific tissue or in a specific context, e.g. wound healing. This mutation is then selectively advantaged over other non-functional mutations and persists. Mutations that are incompatible with the normal functioning of the protein would result in death or compromise of the animal. In this way families or variants of proteins evolve. The overriding principle is that early on it is more important to be compatible than to be novel or useful! With this background, we would expect to find families of molecules where many functional effects are shared, but where in certain contexts or tissues, an individual isoform or variant confers some unique property or response. So it is with TGFβ1, TGFβ2 and TGFβ3. In many assays these isoforms are indistinguishable (Roberts & Sporn 1990), e.g. effects on cell proliferation are functionally interchangeably and so described as evolutionarily redundant. However, our data clearly show that in certain contexts, these molecules can have very different effects, e.g. TGFβ3 facilitates scar-free healing and regeneration, whereas TGFβ1 causes fibrosis and scarring in a healing wound. Interestingly, both isoforms bind to the same receptor, but presumably elicit both common and isoform distinct signalling responses. This is not the normal pharmacological paradigm. However, it is becoming clear from investigations of families of signalling molecules such as the TGFβ family, the FGF family, the interleukin family etc., that evolution of isoforms or spliced variants often results in agonists, partial agonists and antagonists at the receptor and this is the first step in understanding how different molecules binding to the same receptor can elicit different biological responses. Against this molecular evolutionary background, it is easy to understand how both regeneration and scarring can occur in the same organism and indeed in the same tissue depending upon the molecular context: TGFβ3 producing scar-free healing and regeneration, TGFβ1 fibrosis and scarring. This principle that scarring, repair and regeneration share subtly different signalling molecules, is given further strength by the observations, for example, of the key role of thrombin, both in initiating fibrin clotting and scarring repair and in initiating regenerative pathways (Imokawa *et al*. 2004). We might predict that further subtle variants of molecules involved in the healing response, could result in either scarring repair or regeneration.

Fundamentally, there are three principle variables in either scarring repair or regeneration:

(i) the signalling molecules;
(ii) the responding cells; and
(iii) the context in which they are presented.

All experimental and clinical observations can be explained by variations in one or other of these three principle variables.

## 12. GENERATION OF VARIATION AND ROBUST STABLE STATES (SCARRING OR REGENERATION)

In the above paradigm, it might be asked why and how a subtle alteration in one signalling molecule could produce such a dramatic phenotypic difference, i.e. scar or no scar. We believe that the explanation for this lies in understanding the robust basis of genetic signalling networks and the generation of variation (Von Dassow *et al*. 2000; Meir *et al*. 2002; Rockman & Wray 2002; Von Dassow & Odell 2002).

These emerging data from modelling biological regulatory networks such as *Drosophila* segment polarity or neurogenesis have elucidated a number of interesting principles. The genetic (signalling) networks are robust within certain compartments or modules. Within these modules individual signalling molecules can vary widely in their concentrations but still give the same robust anatomical result, although with subtle variations. Indeed, it is the generation of these variations which is at the heart of all biology: which fundamentally depends upon the generation of variation and the effects of natural selection thereon. Take for example, facial morphogenesis, when operating normally, the genetic networks will specify structures such as the eyes, nose, mouth, ears, with the appropriate anatomical form and location, but the same genetic networks will generate so much variation that each face is different from approximately 10 million other faces! So the high-level concept is of a robust stable network where many individual parameters vary widely, but all of which give a similar end result. Typically, however, there are one or two parameters whose regulation is tightly controlled and where variation is either incompatible with life or puts the genetic network into a different stable state with a markedly different outcome. Such genes or molecules are critical for major morphogenetic change.

Applying these principles to a healing wound we would interpret existing data as follows. There are a large number of signalling molecules in a healing wound. The levels of these can vary widely at most times during healing with no major differences in the healing outcome. Thus, for example, TGFβ1 levels may vary widely, both systemically within the blood and at the wound site (depending on

platelet degranulation, inflammatory cell recruitment, etc.), but the end result is a scar with some variation, e.g. very prominent scar, less prominent scar, etc., but no real difference in the underlying process. Conversely other molecules e.g. TGFβ3, may be more tightly controlled at particular times during healing, such that if their levels vary the wound is taken into a different stability state, i.e. scar-free healing or regeneration. It should be noted that unlike TGFβ1, TGFβ3 does not circulate in the blood, is not released by degranulating cells at the wound site and is probably more tightly controlled by the synthetic machinery of the skin keratinocytes and fibroblasts. As indicated earlier, timing of application of this factor is critical to the markedly altered outcome. We would hypothesize that TGFβ3 is one of the master regulators enabling a shift to a different phenotypic outcome. Of course the effectiveness of this molecule will depend on the context in which it is presented, e.g. the amount of TGFβ1 in the system, exposure to appropriate cells early in the wound healing cascade, etc. Often these important contextual elements can only be investigated and understood in complex *in vivo* experiments. *In vivo*, cells are exposed to multiple signalling molecules with varying affinities (usually low) in parallel, which contrasts with the conditions present in most *in vitro* experiments.

## 13. MOLECULAR VARIABLES AND CLINICAL VARIABLES

The above considerations of subtle alterations in signalling molecules, e.g. TGFβ3 versus TGFβ1, cells and context can be used to explain known clinical variables in scarring in man (Bayat *et al.* 2003). Thus for example, it is known that the severity of scarring varies by:

(i) tissue site, e.g. gums (which regenerate) versus the deltoid region of the skin (which scars badly)—see earlier;
(ii) sex (fertile females scar worse than postmenopausal females and males as oestrogen has a major influence on wound healing) (Ashcroft *et al.* 1997*a*);
(iii) race (in general Negroids and Mongoloids i.e. coloured skin races scar worse than Caucasians);
(iv) age, young people, particularly teenagers and those in their twenties scar worse than older people (Ashcroft *et al.* 1997*b*,*c*); and
(v) magnitude of injury and wound contamination (the larger the wound and the more contaminated the wound, the worse the scar).

Explanations for variations in site, magnitude of injury and wound contamination have been provided earlier. The age-related effects correlate with enhanced activity of the immune system and hence TGFβ1 in youth and the dysregulation of the immune system and lower levels of TGFβ1 in old age (Ashcroft *et al.* 1997*b*,*c*). The major influence of sex hormones, e.g. oestrogen and androgen on wound healing might be explained by the skin being a sexual organ, for example, red flushes in the buttocks of great apes when they are in oestrous and/or their importance in the regeneration of the endometrium during the menstrual cycle. Certainly oestrogen levels influence levels of TGFβ1 and inflammatory mediators in healing skin wounds (Ashcroft *et al.* 1997*a*, 1999). Explanations for severe scarring in coloured-skinned races are more problematic, but may represent genetic selection among individuals living in tropical climates (i.e. coloured skin) to wall off parasitic infections of the skin (a similar selective pressure to that on wound healing). Such genetic selection could result in more adverse scarring as similar molecular players are involved. We might expect to see evidence of this in polymorphisms of appropriate genes, e.g. in regulators of the TGFβ family or their receptors or signalling molecules (Rockman & Wray 2002). Such gene polymorphisms could also of course explain why certain individuals irrespective of the above major clinical variables are good or poor healers and good or poor scarrers.

## 14. CONCLUSION

Our experiments in scar-free embryonic wound healing and the experimental and therapeutic manipulation of scarring in animals and man indicate that specific subtle alterations at appropriate times following wounding can result in profoundly different outcomes, i.e. scarring or scar-free healing (regeneration). Our research has focused on the TGFβ family of molecules where TGFβ3 elicits a scar-free or regenerative healing response, whereas TGFβ1 and TGFβ2 elicit a fibrotic scarring response. We argue that the type of wounds commonly encountered in contemporary man and animals are not those to which the cellular and molecular mechanisms of healing have been evolutionarily optimized. We further demonstrate that the differences between scar forming or scar-free healing involve subtle alterations to similar molecular pathways and indeed argue that this must be a general principle as both scarring and regeneration can occur within the same individual and even within the same individual tissue. We have extended our experimental manipulations to the development of novel human pharmaceuticals to prevent scarring and facilitate tissue regeneration. The results from our early clinical trials, together with the new intellectual paradigms of understanding scarring and regeneration in an evolutionary, molecular and cellular context give us great hope for the future. At least in some cases the differences between scarring and regeneration are subtle, involve similar molecular pathways and are experimentally and therapeutically tractable. Such results and approaches suggest that therapeutic regeneration in man, at least in certain clearly defined situations, may be a realizable short-term goal.

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## GLOSSARY

FGF: fibroblast growth factor
PDGF: platelet-derived growth factor
TGFβ: transforming growth factor beta
TNF: tumour necrosis factor