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Epithelial mechanobiology, skin wound healing, and the stem cell niche ☆

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ABSTRACT

Skin wound healing is a vital process that is important for re-establishing the epithelial barrier following disease or injury. Aberrant or delayed skin wound healing increases the risk of infection, causes patient morbidity, and may lead to the formation of scar tissue. One of the most important events in wound healing is coverage of the wound with a new epithelial layer. This occurs when keratinocytes at the wound periphery divide and migrate to re-populate the wound bed. Many approaches are under investigation to promote and expedite this process, including the topical application of growth factors and the addition of autologous and allogeneic tissue or cell grafts. The mechanical environment of the wound site is also of fundamental importance for the rate and quality of wound healing. It is known that mechanical stress can influence wound healing by affecting the behaviour of cells within the dermis, but it remains unclear how mechanical forces affect the healing epidermis. Tensile forces are known to affect the behaviour of cells within epithelia, however, and the material properties of extracellular matrices, such as substrate stiffness, have been shown to affect the morphology, proliferation, differentiation and migration of many different cell types. In this review we will introduce the structure of the skin and the process of wound healing. We will then discuss the evidence for the effect of tissue mechanics in re-epithelialisation and, in particular, on stem cell behaviour in the wound microenvironment and in intact skin. We will discuss how the elasticity, mechanical heterogeneity and topography of the wound extracellular matrix impact the rate and quality of wound healing, and how we may exploit this knowledge to expedite wound healing and mitigate scarring.

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1. Introduction - Skin Structure and Function

The skin is the largest organ in the body. It fulfils a variety of functions, most importantly as a barrier separating the internal organs of the body and the external environment. Critical to this function is the skin's physical robustness—it has evolved to withstand the many mechanical, chemical and biological insults organisms face from the outside world every day, including heat, friction, radiation, pathogenic microorganisms, and toxic chemicals and materials. To achieve this, it has evolved a tough, pliable structure which can remodel and adapt to external conditions and to quickly repair in the event of injury.

In mammals, the two most prominent components of the skin are the dermis and the epidermis, which are attached to (and separated from) each other by a thin layer of extracellular matrix (ECM) proteins called the basement membrane. The dermis, which is usually much thicker than the epidermis, is largely composed of ECM proteins such as collagen type I and elastin, which are responsible for its mechanical strength. Scattered throughout the dermis are cells called fibroblasts which regulate the organisation of the fibrillar dermal matrix. The epidermis, primarily composed of keratinocytes, consists of multi-layered polarised epithelium in close apposition to the underlying dermis. Cells in the basal layer, which contact the basement membrane, continually divide during the lifetime of the organism, providing a source of cells which progressively migrate upwards through the epidermis, differentiating and stratifying to form the barrier layer of the skin.

The higher order structure of the skin varies considerably between species and anatomical location. For example, the skin of the palms of the hands and the soles of the feet in humans is clearly distinguished from that of the trunk or scalp. The latter is characterised by the presence of hair follicles while the former is characterised by the absence of hair follicles and by patterns of bifurcated rete ridges which project deep into the dermis. These differing structures are adaptations for the functions the area of skin has evolved to perform – heavily follicularised areas in mammals are adaptations to minimise heat loss and limit UV exposure, whereas palm skin in humans is highly innervated – an adaptation for improving touch sensitivity and tactility.

2. Skin wound healing

Regardless of anatomical location or function, all breaches in the skin surface must be repaired quickly, not only to prevent pathogens or harmful materials getting into the body but also to prevent fluid loss. Therefore, in mature organisms the process of wound healing is intimately associated with blood clotting and the activity of the immune system. This has been reviewed elsewhere in detail (Singer and Clark, 1999). Although wound healing is a continuous, seamless process, many researchers have found it convenient to divide the process of wound healing into phases, including inflammation, tissue formation and tissue remodelling.

Immediately after a breach in the skin surface, clotting factors are released into the wound bed to prevent loss of blood and to provide a hard fibrous matrix to prevent the ingress of pathogens. Inflammatory cells are then recruited to the wound site by a variety of chemotactic signals and engulf foreign particles, including bacteria. As this process progresses, fibroblastic cells are attracted to the wound bed and begin to secrete collagenous ECM known as granulation tissue, which gradually replaces the fibrin eschar (scab). Concurrently, epithelial cells from the epidermis neighbouring the wound site begin to migrate over the surface of the wound bed – in some cases burrowing a path beneath the hard scab and the underlying granulation tissue – until the two epithelial tongues meet in the centre of the wound, providing a new epithelial coverage (Fig. 1). Soon after, when the nascent epithelium reaches maturity, the fibrous clot separates from the underlying epithelium and is shed. In some managed skin wounds, the eschar may be removed so that wound healing occurs in the absence of a fibrous eschar and the epithelial layer migrates on the surface of the granulation tissue. In fact, some evidence suggests that skin wound healing may be expedited and improved by the provision of such a moist environment (Field and Kerstein, 1994).

Following and during re-epithelialisation, many fibroblastic cells in the granulation tissue undergo programmed cell-death (apoptosis), while others differentiate. These cells remodel the ECM of the skin leading to the formation of a collagenous matrix. In superficial wounds, the skin heals to form a tissue that is largely indistinguishable from the intact skin, but if a wound is sufficiently deep and/or large, the wound will heal with the formation of a scar. Scars are characterised by a complete absence of skin appendages, such as hair follicles and sweat glands, and by a collagenous matrix that differs in structure from that of intact tissue. In general, type I collagen fibrils form a 'basket weave' pattern in intact skin, whereas in scarred skin, these fibrils tend to be organised longitudinally (van Zuijlen et al., 2003). For these reasons, scarred skin tissue is inferior to intact skin and may contribute to pathology. For example, scars may become hypertrophic leading to tissue contracture, making it difficult for affected patients to move joints in nearby affected areas (Tredget et al., 1997).

3. Mitigating skin wounding

Skin wounding results in huge economic and societal burdens, and there are a variety of different challenges with respect to different pathologies. For this reason a number of different approaches are needed for their mitigation. Severe burn wounds often leads to such a comprehensive loss of tissue that, in the short term, coverage of the body is key to the survival of the patient. Here, grafting of either autogenic skin (from another site on the same patient, if enough tissue remains) is the preferred treatment, or alternatively allogenic material (skin or cells from a donor individual) may be used (Balasubramani et al., 2001). Other technologies include dressings based on synthetic or human or animal derived matrix proteins, which can be temporarily applied at the wound site to prevent fluid loss and infection, or tissue

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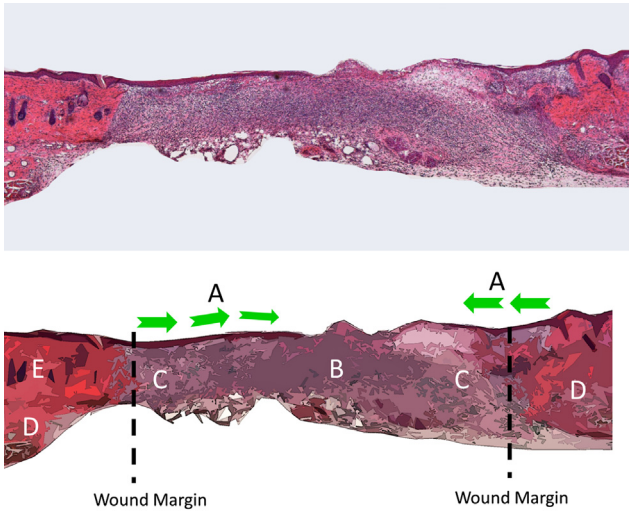

Fig. 1 – Histological cross section of a healing skin wound (upper) with diagrammatic representation (lower). A new layer of epithelium (A) migrates over the highly cellular granulation tissue (B) that fills the wound bed after skin wounding. The granulation tissue is composed of extracellular matrix proteins such as fibrin and type III collagen (C) that can be quickly laid down by the initial surge of fibroblasts that is recruited to the wound site. This is slowly replaced by type I collagen during tissue remodelling to strengthen the wound. Neighbouring intact dermis (D) provides an essential source of nutrients and immune cells via its blood supply network, and is composed of fibrils of collagen and elastin. Skin appendages such as hair follicles and sebaceous glands can also be seen in this cross section (E), but under normal circumstances do not regenerate in large wounds.

engineered substitutes, consisting of a material and a (usually allogeneic) cell source cultured in vitro before being applied at a wound site (Metcalfe and Ferguson, 2007; Place et al., 2009). Different strategies may be employed for other pathologies, such as chronic skin wounds. Diseases or syndromes that affect the microcirculation, such as diabetes or neuropathy, may lead to skin ulcers—areas where skin tissue has undergone necrosis and become lost. Like acute skin wounds, infection must be prevented by coverage of the wound with a suitable dressing. However, the key here is to ensure that wound healing actually occurs – often these wounds are refractory to healing due to the underlying condition (nutrition, metabolic control, persistent pressure etc), so in general the first course of action is to alleviate the root cause – for example in diabetes by tackling persistent hyperglycaemia (Cavanagh et al., 2005).

There remain very few drugs for expediting or improving wound healing. Epidermal growth factor, which stimulates the division and migration of keratinocytes in vitro, was considered a therapy with high potential after promising initial clinical results (Brown et al., 1989), but this promise failed to translate into long-term clinical success. To date, the only growth factor that has reached the clinic is platelet-derived growth factor BB (PDGF-BB), which has had approval for use in treating diabetic foot ulcer (marketed as Regranex (Wieman, 1998; Wieman et al., 1998)). This was shown to stimulate the division of dermal cells at wound sites, speeding up the formation of granulation tissue, although a warning issue has recently been issued after it was shown to elevate cancer risk (Papanas and Maltezos, 2010).

Similarly, there are no drugs or molecules that have been shown to mitigate scarring. A member of the transforming growth factor family (TGFβ3, named Avotermin) had had some initial encouraging results in phase I/II trials (Ferguson et al., 2009), but shares in the company fell significantly after this drug failed at phase III (Pharmatimes.com, 2011).

As the wound healing market is estimated to be worth around $6.7 billion worldwide (VisionGain, 2011) and the scar-reduction market is estimated to be worth greater than $4 billion in the US alone, there is great impetus to develop novel ways of improving these conditions. One promising line of investigation is by modifying the mechanical environment of the wound site.

4. Mechanical properties of skin and its importance in wound healing

Intact skin is subject to tensile stress. This can be demonstrated by the observation that when a small wound is made with a spike, the skin relaxes to form a wound of a greater diameter than the incision. This tensile stress is largely anisotropic, and circular wounds tend to elongate in the direction of the greatest stress. Karl Langer, a nineteenth-century German anatomist, catalogued the pattern of these strains over the entire human body by painstakingly pricking the skin of a cadaver and by measuring the skin's relaxation. He then used the elongation of the incisions to indicate the direction and relative magnitude of this stress (Langer, 1861). Despite the extensive citation of Langer's work, there has been surprisingly scant research to quantify the absolute magnitude of the resting stress in skin, with estimations in the available studies of resting tensions between 12 and 36 Nm^{-1} and pre-stresses of 5.4–24 kPa (de Jong 1995; Diridollou et al. 2000; Jacquet et al., 2008). In a more recent study, Flynn et al. (2011a) showed that the resting tension in the posterior part of the arm of human volunteers increased by approximately a factor of 5 during flexion (in the direction longitudinal to flexion), with forearm values ranging from 1.8 to 11.4 Nm^{-1}, and more recently the same group has estimated pre-stresses in skin to range from 28 to 92 kPa (Flynn et al., 2011b). The magnitude of tension in other regions of the skin's surface remain unexamined, but physiological values must lie below tensions lower than that permitted by the tensile strength of human skin tissue, which has been measured variously between 5 and 30 MPa in various studies (Annaidh et al., 2010; reviewed in Edwards and Marks, 1995), although some authors have suggested that pre-stresses may be as great as 1 MPa (Silver et al., 2003). In particular, when skin is stretched, for example during growth of underlying tissue during pregnancy or weight gain, the skin adapts to reduce this increase in mechanical tension by increasing *its own* mass, volume and area by a process of growth. This phenomenon of 'biological creep' is exploited by plastic surgeons, where skin can be expanded over a period of

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several weeks using a subcutaneously-implanted inflatable device (tissue expander) at one anatomical site to provide skin tissue for autografting at a remote site. (Radovan, 1982; see Johnson et al. (1993) and Marcus et al. (1990) for reviews). The increase in tension that such devices exert in the short term has been modelled by Kuhl and colleagues (Zöllner et al., 2012; Tepole et al., 2012), but there is little data to suggest the quantitative increase in tension that promotes tissue growth. A strain of 10% has been adopted as a critical threshold to promote tissue growth in Zöllner et al.'s simulations, but there is little data on what stresses or strains are necessary *in vivo* to elicit tissue growth. Clearly more work is necessary to quantify such real-life parameters to ensure repeatable surgical results.

In contrast, there has been extensive research into the measurement of the skin's elastic modulus via a multitude of *in vitro* and *in vivo* techniques (see (Edwards and Marks, 1995; Hendriks, 2001) for comprehensive reviews). This is largely due to the multiple layers and sub-layers that make up the skin as previously described; giving rise to an anisotropic, non-homogenous and non-linear viscoelastic organ. This poses experimental challenges to accurately measure the true mechanical properties of the skin. Suction and torsion techniques used to quantify the elastic modulus of the skin have been shown to vary from 0.02 MPa to 57 MPa, a factor of almost 3000 (Diridollou et al., 2000). Confounding factors such as the thickness of the skin, the surface area being tested, the type of forces applied as well as the hydration level of the sample are thought to give rise to the large differences seen (Bhushan et al., 2010; Diridollou et al., 2000; Liang and Boppart, 2010; Bader and Bowker, 1983). To address these problems Pailler-Mattei et al. (2008) developed a novel skin tribometer device to measure the elastic mechanical properties of the skin with a series of indentations to the inner aspect of the forearm. By simplifying the components of the skin to the dermis and hypodermis and underlying muscle layer (acting as a rigid substrate) Pailler-Mattei et al. were able to mathematically model the layers as three springs connected in series each with different stiffnesses and thus deducing that from their experiments the dermis has an $E=35$ kPa, hypodermis $E=2$ kPa and muscle $E=80$ kPa. These values however are not fixed but are dynamic depending on the positioning of the forearm as shown by Iivarinen et al. whereby different stiffnesses of the forearm was measured at rest, at isometric flexor and extensor loading and with venous occlusion. Their study found a resting elastic modulus of 210 kPa that changed according to the different conditions; at isometric flexor loading $E=446$ kPa (112% increase), isometric extensor loading $E=651$ kPa (210% increase) and with venous occlusion $E=254$ (21% increase) (Iivarinen et al., 2011). Not only does the skin adjust its elastic modulus by the active or passive state of the underlying muscle but is also subjected to shear forces during contact with everyday object or materials in the form of friction. An extensive review on the friction coefficient of human skin has been done elsewhere (Derler and Gerhardt, 2012). Derler and Gerhardt concluded that adhesion friction is the main friction mechanism experienced by the skin and that a minimum shear modulus of 13.3 kPa is observed when the skin sticks to a surface and is sheared. As we can see the complexity of the structure of the skin and the multitude of factors that can influence skin mechanics presents an exciting challenge for further research to characterise the skin mechanically.

The skin's tensile stress is also of great importance in wound healing. Several studies have established that skin wounds which are under mechanical tension are more prone to heal with the formation of a scar. This data has led to the prevailing practice, where surgeons aim to reduce tension at incision sites post-surgery. Simple methods for tension reduction, such as adhesive tape, have proved to be successful in improving wound healing, and more hi-tech devices are currently in pre-clinical development (Atkinson et al., 2005; Gurtner et al., 2011).

That the skin's tensile stress has such a profound effect on wound healing indicates that there must be a cellular mechanism at the wound site by which stress is sensed and transduced to a physiological response. Most evidence points to cells in the connective tissue of the wound bed – fibroblasts or their relations, the myofibroblasts – as mediators of this mechanism (see (Sarrazy et al., 2011) for a recent review). These cells act during the course of normal wound healing to actively create tension in order to draw the edges of the wound together. This is especially true in loose skinned mammals (which comprise the majority of mammalian species, such as rodents), but this also occurs in mammals where the dermal tissue is attached more firmly to the underlying fascia (such as humans or pigs). A number of *in vitro* and *in vivo* models have demonstrated that increased tension promotes the proliferation of these cells (Webb et al., 2006), inhibits their apoptosis (Aarabi et al., 2007), and activates many signalling pathways that may promote the irregular deposition of ECM. For example, Hinz et al. (2001) prevented wound contracture in a rat model of wound healing by using a plastic splint. They showed elevated smooth muscle actin expression in wound fibroblasts and myofibroblasts—presumably a cellular response directed at overcoming the splint and closing the wound. This elevated contractile response is also linked to the excessive deposition of ECM, scarring and aberrant scarring, such as hypertrophic scarring and its occasional corollary, keloid scarring. Because of this, some devices that function by mechanically offloading tension at wound sites are in preclinical development (Gurtner et al., 2011). In addition, it is thought that some of the success of vacuum-assisted devices may relate to their effect in reducing wound tension, as well as their other effects (e.g. reducing of swelling and wound exudate) (Orgill and Bayer, 2011). Future work may shed more light on this.

While the role of fibroblasts and cells of the dermal tissue in intact and wounded skin is becoming well established, there is less data on how the mechanical environment of the *epidermis* affects skin wound healing.

5. Epidermal mechanics

The epidermis of the skin is certainly a mechanosensitive tissue. As we have already seen, the skin has to expand to provide coverage for a greater volume of tissue during growth and development, or during artificial tissue expansion, and data confirms that this increase in tension is sensed by

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human epidermal tissues by an increase in mitotic activity ((Olenius et al., 1993), after Austad's experiments using Guinea pigs (Austad et al., 1982)). But it was not until the late 1990s that the molecular mechanisms of this were investigated. This was achieved using devices for cyclically stretching cells in culture to measure the effect of tensile strain on cell behaviour—a technology that had been used to investigate a range of other cell types, including endothelial cells (Letsou et al., 1990), lung cells (Liu et al., 1994) and mesangial cells of the kidney (Harris et al., 1992). This involves culturing cells on flexible (often silicone) surfaces which can be stretched either isotropically by a mechanical device, or by applying a periodic partial vacuum to the underside of the silicone substrate. Since these studies had often reported an increase in proliferation in response to stretch, and as tissue expanders clearly worked in promoting skin expansion, it was somewhat unsurprising when Takei et al. (1997) reported a large increase in DNA synthesis and cell division in human keratinocytes subjected to cyclical stretch with a maximum of 10% strain. But this in vitro system also provided a convenient method for analysing the effect of mechanical stimulation on intracellular molecular signalling pathways. In particular, this study and others found that stretching activated enzymes involved in several signalling pathways, such as mitogen-activated protein kinase (MAPK) and protein kinase C (PKC). Kippenberger et al. (2000) found that activation of MAPK could be attenuated by blocking a protein called β_1 integrin, normally responsible for adhering keratinocytes to the ECM. This provided support for the idea that physically stretched keratinocytes can sense their deformation by switching on intracellular signalling pathways. Subsequent to this study, Yano et al. confirmed the 2-3 fold increase in cell division in normal keratinocytes isolated from human subjects, and also implicated another signalling pathway—the ERK (extracellular signal-related kinase) pathway, which linked mechanosensing to cellular differentiation. Mechanically stimulated cells were shown to increase production of cytokeratin 6 and reduce that of cytokeratin 10, indicating the stimulation of division rather than differentiation (Yano et al., 2004). More recently, this group has shown that protein kinase B (Akt) is activated by stretching, which prevents apoptosis (Yano et al., 2006), while another group has demonstrated that keratinocytes that are stretched produce more matrix metalloprotease 9 (MMP-9) – an enzyme necessary for keratinocyte migration – that those that are not (Reno et al., 2009).

6. Epithelial mechanics and stem cell niches

In addition to dynamic stresses experienced by cells in the epidermis – such as cycling stretching – it is also probable that cells experience a variety of static mechanical 'microenvironments' dictated by the structure of the skin. These microenvironments are likely to be very important in determining the patterning of different cell types in the skin—i.e. specifying which cells appear where. Of particular interest is the putative stem cell 'niche'.

The skin epithelium does indeed have a physical structure which is likely to determine the mechanical environment that a cell experiences. For example, in the palmoplantar regions of human skin there is a pattern of rete ridges, which mark the boundary of the epidermis and dermis. These form a pattern of peaks and troughs where the epithelium overlays structures that project out form the dermis called dermal papillae (Fig. 2B and D). (Note here that, by convention, the peaks – the 'tips' – of rete ridges are those areas where the epithelium projects most deeply into the dermis and do not refer to the areas where the dermis comes closest to the skin surface.) These ridges are responsible for dermatoglyphs, which we rely on for generating fingerprints. But they may also contain mechanical information about where stem cells should reside. Lavker and Sun noted different morphologies and rates of cell division in the keratinocytes of the basal layer of the epidermis, depending on whether they were found in the tips or the troughs of the rete ridges, speculating that stem cells may inhabit the tips of deep rete ridges (Lavker and Sun, 1982). In non-palmoplantar skin, on the other hand, stem-like cells have been found to inhabit the diametrically opposite region—i.e. at troughs of the rete ridges where the dermis comes closest to the skin surface (Jensen et al., 1999). These studies demonstrate that cell populations may be patterned according to the topography of a tissue surface. It is of course probable that signalling by soluble molecules, such as growth factors, initially regulates this physical patterning. Threshold concentrations of signalling molecules have been known for many years to regulate the positioning and patterning of an organism's tissues. Alan Turing and other early theoreticians initially proposed such a mechanism to regulate morphogenesis in plants and animals (Turing, 1952) and it is known that this 'reaction-diffusion' mechanism is responsible for patterning, for example, the spacing of hair follicles in the skin (Sick et al., 2006) and body segmentation in insects (Kauffman et al., 1978).

But it is also possible that the mechanical characteristics of these topographically-patterned environments themselves that can inform resident cells how to behave, and some research now supports this idea. Gut epithelium consists of an undulating topographical pattern of villi, which project out into the gut lumen, and crypts, which extend down into the stromal tissue underlying the gut (Fig. 2A and C). Hannezo et al. (2011) invoked buckling instability to illustrate how such a pattern could arise spontaneously by the exertion of negative tension (caused by a dividing epithelium) on an elastic underlying tissue. This theoretical description has had been given extra biological relevance by a recent experimental study by Buske et al. (2012), which builds on earlier work by Clevers and his colleagues. The Clevers group had demonstrated that stem cells are reproducibly located in very specific regions only at the base of the crypts of the intestine (Barker et al., 2008,, 2007). Progeny of these cells migrate up the walls of the crypt to give rise to all of the many differentiated cell types that line the gut. This suggests that the base of the crypt forms a tightly regulated stem cell niche. Buske et al., by modelling an in vitro organoid system of crypt formation, have suggested that this niche is mechanically self-organised. They provide indirect evidence that the curvature of the surface to which the epithelial cells are attached

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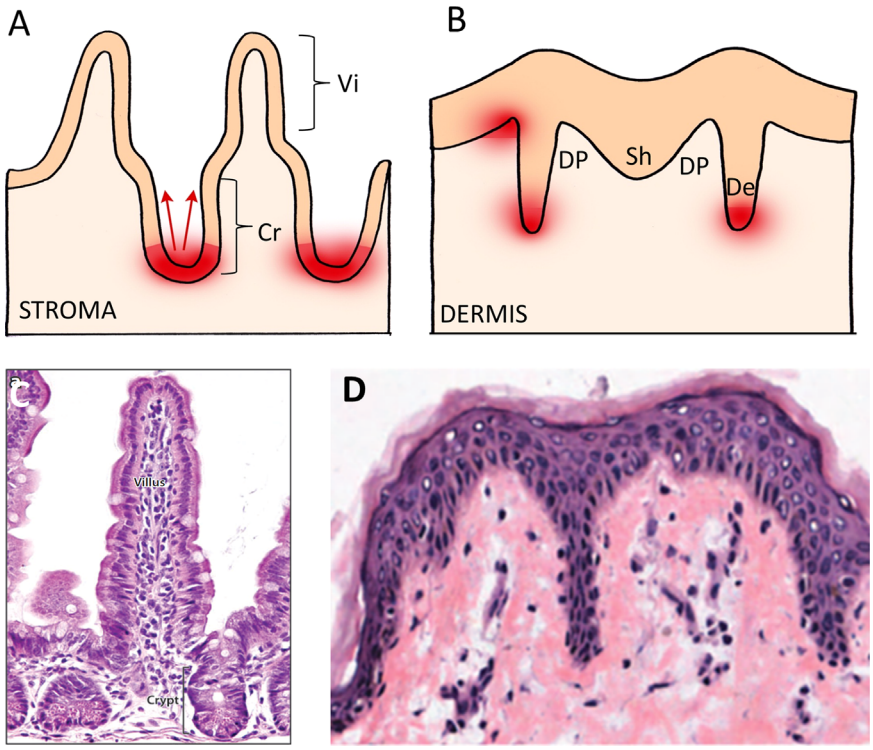

Fig. 2 – The topography of epithelia may give rise to mechanical microenvironments. The epithelium of the intestine is organised into a pattern of villi, which extend out into the lumen of the gut, and crypts, which project into the gut stroma. A population of stem cells is found at the base of the crypts of the gut, as shown in the cartoon schematic (marked in red, (A)), where positive epithelial curvature is greatest. Progeny of these cells migrate upwards out of the crypts to populate the entire gut epithelium (arrows) (Cr and Vi denote crypt and villus, respectively). The basement membrane of the skin is also topographically organised, with a pattern of peaks and troughs called rete ridges, as shown in (B). These are especially evident in the palmoplantar regions (DP denotes dermal papillae; Sh and De denote shallow and deep rete ridges, respectively). Cell populations in these areas may also be specified due to the mechanical microenvironment, which in turn is dictated by the curvature of the epithelium (marked in red). Histological H&E section of the gut and skin palm epithelium is shown in (C) and (D), respectively, which are reproduced from van der Flier and Clevers (2009) and Giangreco et al. (2010)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regulates cell differentiation, with a cell type called Paneth cells being confined to areas of high positive curvature. Since Paneth cells are suspected to nurture stem cells, it is possible that mechanically defined environments in vivo may closely regulate stem cell behaviour.

It is unknown whether such physically defined niches in the skin may affect cells in a similar way. But there is ample evidence to suggest that keratinocytes respond to the morphology of their growth environment. In pioneering experiments, Watt et al. (1988) showed that when keratinocytes were confined to small substrate islands they differentiated more and divided less than those on larger islands. As detachment from the basement membrane is an integral part of keratinocyte differentiation, it was speculated that reduced adhesion and cell spreading might be either the cause or consequence of cell differentiation in the skin. Similar work has been carried out more recently by McBeath et al. (2004) on mesenchymal stem cells. Here, confinement on small islands promoted the differentiation of adipocytes, while growth on large islands, where cells were allowed to spread, promoted the differentiation of osteoblasts. They found that this process was in some part regulated by the Rho kinase (ROCK) signalling pathway, indicating that cell spreading can be transduced to an intracellular molecular signal, which can then direct cell specification. More recently, Nelson et al. (2005) showed similar effects but in multicellular sheets rather than in single cells. By patterning cell colonies on islands of ECM, they were able to show selective cell differentiation depending upon the predicted mechanical tension of areas of the cell sheet. Again using mesenchymal stem cells, they demonstrated adipocyte specification in areas of low tensile stress and osteoblastic differentiation in areas of high tensile stress. These experiments have not yet been extended to epithelial cell differentiation, but Connelly et al. (2010) recently extended Watt's earlier work by demonstrating that cell shape, rather than island size alone, is another important factor in determining differentiation in human keratinocytes. Here, keratinocytes were cultured on ellipsoid cell islands with identical areas, but with different aspect ratios. Those with the highest aspect ratio (i.e. stretched ellipsoids) maintained stemness to a greater degree.

These results raise the interesting possibility that keratinocytes in the basal layer of the skin may be acutely influenced by tissue morphology, which ultimately translates

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into changes in mechanical properties. Topographical properties of the basement membrane and the underlying dermis on length scales greater than that of an individual cell may create mechanical gradients that specify where certain cells should reside, as has been implicated in the gut epithelium. Cell spreading in keratinocytes cultured in two dimensions promotes 'stemness', but it is unknown how basal spreading combined with apical constriction (or the opposite), which may occur in epithelial cells on a curved surface, may affect cell behaviour in the skin. Experiments to determine how epithelial sheets, rather than individual cells, respond to substrate topology and ECM mechanics will help us to answer these questions.

7. Epithelial mechanics in wound healing

Since keratinocytes are mechanosensitive cells that are intrinsically involved with the process of wound healing, one may ask an obvious question: what is the mechanical environment of the healing wound and how does this affect and/or signal to the keratinocyte and epidermal cells which must act to close the wound?

As discussed earlier, wound healing involves the migration of keratinocytes from the wound periphery towards the wound centre, combined with an increase in the division of epidermal cells or keratinocytes in proximity to the wound. Both of these activities are dependent on the ability of keratinocytes to interact and adhere to the ECM at the surface of the wound. The importance of this is demonstrated by mice that lack some of the proteins that are responsible for cell-matrix attachment—deficiency in members of the integrin family in experimental mice, such as integrin β_1 (Grose et al., 2002), leads to severe defects in wound healing. And as we have already seen, these ECM receptors are transducers of mechanical force. The mechanism by which keratinocyte migration occurs, and the signals that regulate it, are still a contentious issue, however. Some models suggest that keratinocytes at the wound edge progressively fall over each other onto the wound surface with minimal cell migration (Krawczyk, 1971)—a process that has been referred to as 'leap-frogging' (Lambert et al., 1984). Others suggest that keratinocytes actively crawl out over the wound bed by either the front few cells – 'leader cells' – dragging those behind them (Gov, 2007; Omelchenko et al., 2003), or by rows of cells collectively migrating towards the wound centre at rates that diminish as a function of distance from the wound (Farooqui and Fenteany, 2005; Matsubayashi et al., 2011). This latter idea has been examined in more detail by groups led by Fredberg and Trepat. These authors have suggested that epithelial cells – which are characterised by strong cell–cell adhesions – act together at very large distance from the wound edge to produce a net movement of cells towards the centre of the wound, even though individual cell movements may be heterogeneous (Tambe et al., 2011; Trepat, 2009). They use the evocative analogy of a 'mosh' at a rock concert to describe this phenomenon, with some cells pulling, some cells pushing, but with ultimately a net movement of cells into a space (Trepat and Fredberg, 2011). In other experiments, for example on embryonic tissue or certain epithelial sheets *in vitro*, a model of wound healing known as 'purse-string' healing has been put forward (Martin and Lewis, 1992). In these studies, a circular actin cable becomes established around the periphery of the wound after mechanical wounding, which gradually shortens, leading to wound closure—hence the 'purse string' analogy.

Regardless of the mechanisms of wound healing, the molecular signalling processes that govern keratinocyte migration and wound closure have been investigated extensively. Growth factors such as fibroblast growth factors (including keratinocyte growth factor) and epidermal growth factors exert strong chemotactic and mitogenic effects at wound sites to ensure rapid tissue growth and wound closure (a discussion of this is beyond the scope of this review, but please see a comprehensive review by Santoro and Gaudino (2005) for further information). But it is also likely that wound mechanics may play an important if often overlooked role in this process.

During wound healing, keratinocytes are likely to experience a change in their mechanical environment, most likely experiencing tensile forces. In all of these models, there is a net movement of cells towards the wound—this movement is likely to be translated to a 'tug' on cells behind them. As we have already seen, tensile forces act to promote keratinocyte proliferation, so one would expect this force to be translated into an increase in cell division, which then would act to produce tissue to replace that lost following the wounding event. In addition, contraction of the underlying granulation tissue and dermis would also be translated to a strain in the overlying epidermis, exerting a similar effect. Direct evidence for a role for epithelial mechanotransduction in wound healing is skanty, however. Whereas, the studies on tissue expanders and similar work by Pietramaggiori et al. (2007) has demonstrated that applied force stimulates cell division and skin remodelling, there is a paucity of studies that directly measure the tensile strains at wound sites and their physiological corollary. To our knowledge, it remains unknown what strains are experienced by epidermal cells at wound sites and in the vicinity of the wound. A method to measure or approximate the strains that epithelial and dermal cells experience at wounds would inform our understanding of mechanical regulation of the wounded environment.

So far, we have only discussed how applied force – such as tensile strain – affects cells and tissues. But there is a large body of evidence to suggest that cells do not just passively respond to force—they actively 'feel' their environment by exerting force on it themselves and by responding accordingly. Their ability to do this is related to the intrinsic material properties of the cells and ECM with which they interact. One such property of the substrate that cells respond to is tissue stiffness, related to the elastic modulus (E) of the material(s) of which the tissue is composed.

8. Substrate stiffness and wound healing

The stiffness of the ECM can have profound effects on the behaviour of the cells that interact with it. Pelham and Wang were the first to directly test this idea by culturing fibroblast cells on the surface of polyacrylamide gels functionalised by covalent binding of collagen type I (Pelham and Wang, 1997).

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They realised that the elastic modulus of polyacrylamide could be varied simply by changing the ratios and concentrations of the monomer and crosslinker used to form these hydrogels. (Note that stiffness and elastic modulus are often used interchangeably, but that they are not directly equivalent. Elastic modulus refers to the intrinsic property of a material and is scale-independent, whereas the stiffness of a material may depend on its dimensions). In doing so, they were able to show that cells on stiffer substrates spread out to a greater degree and formed more focal adhesions than those on soft substrates. Subsequently, a number of groups showed related effects on other cells, such as endothelial cells (Deroanne et al., 2001) and muscle cells (Engler et al., 2004). Interest in the biological effects of ECM stiffness really gathered momentum with the work of Dennis Discher's group. Their 2006 paper in *Cell* showed that the stiffness of the growth substrate alone could direct bone marrow stromal stem cells (alternatively referred to as mesenchymal stem cells, MSCs) to differentiate into cells as diverse as neurons, muscle and bone (Engler et al., 2006). It is now widely appreciated that ECM stiffness can affect a wide variety of functions of many different cell types (please refer to recent reviews (Discher et al., 2009; Eyckmans et al., 2011; Janmey and Miller, 2011) for a detailed description of this field). For example, increasing stiffness has been show to promote cell division (Peyton et al., 2006; Yeung et al., 2005), stimulate tumour growth and fibrosis (Georges and Janmey, 2005; Li et al., 2007) and direct the differentiation of other stem cells, like embryonic stem cells (Evans et al., 2009) (see Fig. 3). But more recently, some of the underpinning assumptions of this large body of work have been called into question. Trappmann et al. (2012) provided evidence to argue that it is the concomitant decrease in ligand density that occurs in softer gels composed of less dense networks of polymers chains rather than the actual stiffness that is responsible for many of the effects. By artificially stiffening soft gels, they were able to show that there was no difference in cell behaviour on a soft gel that had been stiffened, compared to an untreated soft substrate. However it is difficult to reconcile these results with the data on the effect of substrate depth on cell behaviour – other groups have shown that cells on soft gels attached to glass begin to sense the stiffness of the underlying glass when the substrate depth reaches a certain minimum value (Lin et al., 2010). This suggest that such cells are unable to generate a strain field in the gel beneath them, the gel becomes maximally extended, and the cell 'feels' a true stiffness equal to the modulus of the glass, and *not* the gel. If ligand density could explain the effects previously related to substrate stiffness, one would expect no substrate depth-dependent change in cell behaviour. More research is required to clarify these issues.

The field continues to generate interest, and currently many groups are addressing the challenge of how cells and tissues respond to substrate stiffness in three-dimensional tissues (Lutolf et al., 2009; Wozniak and Keely, 2005; Zaman et al., 2007). In spite of these advances, the effect of material stiffness on epithelial cells and on skin epithelial cells, such as keratinocytes, has only recently begun to be explored. This is particularly surprising since the skin epithelium consists of polarised layer of cells in close contact to an elastic substrate (the basement membrane and dermis)—a tissue especially suited to this type of investigation. Wang et al. (2012) recently showed that HaCaT keratinocytes proliferate to a greater degree on stiff surfaces (in this instance made of polydimethoxysilane [PDMS]). They also found that when a confluent layer of these cells, growing on a polymer surface, was 'wounded' by mechanically scratching away a small channel, the rate of cell migration into the space was more rapid on the surface of harder PDMS surfaces than on soft surfaces. This study indicates that a stiffer wound bed may be more favourable to rapid wound healing. These results are supported by work from Anon et al. (2012), who studied wound closure in epithelial Madin–Darby canine kidney (MDCK) cells. In this study, cell sheets were patterned around a series of PDMS pillars to create islands of various dimensions and geometries (circular, ellipsoid and polygonal). By also using mechanically-tuned PDMS substrates, they showed that on removal of these pillar stencils, cells migrated to close the gaps more rapidly on stiff as opposed to soft substrates, with a complete absence of wound closure on the softest substrates (estimated at ~20 kPa). Since Goffin et al. (2006) found that wound stiffness increases from 18 to 40 kPa during wound healing, this may indicate that an increase in the fibrosity of the wound bed with a concomitant increase in stiffness may act as a mechanism to ensure wound coverage. Technologies or drugs that can control the mechanical properties of the wound materials that cells interact with may be a promising avenue for future exploration.

9. Conclusions and future directions

Skin homeostasis and wound healing is intrinsically linked to the mechanical properties of the epithelium of the skin. Changes in properties such as epithelial tension, topography and stiffness may all combine to regulate how the skin behaves in both physiological and pathological circumstances (see Fig. 4). As we have seen, changes in tensile stress within the skin epithelium can regulate cell division and the formation of new tissue, and it is likely – but has not yet been shown – that similar processes may operate during the disruption of the skin's mechanical environment that occurs during wound healing. It is not yet known how the topography of the basement membrane of the skin (on length scales greater than the cell) may affect cells that inhabit the epithelium, although there is evidence to suggest that stem cells are patterned in different topographical microenvironments both in the skin and in other epithelia, such as the gut epithelium. As the skin consists of a multi-layered epithelium, the contribution of the cells in terminal layers of the skin to the mechanical environment of basal cells will need to be taken into account to clarify this question. Finally, the mechanical properties of the underlying matrix – the dermis in intact skin and the dynamic granulation tissue of the healing wound – may play important roles in skin healing and homeostasis.

Resolving these questions will depend on fundamental studies on how collective groups or sheets of cohesive epithelial cells respond to the mechanical properties of their growth environment. Recent work has shown that

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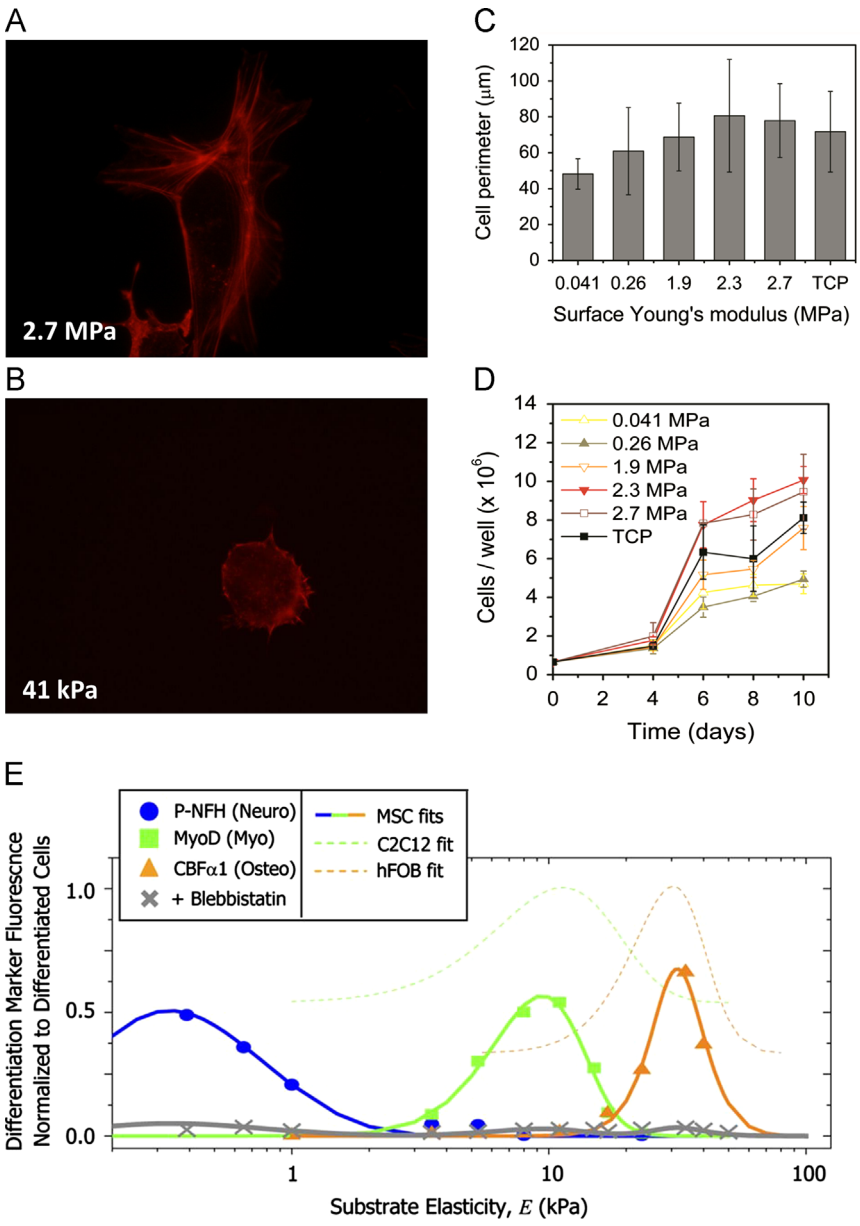

Fig. 3 – Substrate stiffness affects the morphology, division and differentiation of stem cells. Cells, such as differentiating embryonic stem cells, spread to a greater degree and form prominent intracellular actin stress fibres (marked in red, by fluorophore-linked phalloidin) on stiff substrates (elastic modulus=2.7 MPa; (A) compared to soft subtrates (41 kPa; (B). This phenomenon can be quantified by measurements of, for example, cell perimeter (C). In addition, cells cultured on stiff substrates proliferate more quickly than those on softer substrates (D). Cell differentiation is also affected by substrate stiffness; for example, mesenchymal stem cells (MSCs) cultured on polyacrylamide substrates have been shown to differentiate into cells as diverse as neurons or osteoblasts, purely as a function of ECM stiffness (E). ((C) and (D) are reproduced from Evans et al. (2009) and (E) is from Engler et al. (2006)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cohesive groups of cells show complex mechanical behaviours that are difficult to predict by studying individual cells. For example, Trepat's group has shown that epithelial movements are analogous to the behaviour of closely packed particulate systems (Angelini et al., 2011), while Gjorevski and Nelson (2012) showed that the shape and size of the epithelium, as well as the thickness of the substrate, can have profound effects on the mechanical behaviour of the epithelium as a whole. Similar, controlled experiments where the stiffness, topography and dimensions of the ECM to which cells attach, combined with techniques to create artificial wounds of defined sizes (Nikolic et al., 2006;

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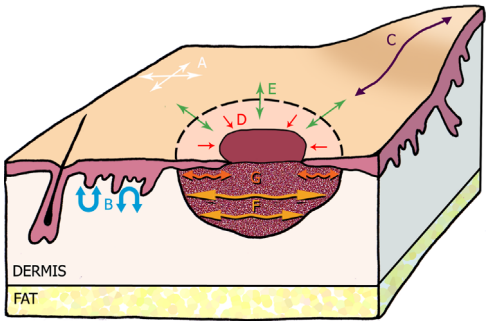

Fig. 4 – A variety of mechanical microenvironments exist in the skin epidermis during homeostasis and growth, and following injury. Under normal homeostasis, the epidermis of the skin is under tensile stress (denoted by white arrows in the diagram; (A), as Karl Langer's early experiments showed. The skin epidermis (pink) is attached to the basement membrane, which in turn contacts the underlying dermis. Throughout the skin, but especially in the palmoplantar regions, the basement membrane forms a pattern of undulating peaks and troughs, called rete ridges. Cells contacting the basement membrane may experience tensile or compressive stresses due to the curvature of this membrane, which may influence cell differentiation (blue arrows, (B). During tissue growth or following implantation of subcutaneous tissue expanders, the epidermis may experience tensile stress, which causes and increase in cell proliferation (purple arrow; (C). Following wounding, epidermal cells migrate over the surface of the wound (red arrows; (D) which may result in tensile stress around the wound site (green arrows; (E)). Myofibroblasts in the granulation tissue of the wound bed exert contractile forces to help close the wound (F) which may also affect epidermal cells at the wound surface. The stiffness of the granulation tissue or the intact dermis may affect the ability of epidermal cells to exert tension and close the wound, and may also direct cellular differentiation (G). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Poujade et al., 2007), will be key to finding out how these processes operate in the skin. Exciting times lie ahead in skin mechanobiology.

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