

# Paracrine regulation of keratinocyte proliferation and differentiation

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The histoarchitecture and function of the epidermis depend on a well-controlled balance between keratinocyte proliferation and differentiation. This balance is perturbed after skin injury, and imbalance is a characteristic feature of major human skin diseases such as psoriasis and epidermal cancers. Recent studies have highlighted the importance of fibroblast-derived soluble factors for the regulation of keratinocyte proliferation and differentiation. Therefore, identification of these paracrine-acting factors and the elucidation of their mechanisms of action are necessary for understanding epidermal homeostasis, repair and disease, and these approaches will offer new potential targets for drug therapy. Here, we review exciting recent findings on the identification, regulation and function of paracrine-acting cytokines in the skin. In particular, we describe the role of fibroblast-derived mitogens as regulators of keratinocyte proliferation and differentiation, and we summarize the regulation of these factors by keratinocyte-derived interleukin 1 that involves the transcription factors c-Jun and JunB.

Interactions between epithelial and mesenchymal cells play a crucial role in the regulation of tissue development, homeostasis and repair. For skin, this was first revealed by the pioneering studies of Rheinwald and Green<sup>1</sup>, who demonstrated that normal human epidermal

keratinocytes depend on the presence of fibroblasts for efficient growth in tissue culture at clonal seeding densities. Pursuing this further, Rubin *et al.*<sup>2</sup> characterized a fibroblast-derived growth factor from conditioned media that strongly stimulated the proliferation of a keratinocyte cell line. Owing to its unique activity, the new mitogen, which was shown to belong to the fibroblast growth factor (FGF) family, was termed keratinocyte growth factor (KGF, FGF-7). Subsequently, other fibroblast-derived keratinocyte mitogens were identified, but their role in the paracrine stimulation (see Box 1) of keratinocyte proliferation and differentiation, as well as their regulation, remained unknown. Here, we highlight recent findings on the roles of KGF and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the regulation of epidermal homeostasis and on the regulation of these growth factors by keratinocyte-derived interleukin 1 (IL-1) through the c-Jun and JunB transcription factors. Finally, we report on a novel role of the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily member activin in the regulation of skin morphogenesis and cutaneous wound repair.

## Model systems to analyze regulation and function of paracrine-acting factors

For the detailed analysis of epithelial-mesenchymal interactions, there was a need for appropriate test systems. *In vivo* models are difficult owing to the high complexity of the experimental conditions, but tissue-culture models offer a valuable complementary approach. The tissue-culture models are of increasing complexity, ranging from simple monolayer cultures of one cell type to co-cultures of epithelial and mesenchymal cells and finally to three-dimensional skin equivalents – organotypic cultures – that form a highly organized epithelium under the control of co-cultured fibroblasts<sup>3</sup>. In skin equivalents, epidermal keratinocytes grow exposed to air on a matrix of type I collagen in which either primary dermal fibroblasts or heterologous mouse

fibroblast lines are embedded. Keratinocyte proliferation is supported in the basal layer – whereas, suprabasally, characteristic markers of epidermal differentiation appear, giving rise to an almost normal tissue architecture<sup>4</sup>. These tissue-engineered skin equivalents are currently employed for the treatment of acute and delayed-healing wounds, where they provide a temporary biological wound cover.

## Discovery of a double paracrine loop

This organotypic culture system, as well as conventional feeder-layer cultures, was first used by Smola *et al.*<sup>5</sup> to study paracrine cellular interactions operating through cytokine expression networks. Interestingly, analysis of the different cell compartments revealed that the presence of keratinocytes stimulated the expression of mRNA encoding KGF, GM-CSF and interleukin 6 in fibroblasts, indicating that keratinocytes produce factors that, in turn, induce growth-factor expression in the underlying mesenchymal cells. One of the keratinocyte-derived factors was identified as the cytokine IL-1, which is present in large amounts in the epidermis and stimulates KGF expression in monocultures of fibroblasts<sup>6</sup>. KGF and other fibroblast-derived mitogens are obviously important paracrine-acting factors because the presence of fibroblasts in the organotypic culture model strongly stimulated keratinocyte proliferation and allowed differentiation of these cells. Taken together, keratinocytes seem to produce factors that, in turn, induce growth-factor expression in fibroblasts. KGF and other fibroblast-derived mitogens might then regulate keratinocyte proliferation and differentiation in a paracrine manner (Fig. 1a).

A follow-up study demonstrated the functional importance of this double paracrine loop for the induction of KGF expression and subsequently for keratinocyte proliferation. Neutralization of interleukin-1 $\alpha$  and -1 $\beta$  in the culture medium reduced KGF production by human fibroblasts, resulting in a significant inhibition of keratinocyte proliferation, and neutralizing antibodies against KGF had

### Box 1. Autocrine and paracrine regulation

#### Autocrine regulation:

Signaling molecules that a cell secretes activate receptors on the same cell.

#### Paracrine regulation:

Signaling molecules that a cell secretes activate receptors on cells in the immediate neighborhood. In our example, KGF and GM-CSF are produced by fibroblasts in the dermis and act locally on epidermal keratinocytes.

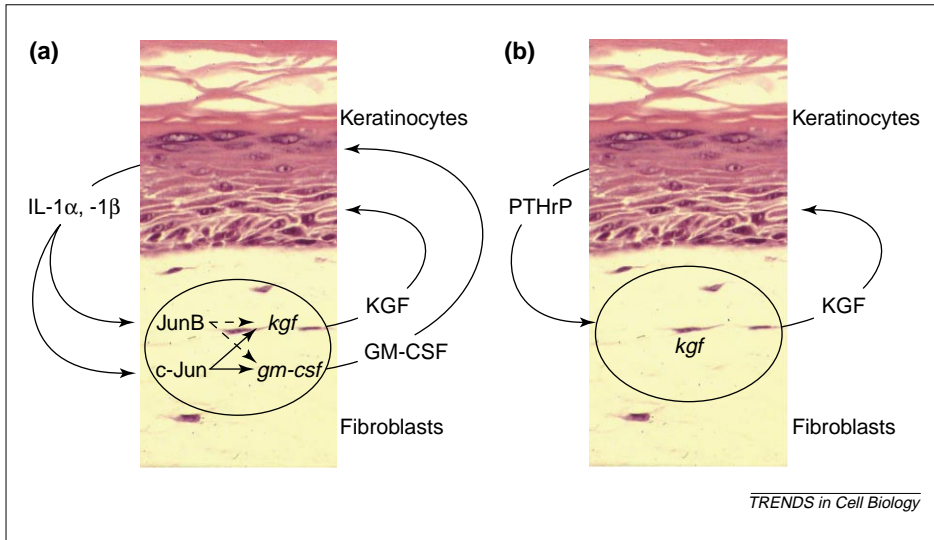


Fig. 1. (a) Schematic illustration of the mesenchymal-epithelial cross-talk in skin involving interleukin 1 (IL-1), keratinocyte growth factor (KGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Keratinocyte-derived IL-1 $\alpha$  and -1 $\beta$  stimulate expression of KGF and GM-CSF in fibroblasts through the c-Jun and JunB transcription factors. KGF and GM-CSF are then released by fibroblasts and stimulate keratinocyte proliferation and differentiation in a paracrine manner. (b) Keratinocyte-derived parathyroid hormone-related protein (PTHrP) stimulates KGF expression in fibroblasts. KGF is then released by fibroblasts and stimulates keratinocyte proliferation and differentiation in a paracrine manner.

comparable effects in the same co-culture system<sup>7</sup>. Thus, IL-1, which is rapidly released from keratinocytes upon stress, such as UV irradiation, surface damage and wounding, is not only a major pro-inflammatory cytokine but also an inducer of epidermal regeneration by inducing KGF expression in fibroblasts. However, IL-1 is unlikely to be the only regulator of KGF expression: first, IL-1 inhibition did not completely suppress expression of KGF in fibroblasts; furthermore, other keratinocyte-derived factors, such as parathyroid hormone-related protein (PTHrP), induce KGF expression<sup>8</sup>. This paracrine regulation of KGF expression by PTHrP (Fig. 1b) might at least partially explain the epidermal atrophy seen in the skin of PTHrP-null mice and correlates well with the epidermal hyperplasia seen in transgenic mice overexpressing PTHrP in basal keratinocytes<sup>9</sup>. Finally, during wound healing, growth factors released from platelets and from serum upon hemorrhage, and inflammatory-cell-derived pro-inflammatory cytokines, are likely candidates to trigger the immediate and particularly strong expression of KGF observed in wounded skin<sup>6</sup>.

#### Transcriptional control of KGF and GM-CSF expression

To gain insight into the mechanisms underlying the regulation of KGF expression by keratinocyte-derived IL-1,

Szabowski *et al.*<sup>10</sup> determined the potential role of the AP-1 transcription factors c-Jun and JunB in this regulation. There are no *in vivo* knockout mouse models to study c-Jun and JunB function in the mesenchymal compartment because constitutive-homozygous knockout mice suffer embryonic lethality and appropriate fibroblast-specific promoters for conditional gene-inactivation strategies are as yet unavailable. Instead, immortalized fibroblasts derived from *c-jun*<sup>-/-</sup> and *junB*<sup>-/-</sup> embryos were combined with human primary keratinocytes in the three-dimensional co-culture system. Interestingly, the lack of either one of the transcription factors severely affected proliferation and differentiation of the overlying normal human keratinocytes (Fig. 2). Epithelia of co-cultures containing *c-jun*<sup>-/-</sup> fibroblasts were atrophic and consisted of fewer keratinocyte layers, basal cell proliferation was remarkably reduced and the process of terminal differentiation was altered. The opposite effect was observed with *junB*<sup>-/-</sup> fibroblasts. In this case, the basal cell proliferation rate was increased, and severe epithelial thickening resulted owing to an increased number of nondifferentiated and also of differentiated cell layers (Fig. 2). These phenotypic abnormalities reverted by transgenic re-expression of *c-jun* or *junB*,

thus confirming that the epithelial defects were indeed caused by the loss of these transcription factors in the collagen-embedded fibroblasts.

These findings suggest that c-Jun and JunB regulate differently the expression of the soluble factors in fibroblasts that modulate keratinocyte proliferation and differentiation in a paracrine manner. KGF appeared to be a likely candidate, and indeed, *c-jun*<sup>-/-</sup> fibroblasts produced very low levels of KGF mRNA and protein, even in the presence of co-cultivated keratinocytes. By contrast, *junB*<sup>-/-</sup> fibroblasts revealed high basal levels of KGF, which were further increased by co-cultivation with keratinocytes. These results demonstrated that c-Jun is required for KGF expression in fibroblasts, whereas JunB suppresses expression of KGF (Fig. 1a).

The potent mitogenic activity of KGF for keratinocytes<sup>2</sup> and the abnormal levels of KGF in the co-cultures with *c-jun*<sup>-/-</sup> and *junB*<sup>-/-</sup> fibroblasts correlate well with the epithelial atrophy or hypertrophy, respectively. Indeed, in co-cultures with *c-jun*<sup>-/-</sup> fibroblasts, keratinocyte proliferation was completely restored upon addition of KGF to the culture medium, arguing that KGF regulates at least in part the normal proliferation rate of keratinocytes. The lack of obvious phenotypic abnormalities in normal and wounded skin of KGF-deficient mice, however, points to compensatory mechanisms by other growth factors *in vivo*<sup>11</sup>. A potential candidate is FGF-10, another FGF-family member, which has high homology to KGF and also binds to the KGF receptor with high affinity<sup>12</sup>. Like KGF, FGF-10 is expressed in the dermal compartment of the skin<sup>13</sup>. Mice lacking the gene encoding FGF-10 but with a functional *FGF-7* gene are characterized by defective terminal differentiation and hypoplasia of the epidermis, strongly favoring the view that FGF-10 has the potential to display compensatory activities<sup>14</sup>. Furthermore, severe epidermal atrophy and a strong inhibition of wound re-epithelialization were observed in transgenic mice expressing a dominant-negative mutant of the KGF/FGF-10 receptor in the epidermis<sup>15</sup>, rendering them unable to respond to either of these ligands.

**A novel regulator of keratinocyte proliferation and differentiation: GM-CSF**  
In addition to FGF-family members, recent studies have provided evidence for a particularly important role of GM-CSF

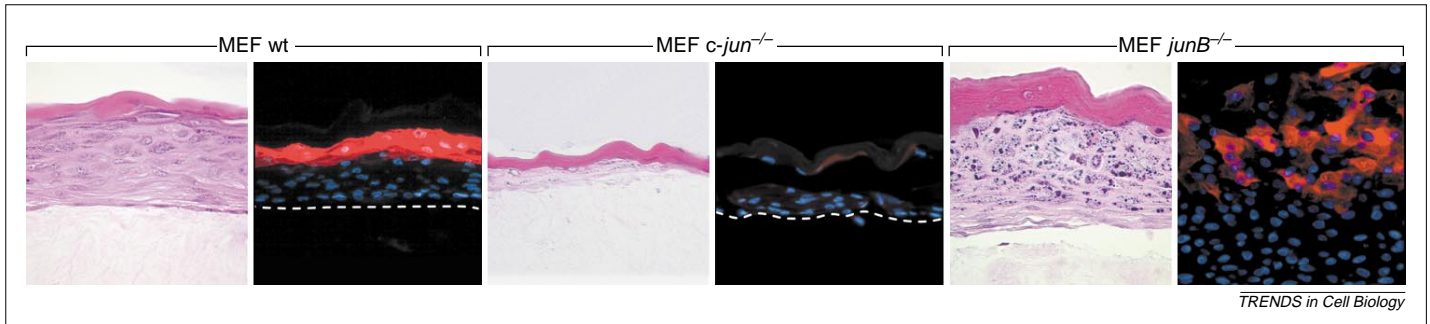


Fig. 2. Epidermis formation in organotypic cultures depends on the genotype of co-cultured fibroblasts. Epidermal tissue morphology of *in vitro* co-cultures of primary human keratinocytes and immortalized mouse embryonic fibroblasts (MEFs) with wild-type (wt), *c-jun*<sup>-/-</sup> and *junB*<sup>-/-</sup> genotype, respectively, in the collagen gel. Cross-sections were stained with hematoxylin–eosin (HE) or analyzed for the late differentiation marker loricrin (red) by immunofluorescence. Nuclei were counterstained with bisbenzimidazole (blue). The keratinocytes–collagen matrix boundaries are indicated by a dotted line. The lack of *c-jun* causes epidermal atrophy and altered keratinocyte differentiation as demonstrated by reduced expression of loricrin. By contrast, lack of *junB* resulted in epithelia with an increased number of cell layers. Expression of loricrin was scattered over a thicker upper cell compartment. (Image reproduced, with permission, from Ref. 10.)

in the regulation of keratinocyte proliferation and differentiation. Thus, expression of this factor is induced in dermal fibroblasts in the presence of keratinocytes<sup>5</sup>, and keratinocyte-derived IL-1 appears to be responsible for this induction (Fig. 1a)<sup>10</sup>. Indeed, GM-CSF is a target of c-Jun and JunB as its expression was regulated in the same way as that of KGF in *c-jun*<sup>-/-</sup> and *junB*<sup>-/-</sup> embryonic fibroblasts<sup>10</sup>. In contrast to KGF, however, addition of GM-CSF to co-cultures with *c-jun*<sup>-/-</sup> fibroblasts not only stimulated keratinocyte proliferation but also caused enhanced terminal differentiation in suprabasal cells, reminiscent of the *junB*<sup>-/-</sup> phenotype. Furthermore, addition of neutralizing antibodies against GM-CSF reduced the epidermal hyperthickening and reverted the irregular organization of the keratinocytes seen in the *junB*<sup>-/-</sup> co-cultures. Addition of the neutralizing GM-CSF antibodies to co-cultures with wild-type murine fibroblasts caused epidermal atrophy resembling the phenotype of the *c-jun*<sup>-/-</sup> co-cultures<sup>10</sup>. Together, these results demonstrate that KGF and GM-CSF regulate specific programs of keratinocyte proliferation and differentiation. Similar differences between the effects of KGF and GM-CSF are also observed *in vivo*. Thus, overexpression of KGF in the basal epidermal layer of transgenic mice resulted in epidermal hyperplasia and early papilloma formation, demonstrating that KGF induces proliferation but not differentiation of keratinocytes<sup>16</sup>. These findings correlate with *in vitro* results from Hines and Allen-Hoffmann, who

demonstrated that KGF reduces the ability of keratinocytes to initiate terminal differentiation and to undergo programmed cell death<sup>17</sup>. By contrast, targeted expression of GM-CSF in basal keratinocytes of transgenic mice resulted in a higher mitotic activity of these cells, but the epidermal thickness and differentiation were normal in the skin of mice overexpressing GM-CSF. The homeostasis was in fact being maintained by increased apoptosis in the epidermis<sup>18</sup>. It remains to be seen whether endogenous GM-CSF is indeed important for epidermal morphogenesis, homeostasis and repair *in vivo*.

#### The role of activin

In addition to KGF and GM-CSF, which might act synergistically in the control of keratinocyte proliferation and differentiation<sup>10</sup>, additional cytokines are likely to be involved in these processes *in vivo*. For example, members of the TGF- $\beta$  superfamily of growth and differentiation factors seem to influence epidermal morphogenesis not only in an autocrine but also in a paracrine fashion. Although the roles of TGF- $\beta$ 1, - $\beta$ 2 and - $\beta$ 3 in epidermal development and homeostasis are still controversial, the TGF- $\beta$  superfamily member activin seems to play an important role in these processes. The latter has been shown to affect growth and differentiation of many different target cells during embryonic development and in the adult organism<sup>19</sup>, but a role for activin in skin morphogenesis has only been demonstrated recently<sup>20</sup>. Activin is expressed in the dermis of embryonic but not of adult skin. It is strikingly re-expressed in the granulation

tissue and also in the hyperproliferative epithelium during cutaneous wound healing<sup>20</sup>. This upregulation is likely to be functionally important as recombinant activin reduces keratinocyte proliferation and induces differentiation *in vitro*<sup>21,22</sup>. The situation is more complex *in vivo* as overexpression of activin in the epidermis of transgenic mice resulted in increased proliferation of basal keratinocytes and in the formation of a hyperthickened, disorganized epidermis<sup>23</sup>. One possibility to explain the discrepancies between the *in vitro* and *in vivo* data could be that highly diffusible activin stimulates the underlying mesenchyme. That activin might have this paracrine action is further supported by the severe fibrosis that occurs in the dermis of mice that overexpress activin in the epidermis. Thus, it seems likely that keratinocyte-derived activin induces the expression of epithelial mitogens in fibroblasts, which then stimulate keratinocyte proliferation. The analysis of the autocrine and fibroblast-mediated effects of activin on keratinocytes grown in a three-dimensional organotypic culture (see above) will help to further define the role of activin in the regulation of keratinocyte proliferation and differentiation.

#### Concluding remarks

In summary, three-dimensional organotypic skin cultures allow the detailed analysis of epithelial–mesenchymal interactions, with particular emphasis on the role of the underlying mesenchyme for epithelial homeostasis. Using genetically modified fibroblasts in these models can help to minimize the complexity of *in vivo* models or even allow analysis of regulatory processes for which appropriate animal models are not yet available. The above-described identification of the IL-1–KGF/GM-CSF double paracrine loop and its regulation by c-Jun and JunB is a particularly nice example of the usefulness of this model. The roles of paracrine-acting

fibroblast-derived factors on keratinocyte proliferation and differentiation, and particularly compensatory mechanisms, however, can only be identified by a complementary approach integrating *in vivo* and *in vitro* data.

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