

Keratinocyte–Fibroblast Interactions in Wound Healing

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Cutaneous tissue repair aims at restoring the barrier function of the skin. To achieve this, defects need to be replaced by granulation tissue to form new connective tissue, and epithelial wound closure is required to restore the physical barrier. Different wound-healing phases are recognized, starting with an inflammation-dominated early phase giving way to granulation tissue build-up and scar remodeling after epithelial wound closure has been achieved. In the granulation tissue, mesenchymal cells are maximally activated, cells proliferate, and synthesize huge amounts of extracellular matrix. Epithelial cells also proliferate and migrate over the provisional matrix of the underlying granulation tissue, eventually closing the defect. This review focuses on the role of keratinocyte–fibroblast interactions in the wound-healing process. There is ample evidence that keratinocytes stimulate fibroblasts to synthesize growth factors, which in turn will stimulate keratinocyte proliferation in a double paracrine manner. Moreover, fibroblasts can acquire a myofibroblast phenotype under the control of keratinocytes. This depends on a finely tuned balance between a proinflammatory or a transforming growth factor (TGF)- β -dominated environment. As the phenotype of fibroblasts from different tissues or body sites becomes better defined, we may understand their individual contribution in wound healing in more detail and possibly explain different clinical outcomes.

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Epithelial–mesenchymal interactions in tissue repair

After cutaneous injury a cascade of events is observed, which mediates tissue repair and eventually the re-establishment of the barrier function of the skin. Tissue repair has been divided into an inflammatory phase, a granulation phase with synthesis of new connective tissue and epithelial wound closure, and finally a scar-remodeling phase once the epidermal barrier has been re-established.

Throughout the whole repair process interaction between different cell types provides coordination of the individual events, allowing for a temporal and spatial control. In the mid- and late phase of wound healing, cellular interactions become dominated by the interplay of keratinocytes with fibroblasts, which gradually shift the microenvironment away from an inflammatory to a synthesis-driven granulation tissue. Epithelial–mesenchymal interactions are also crucial for pre- and postnatal skin development, and they play an important role in tumor biology. During development, tightly controlled mutual interactions between the epidermis and the mesenchyme control the formation of limbs, skin architecture, and appendages through gradients of morphogens and differential sensitivity of target cells for these diffusible mediators. Thus, highly complex, cell-type-specific re-

sponses can be achieved. After a series of these interactions, limb or skin appendages eventually form (reviewed by Schmidt-Ullrich and Paus, 2005; Tickle, 2006). There is strong evidence that these patterns of epithelial–mesenchymal interactions persist in adult life and regulate skin homeostasis, part of the wound-healing response (Martin and Parkhurst, 2004), or become critically involved in tumor cell invasion and tumor progression (Bhowmick *et al.*, 2004; Mueller and Fusenig, 2004).

The epidermal phenotype

Keratinocytes in skin represent a constantly renewing cellular compartment, and stem cells have raised continuous interest from a scientific as well as therapeutic aspect. Epidermal stem cells have been characterized largely on their functional properties and

Editor's Note

The following 3 articles by Werner *et al.*, Stramer *et al.*, and Clark *et al.* continue the Wound Healing Perspectives Series which began in the March 2007 issue of the journal.

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Abbreviations: α -SMA, α -smooth muscle action; EGF, epidermal growth factor; KGF/FGF7, keratinocyte growth factor/fibroblast growth factor 7; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor

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marker expression (Barrandon and Green, 1987; Cotsarelis *et al.*, 1990; Oshima *et al.*, 2001; Tumber *et al.*, 2004; Jensen and Watt, 2006), nevertheless their exact nature still remains somewhat elusive. The role of epithelial–mesenchymal interactions for the keratinocyte stem cell phenotype was demonstrated early (Rheinwald and Green, 1975). The feeder cell–culture system demonstrated clearly that the epidermal stem cell phenotype depends on the interaction with mesenchymal cells. Primary keratinocytes are seeded at clonal density onto growth-inhibited fibroblasts, which serve as feeder cells. Within 7–10 days, the cultures become confluent and keratinocytes can be passaged with high efficiency. There appears to be flexibility concerning the source of the fibroblasts, as murine fibroblasts can be substituted by human skin fibroblasts (Limat *et al.*, 1990). Apparently, the mesenchymal feeder cells provide a microenvironment, which supports the stem cell phenotype and augments epidermal proliferation. Growth factors play a critical role and are added to the culture medium to support epidermal proliferation. However, even complex growth factor mixtures can hardly replace the net effect of mesenchymal feeder cells when keratinocytes are seeded at clonal densities. It became apparent that keratinocytes in these cultures instruct fibroblasts to synthesize and secrete growth factors and cytokines, such as keratinocyte growth factor (KGF)/fibroblast growth factor-7 (FGF7), IL-6, and GM-CSF (Waelti *et al.*, 1992; Smola *et al.*, 1993; Boxman *et al.*, 1996). Expression of these factors can be induced by IL-1, and indeed, keratinocyte-derived IL-1 was identified as primary inducer (Waelti *et al.*, 1992; Maas-Szabowski and Fusenig, 1996; Maas-Szabowski *et al.*, 1999). Szabowski *et al.* (2000) had elegantly shown that in fibroblasts, the transcription factor activator protein-1 plays a central role in this paracrine growth factor loop. Activator protein-1-dependent KGF/FGF7, GM-CSF, pleiotrophin, and stromal cell-derived factor 1 expression regulate keratinocyte proliferation and differentiation in keratinocyte–fibroblast cocultures (Florin

et al., 2005). The concept of double paracrine signaling was proposed where keratinocytes initiate growth factors in fibroblasts, which themselves stimulate keratinocyte proliferation.

Apart from paracrine growth factor regulation, the formation of a new basement membrane zone is another example where interaction between keratinocytes and fibroblasts are crucially involved. Basement membrane constituents and a fully organized basement membrane can influence the keratinocyte phenotype (El Ghalbzouri *et al.*, 2004). Recombination experiments have detailed the expression pattern and cellular origin of basement membrane and extracellular matrix constituents in epidermal–dermal cultures (Regauer *et al.*, 1990; Fleischmajer *et al.*, 1997; Smola *et al.*, 1998). Laminin-5 is of epithelial origin, collagen VII primarily produced by keratinocytes and to a lesser extent by fibroblasts (Marinkovich *et al.*, 1993), whereas nidogen is exclusively derived from fibroblasts. The other basement membrane components are produced in both cell types with mutual induction patterns. Apart from matrix-derived signals, which help to maintain the epidermal stem cell phenotype, proteolytic processing can generate laminin-5 fragments, which can stimulate epidermal growth factor (EGF) receptor signaling and proliferation (Schenk *et al.*, 2003). Extracellular matrix also modulates cellular functions directly and this has been extensively studied in fibroblasts (Eckes *et al.*, 2006). This involves binding of matrix molecules to adhesion receptors, and mechanical tension plays a critical role in regulating the *de novo* synthesis of collagens. Interestingly, fibroblasts in collagen gels and also in the granulation tissue form gap junctions, which are also involved in the regulation of extracellular matrix production (Ehrlich and Diez, 2003; Ehrlich *et al.*, 2006). In a polyvinyl alcohol sponge implant model, a gap junction uncoupler reduced the number of cells migrating into the sponges, the myofibroblast density and collagen deposition and organization (Ehrlich and Diez, 2003). Thus, epithelial–mesenchymal cocultures provide an environ-

ment where the epidermal stem cell phenotype and keratinocyte proliferation are supported by a synergistic mix of growth factors, extracellular matrix components, and direct cell–cell contacts. This concept has been excellently reviewed by Nelson and Bissell (2006).

Growth factor-mediated cross talk between keratinocytes and fibroblasts

Growth factor networks have been identified in the above-described tissue culture systems. In the meantime, the existence of at least some of these networks has been verified *in vivo* in normal and wounded skin. In particular, studies with genetically modified animals have advanced our understanding in this field, and the wound-healing phenotypes of different transgenic and knockout mice has been reviewed previously (Grose and Werner, 2004). Furthermore, a website has been set up, which summarizes these phenotypes (<http://icbxw.ethz.ch/woundtransgenic/home.html>).

One of the first growth factors, which was shown to be involved in mesenchymal–epithelial interactions, is KGF/FGF7, which is rapidly induced in fibroblasts after wounding and which exerts its effects through binding to its receptor FGFR2IIIb on keratinocytes. Some growth factors are predominantly expressed in epidermal cells and exert their actions on mesenchymal cells, such as platelet-derived growth factor (PDGF). For other growth factors, expression was detected in both keratinocytes and the mesenchymal cell compartment and the effects were observed in both cell compartments in an autocrine and paracrine fashion. Activins are typical examples (for review, see Werner and Grose, 2003).

Paracrine acting mesenchymally derived growth factors

As mentioned above, KGF/FGF7 is the prototype of a mesenchymally derived growth factor, which stimulates epidermal proliferation (Finch *et al.*, 1989; Rubin *et al.*, 1989). On injury, KGF/FGF7 expression is strongly upregulated in mesenchymal cells (Werner *et al.*, 1992; Marchese *et al.*, 1995). IL-1, tumor necrosis factor- α (TNF- α),

PDGF, and serum were shown to stimulate KGF expression, and all these inducers are present at the early stages of wound healing (Brauchle *et al.*, 1994; Chedid *et al.*, 1994). Expression of a dominant-negative FGFR2IIIb mutant in basal keratinocytes of transgenic mice caused epidermal thinning, hair follicle abnormalities, dermal fibrosis, and a severe delay in wound re-epithelialization (Werner *et al.*, 1994). However, the dominant-negative mutant blocks signaling by all FGF receptors in response to common ligands, and the type of FGF and FGFR, which is involved in wound healing, remained therefore unclear. Subsequent analysis of mice deficient for FGF7/KGF showed that healing of incisional wounds is not impaired (Guo *et al.*, 1996), suggesting that other factors like fibroblast-derived FGF10, which also signals through the FGFR2IIIb receptor, may compensate in this experimental model, which requires little epidermal healing. FGF10 is also expressed in fibroblasts, but unlike FGF7/KGF, FGF10 expression is not induced during wound healing. Transforming growth factor- β (TGF- β) and TNF- α repress FGF10 expression, suggesting a differential regulation of FGF10 and FGF7 (Beer *et al.*, 1997a). Recently, another FGF family member, FGF22, has been identified, which also can activate FGFR2IIIb (Zhang *et al.*, 2006). Interestingly, the predominant cell type expressing FGF22 are keratinocytes (Nakatake *et al.*, 2001; Beyer *et al.*, 2003). Therefore, FGF22 is considered to act in an autocrine fashion, and high expression of this FGF was seen in the late stages of the wound-healing response. The activity of these FGFR2IIIb ligands may be enhanced by the FGF-binding protein. FGF-binding protein is synthesized in the epidermis, and it is upregulated during wound healing. It binds FGF7, -10, -22 and enhances activity of ligands at low concentrations (Beer *et al.*, 2005).

IL-6 has been shown to stimulate epidermal cell proliferation (Grossman *et al.*, 1989), and its expression is strongly upregulated in keratinocyte-fibroblast cocultures in comparison to monocultures. In mice deficient for IL-6, excisional wound healing was se-

verely retarded. This is very well in line with its presumed role in mesenchymal-epidermal cell-cell communication (Gallucci *et al.*, 2000; Lin *et al.*, 2003). In addition, fewer leukocytes were observed in the wounds of IL-6-deficient mice as well as decreased angiogenesis and collagen accumulation. The phenotype could be mimicked by injecting a neutralizing mAb into wild-type mice (Lin *et al.*, 2003) and rescued by injection of a single dose of IL-6 into IL-6-deficient mice (Gallucci *et al.*, 2000).

In addition to KGF/FGF7 and IL-6, other factors, such as hepatocyte growth factor and GM-CSF are primarily produced by mesenchymal cells and act on keratinocytes following the same paracrine pattern (Smola *et al.*, 1993; Maas-Szabowski and Fusenig, 1996; Szabowski *et al.*, 2000; Gron *et al.*, 2002).

Keratinocyte-derived growth factors

KGF/FGF7 and IL-6 are predominantly expressed in the mesenchyme and target keratinocytes. There are also examples where keratinocytes are the primary source of growth factors, which may stimulate not only keratinocytes themselves but also fibroblasts or other mesenchymal cell types.

TGF- α is predominantly expressed in keratinocytes and has a profound autocrine effect on keratinocytes in wound healing. Expression of this growth factor is upregulated in keratinocytes after skin injury (Antoniades *et al.*, 1993). EGF as well as TGF- α activity were detected in wound fluid from rats (Grotendorst *et al.*, 1989) and humans (Ono *et al.*, 1995). Furthermore, keratinocytes bordering the epithelial defect as well as epithelial cells in hair follicles were found to express high levels of TGF- α , particularly in the strongly proliferating cells. High TGF- α expression is also a hallmark of psoriatic epidermis and appears to be associated with keratinocyte hyperproliferation (Gottlieb *et al.*, 1988; Elder *et al.*, 1989). Surprisingly, wound-healing experiments in TGF- α -deficient mice showed no abnormal wound-healing phenotype (Luetteke *et al.*, 1993; Mann *et al.*, 1993) and only on closer examination and in wound-

healing models where epithelial migration is critical for healing small differences were observed (Kim *et al.*, 2001). Less clear is the role of TGF- α on mesenchymal cells in wound healing. It is a strong mitogen for fibroblasts (Rosenthal *et al.*, 1986) and augments angiogenesis (Schreiber *et al.*, 1986). However, the lack of an obvious mesenchymal phenotype in TGF- α -deficient mice suggests that this growth factor is dispensable for fibroblast proliferation and/or angiogenesis and that its loss can be compensated by other EGFR ligands. Heparin-binding EGF might be a possible candidate, since it was also detected in wound fluid of partial thickness wounds and of pediatric burn patients (Marikovsky *et al.*, 1993) as well as in migrating keratinocytes in a mouse burn wound model (Cribbs *et al.*, 2002). Heparin-binding EGF is mitogenic for both keratinocytes (Cribbs *et al.*, 2002) and fibroblasts and can synergize with IGF I (Marikovsky *et al.*, 1993). Furthermore, keratinocyte-specific inactivation of the *hb-egf* gene resulted in delayed epithelial wound closure in mice, suggesting that heparin-binding EGF is involved in epithelialization by accelerating keratinocyte migration, rather than proliferation (Shirakata *et al.*, 2005).

PDGF is another predominantly keratinocyte-derived growth factor acting in a paracrine manner. This growth factor is immediately released after wounding by degranulating platelets. Without the time-consuming *de novo* synthesis at the wound site, PDGF activity is readily available within minutes after injury. Later during tissue repair, PDGF is synthesized in the wound tissue. PDGF transcripts and protein were detected in the epithelium (Antoniades *et al.*, 1991; Ansel *et al.*, 1993; Beer *et al.*, 1997a,b), whereas transcripts in mesenchymal cells were described by Antoniades *et al.* (1991) also. The corresponding receptors were detected in mesenchymal cells (Ansel *et al.*, 1993) and in one report on keratinocytes as well (Antoniades *et al.*, 1991). Fibroblast- and keratinocyte-derived PDGF-AA has little effect on these cells, as the predominant PDGF receptor- β is little responsive to the PDGF-AA isoform (Bonner *et al.*, 1991)

Table 1. Differential mRNA expression pattern of fibroblasts cocultured with HaCaT keratinocytes for 4 days

Genes induced in cocultured fibroblasts			
Proteases and inhibitors	Extracellular matrix components	Cell – Cell signaling associated	Cytoskeleton associated
PAI-1	Collagen V alpha 3	ENA 78	SM22 alpha
	Collagen IV alpha 1	MCP1	Integrin alpha 1
	Hyaluronan synthetase 2	Activin A	Tropomyosin 2 beta
	Lysine hydroxylase 2	LTBP 1	Tropomyosin 1 alpha
	Transglutaminase 2	Interleukin 6	VASP
	Collagen V alpha 1	CTGF	Calponin 1 (basic)
	Collagen I alpha 1	HB-EGF	Smooth muscle actin alpha2
	Tenascin C (GP)	Endothelin 1	n-chimaerin
	Decorin (PG)	Angiopoetin 1	MLCK
	Collagen VI alpha 3	WNT 5A	Integrin alpha 5
	Syndecan 2 (PG)	LIF	
		COX 2	
		G-CSF	
	WNT 2		
	Dickkopf 3		
Genes downregulated in cocultured fibroblasts			
Proteases and inhibitors	Extracellular matrix components	Cell – Cell signaling associated	Cytoskeleton associated
Cathepsin K	Fibulin 4	TGF beta receptor III	Integrin alpha 4
Cathepsin L	Testican	Prostaglandin F Receptor	
Cathepsin L2	Tenascin XA	Follistatin	
	Fibulin 1	gp130 Receptor	
	Glypican 1	Osteoprotegerin Receptor	
	Thrombospondin 2	Toll Like Receptor 4	
		EGF Receptor	
	HGF Receptor		
	MCSF		
	Stromal cell-derived factor		

The expression pattern has been clustered according to their potential function (Figure 1). Adapted from Shephard *et al.* (2004b).

also. On the other hand, different cell types in healing skin wounds express the PDGF-BB isoform for which the fibroblasts are responsive and which is able to induce KGF in mesenchymal cells (Brauchle *et al.*, 1994; Beer *et al.*, 1997a, 1997b). The expression pattern suggests a paracrine signaling from the epidermis to mesenchymal cells as well as autocrine signaling in the granulation tissue. Moreover, neutralizing PDGF antibodies reduced the mitogenic activity of acute wound fluid by 45% when tested on fibroblasts (Katz *et al.*, 1991), indirectly supporting this assumption.

Growth factors expressed in keratinocytes and mesenchymal cells

Activins are members of the TGF- β family of growth and differentiation factors. They are homo- or heterodimeric polypeptides consisting of β_A , β_B , β_C , β_D , and β_E chains. The β_A and β_B chains are expressed in keratinocytes as well as in the mesenchyme on wounding (Hubner *et al.*, 1996b). The β_C and β_E chains are predominantly found in other tissues (Vejda *et al.*, 2002) and the β_D subunit is present only in *Xenopus laevis* but not in mammals. Epidermal overexpression of the activin β_A under the control of a keratin 14

promoter caused a dermal phenotype mostly characterized by a loss of adipose tissue, which was replaced by a fibrosing dermis, and a hyperproliferating epidermis (Munz *et al.*, 1999). The epidermal phenotype was unexpected, as activin was shown to be growth inhibitory for keratinocytes *in vitro* (Seishima *et al.*, 1999). Therefore, it seems likely that the phenotype is at least in part mediated via mesenchymal cells. Of particular interest was the enhanced wound healing in activin-overexpressing mice. Granulation tissue formation was particularly affected, but faster re-epithelialization was also

observed. As a consequence, however, the mice also showed an extended scar area (Munz *et al.*, 1999; S.W. unpublished data). To study the role of endogenous activin in wound repair, mice overexpressing the soluble activin antagonist follistatin in the epidermis were generated (Wankell *et al.*, 2001). These mice showed a severe delay in wound healing, and granulation tissue formation was particularly affected. However, the wounds finally healed, and the resulting scar was smaller than in control animals. These findings demonstrated that the levels of bioactive activin in a wound determine the speed of wound repair but also the scarring response. To determine if activin affects wound healing via keratinocytes or mesenchymal cells or both, transgenic mice expressing a dominant-negative activin receptor mutant in keratinocytes were generated. Their skin architecture was almost unchanged compared with control mice. Wound re-epithelialization was slightly delayed owing to reduced keratinocyte migration. However, the strong inhibition of granulation tissue formation seen in the follistatin-overexpressing mice was not observed in the transgenic mice expressing the dominant-negative activin receptor mutant in keratinocytes, demonstrating that activin exerts its effect on wound healing predominantly via stromal cells (Bamberger *et al.*, 2005).

The fibroblast phenotype in epidermal-mesenchymal interactions

Most of the studies described above analyzed the phenotype of the epidermis, as delayed wound closure is often due to reduced keratinocyte migration or proliferation. By contrast, much less is known about the changes that mesenchymal cells undergo in healing skin wounds. From wound-healing studies, it has long been known that myofibroblasts start to appear in the granulation tissue around the mid-phase of wound healing. This coincides with a strong induction of contractile properties so that cells align parallel to mechanical tension that is building up in the granulation tissue. Myofibroblasts appear to differentiate from fibroblasts by acquiring the smooth

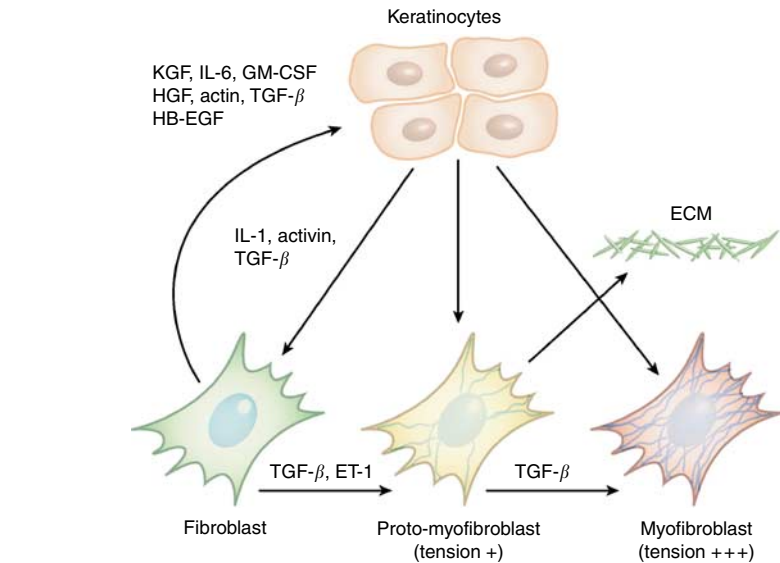


Figure 1. Proposed model for the modulation of fibroblast differentiation in keratinocyte-fibroblast cocultures. At early stages after injury proinflammatory mediators, such as keratinocyte-derived IL-1, dominate keratinocyte-fibroblast interactions. At this time, TGF- β activity is strongly upregulated in keratinocyte-fibroblast cocultures but NF κ B activation in fibroblasts blocks TGF- β signaling. IL-1-responsive genes, such as the genes for KGF, IL-6, endothelin-1 (ET-1), heparin-binding EGF and GM-CSF, are upregulated in fibroblasts. These factors can then stimulate keratinocyte proliferation and differentiation. Basement membrane constituents are expressed by both keratinocytes, and to a lesser extent, by fibroblasts. ET-1, besides, TGF- β initiates early contractile activity and mechanical tension starts to build up. Fibroblasts are negative for α -SMA expression at this stage. In the mid-phase of keratinocyte-fibroblast interactions, the balance of keratinocyte-derived IL-1 and TGF- β effects starts to shift. NF κ B activation in fibroblasts is decreasing and TGF- β -dependent gene expression is becoming upregulated. Fibroblasts start to increase their synthesis of basement membrane constituents. At this stage, fibroblasts are still largely negative for α -SMA expression, but mechanical tension is building up in a second peak. They may be best described as proto-myofibroblasts. In mature keratinocyte-fibroblast interactions, TGF- β effects fully govern the fibroblast phenotype. ET-1 and TGF- β synergize in the induction of α -SMA, and the majority of fibroblasts has acquired α -SMA expression and differentiated into myofibroblasts. Mechanical tension is highly developed in fibroblasts in close vicinity to keratinocytes. Keratinocyte-derived IL-1 is still present, but the remaining NF κ B activation in fibroblasts appears to block TGF- β signaling only mildly. Basement membrane constituents are strongly expressed in fibroblasts and contribute to the formation of an organized basement membrane zone.

muscle cell actin isoform α -smooth muscle action (α -SMA). Several studies investigated the mechanisms on how fibroblasts become myofibroblasts, and it seems that the two most important stimuli are mechanical tension (Hinz *et al.*, 2001) and TGF- β activity (Desmouliere *et al.*, 1993).

To analyze how fibroblasts respond to keratinocyte-derived stimuli, the messenger RNA (mRNA) expression pattern of fibroblasts in monoculture was compared with the fibroblasts cocultured with keratinocytes (Shephard *et al.*, 2004b). To reduce the complexity of the experimental system HaCaT keratinocytes were cultured on a feeder layer of fibroblasts. HaCaT cells in these experiments had the advantage that the epidermal pheno-

type was relatively stable although normal epidermal architecture is observed only after transplantation onto nude mice (Boukamp *et al.*, 1997) or after addition of TGF- α in organotypic cultures (Maas-Szabowski *et al.*, 2003). When HaCaT keratinocytes reached 50% confluence, cocultured fibroblasts were isolated by differential EDTA treatment. Comparing the RNA expression pattern of cocultured and control fibroblasts revealed differential expression of growth factor, extracellular matrix, protease, and intracellular structural protein transcripts (Table 1). The intracellular structural proteins suggested that fibroblasts differentiated into myofibroblasts as shown by a time-dependent increase of α -SMA-positive mesenchymal cells. The expression

data showed that several genes characteristic of TGF- β signaling were upregulated. They encode, among others, plasminogen-activator inhibitor-1, α -SMA and collagen type 1. HaCaT keratinocytes as well as fibroblast monocultures produced low amounts of TGF- β , which was mostly in the latent form. In cocultures, however, there was a strong increase in latent and active TGF- β . Surprisingly, high levels of active TGF- β protein were already detected in cocultures after 24 and 48 hours, whereas the differentiation into myofibroblasts started to be seen only after 4 days and later. This raised the question whether there were inhibitors in the keratinocyte-fibroblast cocultures, which may delay the TGF- β effects. And indeed, NF κ B was strongly induced in cocultured fibroblasts within the first 2 hours, persisting for the first 2 days, and slowly waning off until day 7. NF κ B activation was shown to interfere with TGF- β signaling (Bitzer *et al.*, 2000; Nagarajan *et al.*, 2000; Verrecchia *et al.*, 2000). Further experiments supported the assumption of a role of NF κ B as an inhibitor of TGF- β -mediated effects in fibroblasts, as exogenously added IL-1 was able to completely suppress α -SMA induction in cocultures. Further, inhibition of keratinocyte-derived IL-1 increased α -SMA expression (Shephard *et al.*, 2004b). When keloid keratinocytes and fibroblasts were used in cocultures, increased expression of TGF- β was observed, which is responsible for the enhanced proliferation rate of the mesenchymal cells (Xia *et al.*, 2004). In these cocultures keloid keratinocytes expressed more TGF- β 1 and - β 3 than normal keratinocytes. Keloid fibroblasts had increased the levels for TGF- β 1- and β 2 mRNA and produced more collagen I, connective tissue growth factor, and IGF-II/mannose-6-phosphate receptor. Interestingly, keloid fibroblasts also showed a constitutive increase in NF κ B-binding activity with and without TNF- α treatment when compared with normal skin fibroblasts (Messadi *et al.*, 2004). Whether targeting of the NF κ B pathway represents a novel therapeutic intervention for the treatment of keloid scarring remains to

be elucidated. At present, the *in vitro* data suggest that keratinocytes and fibroblasts in cocultures produce significant amounts of active TGF- β , but proinflammatory cytokines determine the degree of cellular responsiveness in fibroblasts.

Keratinocyte-fibroblast interactions increase contractile activity in mesenchymal cells

Apart from TGF- β signals, myofibroblast differentiation is also regulated by mechanical tension (reviewed by Tomasek *et al.*, 2002). In cocultures, fibroblasts readily acquired contractile properties already after 1 and 2 days when compared with control cultures (Shephard *et al.*, 2004a). In these experiments, the cells were cultured on deformable silicone substrates. When cells start to exert contractile forces, the underlying substrate forms wrinkles and this can be observed in phase contrast microscopy. Contractile activity persisted in cocultures for the observational period of 7 days. Wrinkles were predominantly formed where keratinocytes and fibroblasts were in close proximity, reminiscent of α -SMA induction, which also required close proximity of both cell types. At early time points, the underlying mechanism was unclear, as fibroblasts had not yet acquired α -SMA expression. The initial complementary DNA array data suggested that endothelin-1 might have induced contraction of fibroblasts in the cocultures. Endothelin-1 peptide was upregulated in cocultures from early time points on, and inhibition with PD156252, a specific inhibitor at the receptor level, was able to prevent contraction in cocultured fibroblasts. Interestingly, inhibition occurred only at early time points. At later time points when α -SMA was induced, contraction was unaffected by endothelin inhibition. PD156252 inhibited induction of α -SMA expression in cocultured fibroblasts to some extent though not completely. Only the combination of endothelin and TGF- β inhibition suppressed α -SMA induction. Thus there appears to be a cooperative effect of mechanical tension and TGF- β sensitivity in keratinocyte-fibroblast cocultures.

Apart from whole cells, a number of growth factors were shown to modulate contractile activity of fibroblasts in collagen gels. The PDGF-AA and -BB isoforms and TGF- β 1 led to efficient collagen-gel contraction, although TGF- β treatment resulted in a delayed onset and a slower rate of contraction. IL-1 α on the other hand inhibited collagen-gel contraction and caused degradation of the collagen gels at later time points, most likely due to enhanced matrix metalloproteinase activity (Tingstrom *et al.*, 1992). Furthermore, mechanical tension was shown to inhibit the normally observed apoptosis in collagen gel-embedded fibroblasts (Fluck *et al.*, 1998; Grinnell *et al.*, 1999; Niland *et al.*, 2001), and the gene expression pattern was differentially regulated by mechanical tension in normal fibroblasts (Kessler *et al.*, 2001).

IL-1, TNF- α and TGF- β expression during wound healing

The findings described above raise the question how the key players for the interaction between keratinocytes and fibroblasts are regulated in wounded skin. IL-1 and TNF- α expression were analyzed in excisional wound-healing experiments in mice. Within 8 hours there was a strong induction of IL-1 β and slightly later TNF- α mRNA, which persisted until the wounds were in the mid-phase of healing (Hubner *et al.*, 1996a). Granulocytes are the first cells to appear at the wound site and produce high levels of these proinflammatory cytokines (Feiken *et al.*, 1995). Later, macrophages in the granulation tissue and keratinocytes become the predominant source (Hubner *et al.*, 1996a). Blocking TNF signaling resulted in accelerated healing of excisional wounds. In TNF-receptor p55 knockout mice, enhancement of angiogenesis, collagen production, and epithelial wound closure was observed. Moreover, the expression of TGF- β 1 and connective tissue growth factor were enhanced and higher levels of vascular endothelial growth factor, vascular endothelial growth factor receptor-1, and vascular endothelial growth factor-2 were also observed. Overall, the inflammatory activity of

these wounds was markedly reduced compared with controls (Mori *et al.*, 2002). These findings suggest that inflammatory cytokine signals can balance TGF- β expression and TGF- β -mediated effects on healing wounds.

TGF- β expression was analyzed in full-thickness excisional mouse wounds at different stages after injury, and all three isoforms were detected. Nevertheless, each isoform had a distinct and characteristic expression pattern and time course in wound healing. TGF- β 1 and - β 2 expressions were rapidly induced in suprabasal wound keratinocytes and in the stroma, followed later by the expression of TGF- β 3 throughout the hyperproliferative epithelium, and also in the granulation tissue (Kane *et al.*, 1991; Levine *et al.*, 1993; Schmid *et al.*, 1993; Frank *et al.*, 1996). There is also a rapid release of TGF- β at the site of injury from degranulating thrombocytes (Assoian *et al.*, 1983), suggesting that at early time points, TGF- β is supplied by thrombocytes, whereas later, keratinocytes, macrophages, and fibroblasts become the predominant source of TGF- β in wounds. *In situ* measurement of TGF- β bioactivity identified a second peak of active TGF- β (Yang *et al.*, 1999), which coincides with the appearance of myofibroblasts.

The phenotype of TGF- β inhibition in wound healing is complex. Neutralization of TGF- β 1 and - β 2 with neutralizing antibodies and antisense-oligonucleotides resulted in reduced scarring (Shah *et al.*, 1994; Choi *et al.*, 1996). The analysis of wound healing in TGF- β 1-deficient mice was very difficult, as these mice show a severe multifocal inflammatory phenotype after 3 weeks of age, leading to multiple organ failure and death at <30 days of life (Kulkarni *et al.*, 1993). When mice were wounded at day 10, initial wound healing was normal before high numbers of inflammatory cells accumulated, and vascular density and collagen deposition were reduced, and epithelial closure was delayed (Brown *et al.*, 1995). The conclusion that other TGF- β s were compensating at early time points is difficult to draw. Maternal transmission of TGF- β through the milk – before

complete weaning (Letterio *et al.*, 1994) – may have ameliorated inflammation at early time points. Later, inflammation and the subsequent wasting may have prevailed and perturbed wound healing. Blocking TGF- β signaling in keratinocytes or fibroblasts was achieved by tissue-specific expression of a dominant-negative receptor approach or by using a conditional knockout strategy, respectively. Wound closure occurred faster through increased keratinocyte proliferation and decreased apoptosis in transgenic animals, which overexpressed a dominant-negative type II TGF- β receptor mutant in basal keratinocytes (Amendt *et al.*, 2002). In these animals, signaling of all TGF- β isoforms is abolished in basal keratinocytes. When the TGF- β type II receptor was inactivated in fibroblasts, the granulation tissue in excisional wounds had less tensile strength at day 7. The epidermal wound closure rate was unaffected, but, interestingly, the number of suprabasal keratinocytes was increased, suggesting indirect paracrine effects. In these mice, the cell type-specific inactivation was achieved by crossing mice with two floxed TGF- β type II receptor alleles with mice expressing the Cre recombinase under the control of the fibroblast specific protein-1 promoter, which becomes active in cells of the fibroblastic lineage after day 9 of embryonic development (Bhowmick *et al.*, 2002). These studies demonstrated that TGF- β activity is peaking early after wounding and later in the wound-healing process. Major TGF- β effects are seen in the granulation tissue with increased extracellular matrix production; however, it appears that they occur at the expense of epidermal wound closure rates. Thus the balance of proinflammatory cytokines and TGF- β activity appears to control major aspects of keratinocyte–fibroblast interactions *in vivo*.

Diversity of fibroblasts

Fibroblasts are poorly characterized, and there is a large variability of fibroblast phenotypes. Fibroblasts can be derived from many organs; several mesenchymal cells such as adipocytes can de-differentiate into fibroblastic

cells, and dermal fibroblasts can acquire a new differentiation state, for example, they can differentiate into myofibroblasts. In keloids, benign tumors characterized by excessive production of extracellular matrix, and in hypertrophic scars, lesional fibroblasts and keratinocytes differ from those of nonlesional skin (Ehrlich *et al.*, 1994; Machesney *et al.*, 1998). mRNA for activin is increased in keloids, and activin A and follistatin were found in basal keratinocytes (Mukhopadhyay *et al.*, 2006). On the mesenchymal side, keloid and hypertrophic scar fibroblasts show a strong expression of α -SMA (Ehrlich *et al.*, 1994). The phenotype of keloid and control fibroblasts is also evident in tissue culture. High baseline expression of activin A, follistatin, α -SMA, collagen I, thrombospondin-1, and the ectodermal dysplasia-A fibronectin variant distinguishes keloid fibroblasts from normal fibroblasts in culture (Chipev *et al.*, 2000; Chipev and Simon, 2002; Mukhopadhyay *et al.*, 2006). Moreover, hypertrophic scar fibroblasts showed increased fibrin gel (Younai *et al.*, 1996) and collagen–gel contraction (Phan *et al.*, 2003). There is also evidence that neutralizing anti-TGF- β antibodies can partially revert the keloid fibroblast phenotype (Younai *et al.*, 1994). To complicate the situation, several groups have shown that lesional keratinocytes also differ from normal keratinocytes. Keloid keratinocytes are more effective in inducing fibroblast proliferation and matrix production in both keloid and control fibroblasts (Xia *et al.*, 2004). Moreover, keratinocytes from keloids are characterized by a higher expression of TGF- β 1, - β 2, TGF- β receptor 1, and Smad2 in cocultures, and they express the hyperproliferative keratins K6, K16, and K17 with premature expression of filaggrin (Machesney *et al.*, 1998). At present, it is unclear how keratinocyte–fibroblast interactions in keloids or hypertrophic scars differ from normal cells or whether keratinocytes or fibroblasts are the primary cause for the keloid or hypertrophic scar phenotype. Still, it appears that the phenotype or differentiation state is stable for a long time in patients and in tissue culture.

Differences in fibroblast differentiation may be more subtle. Comparing dermal fibroblasts from different layers of human skin showed differences in collagen type I and III mRNA expression (Ali-Bahar *et al.*, 2004). Fibroblasts derived from deeper layers showed reduced collagen expression compared with fibroblasts derived from more superficial layers. Fibroblast diversity was further demonstrated by expression data for fibroblasts derived from different body sites (Chang *et al.*, 2002; Rinn *et al.*, 2006). The analysis showed that fibroblasts retain positional information and their topographic differentiation in tissue culture. The *HOX* gene expression pattern in fibroblasts appears to be a signature from which body site the cells were derived (Chang *et al.*, 2002). It is unclear at present how these differences may influence keratinocyte-fibroblast interactions. Recombination experiments have shown that keratinocytes maintain their gross anatomical site-specific differentiation pattern (Limat *et al.*, 1996; Okazaki *et al.*, 2003). On closer examination, there is evidence that keratinocyte differentiation can be modulated by fibroblast-derived information cues (Okazaki *et al.*, 2003). When comparing the effects of different fibroblast lineages, the effects on the epithelial phenotype become more pronounced (Reynolds and Jahoda, 1992; Aoki *et al.*, 2004). For rodent dermal papilla cells isolated from whiskers, the dermal papilla cell differentiation is sufficiently stable to survive several passages in tissue culture, and the cells are still able to induce hair follicle development with the necessary reprogramming of inter-follicular keratinocytes into hair follicles (Reynolds and Jahoda, 1992). In the other example, Aoki *et al.* (2004) compared the effect of bone marrow stromal cells, subcutaneous preadipocytes, and dermal fibroblasts on the epidermal architecture of keratinocytes in three-dimensional cultures. All mesenchymal cell types supported epidermal proliferation and differentiation. Bone marrow stromal cells and preadipocytes induced in growth of keratinocytes into the fibroblast-populated dermal equivalents, a process the authors describe as "rete ridge-like"

and "epidermal ridge-like" structure formation.

The last years have seen significant advances in the definition of the cells currently summarized as fibroblasts. It will be interesting with this knowledge in mind to review observations on anatomical site variances on wound healing rates and skin transplantation outcomes in patients. Fibroblast differentiation and resulting differences in keratinocyte-fibroblast interactions may have a central role in the clinics of wound-healing and could represent a novel target for therapeutic interventions (Table 1).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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