Organotypic Cocultures with Genetically Modified Mouse Fibroblasts as a Tool to Dissect Molecular Mechanisms Regulating Keratinocyte Growth and Differentiation

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Organotypic cocultures of keratinocytes and fibroblasts generate a normal epidermis irrespective of the species and tissue origin of fibroblasts. The use of mouse fibroblasts and human keratinocytes facilitates the identification of the origin of compounds involved in epidermal tissue reconstitution and growth regulation. Moreover, the functional significance for the keratinocyte phenotype of genetically modified fibroblasts from transgenic or knockout mice, even those exhibiting an embryonic lethal phenotype, can be studied in such heterologous *in vitro* tissue equivalents. Here we communicate results of such studies revealing the antagonistic function of mouse fibroblasts defective in the AP-1 constituents c-Jun and JunB, respectively, on human keratinocyte growth and differentiation. Furthermore, the hematopoietic growth factor granulocyte macrophage-colony stimulating factor has been identified as a novel regulator of keratinocyte growth and differentiation. As will be reported in detail elsewhere both granulocyte macrophage-colony stimulating factor and keratinocyte growth factor have been identified as major mediators of fibroblast–keratinocyte interactions and their expression is induced via AP-1 by interleukin-1 released by the epithelial cells. Thus, these heterologous cocultures provide a novel promising tool for elucidating molecular mechanisms of epithelial–mesenchymal interactions and their consequences on epithelial cell proliferation and differentiation. ***Key words: AP-1/fibroblasts/human/keratinocytes/mouse/skin equivalent. J Invest Dermatol 116:816–820, 2001***

Formation and maintenance of the mature epidermis rely on a tight balance of keratinocyte proliferation and terminal differentiation (Fuchs and Green, 1980; Schröder, 1995; Werner, 1998). There is accumulating evidence that epidermal tissue homeostasis is regulated by a cytokine network between keratinocytes and dermal fibroblasts (Luger and Schwarz, 1990; Fusenig, 1994; Werner *et al*, 1994; Maas-Szabowski *et al*, 1999). Comparably, there is a strong dependence of keratinocyte growth *in vitro* on mesenchymal interactions as first demonstrated in two-dimensional feeder-layer cocultures of keratinocytes and postmitotic 3T3 cells (Rheinwald and Green, 1975). This mesenchymal support is based on matrix components and diffusible factors produced by fibroblasts (Waelti *et al*, 1992; Smola *et al*, 1993; Fusenig *et al*, 1994; Maas-Szabowski and Fusenig, 1996; Maas-Szabowski *et al*, 1999).

The molecular basis of dermal–epidermal interactions has been further elaborated in a more physiologic context *in vitro*, in skin equivalent models. Here, normal keratinocyte proliferation, differentiation and tissue architecture are maintained under the control of cocultured matrix-embedded dermal fibroblasts (Bell *et al*, 1981; Mackenzie and Fusenig, 1983; Asselineau and Prunieras, 1984; Parenteau *et al*, 1991; Stark *et al*, 1999), the latter being either of homologous or of heterologous origin, e.g., mouse NIH 3T3 cells (Turksen *et al*, 1991; Kaur and Carter, 1992).

In the homologous organotypic skin cocultures, including human keratinocytes and fibroblasts, further aspects of epidermal biology became accessible, e.g., reepithelialization after wounding, the relation of proliferation and integrin expression pattern and development of the stratum corneum barrier (Garlick and Taichman, 1994; Ponec *et al*, 1997; Rikimaru *et al*, 1997). Moreover, in this *in vitro* tissue model we have recently documented a novel double paracrine mechanism by which keratinocytes control their proliferation. Through release of interleukin-1 (IL-1), they induce growth factors such as keratinocyte growth factor (KGF) in dermal cells, which in turn stimulate keratinocyte proliferation (Smola *et al*, 1993; Maas-Szabowski *et al*, 2000).

The identification of further components in the culture medium secreted either by fibroblasts or by keratinocytes remains difficult in a homologous skin model, i.e., containing human cells in both

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Abbreviations: bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; GM-CSF, granulocyte macrophage-colony stimulating factor; HDF, human dermal fibroblasts; IL-1RA, IL-1 receptor antagonist; KGF, keratinocyte growth factor; K1/10, keratin 1 and 10; MEF, mouse embryonic fibroblasts; NEK, normal epidermal keratinocytes.

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compartments. To facilitate the unraveling of the origin of diffusible and/or structural components and to get further insight into the signal transduction mechanisms we cocultured embryonic fibroblasts from genetically deficient mice with normal human keratinocytes.

Our studies revealed that mouse embryonic fibroblasts genetically deficient in the AP-1 constituents c-Jun and JunB, respectively, dramatically and antagonistically affected keratinocyte growth and differentiation (Szabowski *et al*, 2000). Here we provide further evidence of the antagonistic role of c-Jun and JunB in fibroblasts in regulating keratinocyte proliferation and differentiation in response to keratinocyte-derived IL-1. In organotypic cocultures containing *c-jun*$^{-/-}$ fibroblasts, keratinocyte proliferation is reduced and differentiation inhibited. In contrast, the effect of *junB*$^{-/-}$ fibroblasts results in hyperproliferative epithelia with an altered expression pattern of differentiation markers. With these as well as other genetically modified mouse fibroblasts an excellent tool has been established to dissect molecular mechanisms regulating epithelial growth and differentiation in normal, but also in diseased skin.

MATERIALS AND METHODS

Cell culture Normal human skin keratinocytes (NEK) and dermal fibroblasts (HDF) were derived from adult skin (Smola *et al*, 1993; Stark *et al*, 1999). HDF obtained from outgrowth of explant cultures, were grown in Dulbecco's modified Eagle's medium (DMEM; Bio Whittaker, Taufkirchen, Germany) supplemented with 10% fetal calf serum, and cells from passages 4–8 were used. NEK were plated on X-irradiated feeder cells (HDF, 70 Gy; MEF, 20 Gy) in FAD medium (DMEM:Hams F_{12}/3:1) with 100 U penicillin per ml, 50 μg streptomycin per ml and supplemented with 5% fetal calf serum, 5 μg insulin per ml, 0.1 ng recombinant human epidermal growth factor (EGF) per ml, 10^{-10} M cholera toxin, 10^{-4} M adenine, and 0.4 μg hydrocortisone per ml (Sigma, Deisenhofen, Germany) as described (Smola *et al*, 1993).

Mouse wild-type and *junB*$^{-/-}$ fibroblasts were isolated from mouse embryos (day 9.5; Schorpp-Kistner *et al*, 1999) and immortalized according to the 3T3 protocol (Todaro and Green, 1966). *c-jun*$^{-/-}$ fibroblasts have been described previously (Schreiber *et al*, 1999; Kolbus *et al*, 2000). Mouse embryonic fibroblasts (MEF) were grown in DMEM (Bio Whittaker) supplemented with 10% fetal calf serum.

Organotypic cocultures Collagen gels (type I, rat tendon, 4 mg per ml) containing 2×10^5 human or mouse fibroblasts per ml were cast in cell culture inserts (porous size 3 μm, polycarbonat; Falcon, Becton Dickinson, Heidelberg, Germany) and precultured for 24 h in DMEM medium. NEK of passage 2 (1×10^6 for cocultures) were seeded onto the gels as described (Stark *et al*, 1999). After 24 h, medium was replaced by DMEM with 50 μg L-ascorbic acid per ml (Sigma) and cultures were raised to the air–liquid interface. Medium with or without additives (human recombinant bFGF 10 ng per ml, EGF 1 ng per ml, GM-CSF

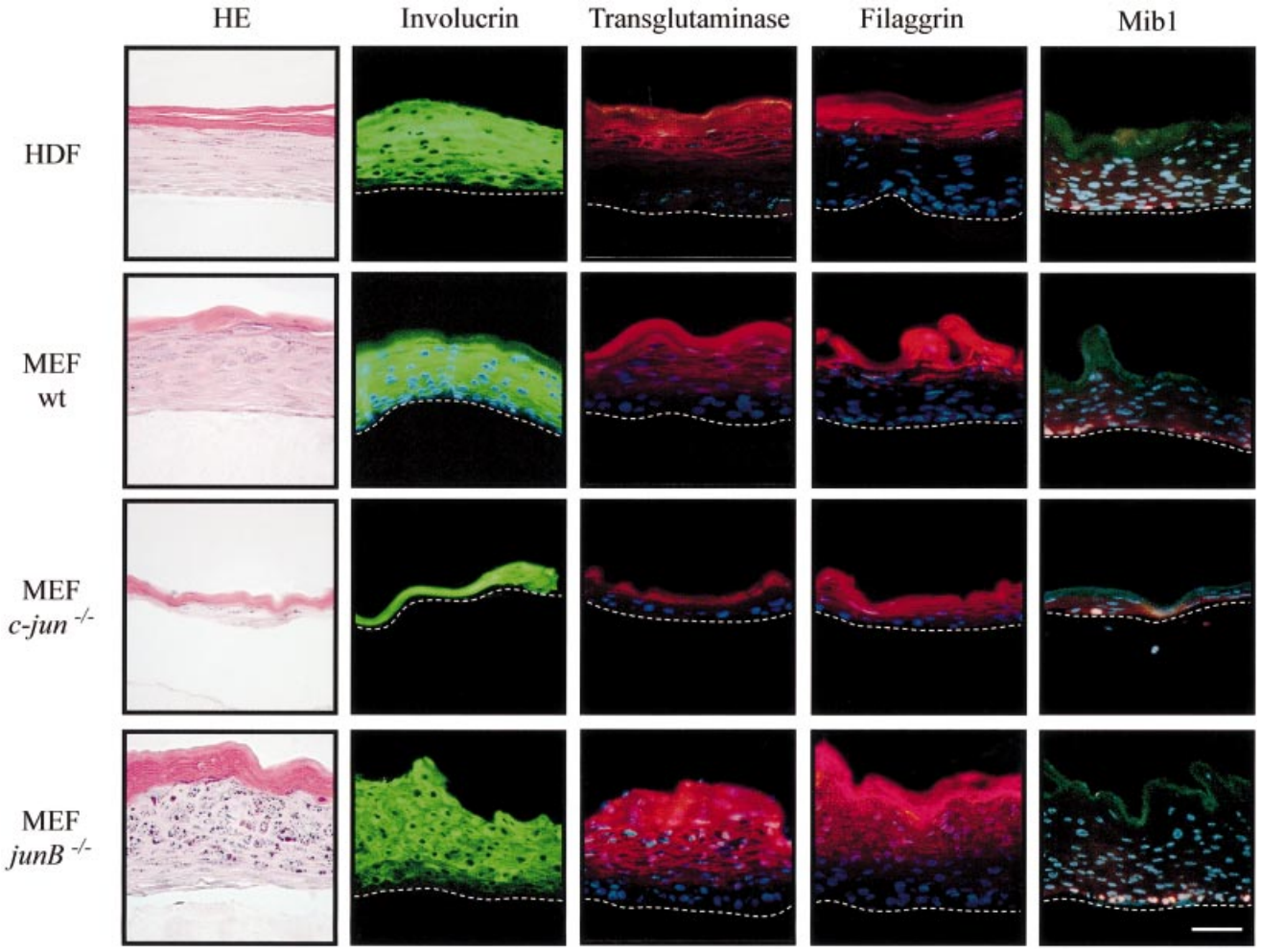

Figure 1. Epithelial architecture and differentiation of organotypic cultures depends on the genotype of cocultured fibroblasts. Epidermal tissue morphology of 7 d organotypic cultures combining human keratinocytes with human dermal fibroblasts (HDF), mouse embryonic wild-type (MEF wt), *c-jun*-deficient (MEF *c-jun*$^{-/-}$), and *junB*-deficient fibroblasts (MEF *junB*$^{-/-}$) (hematoxylin and eosin staining). By indirect immunofluorescence on cross-sections the epidermal differentiation markers involucrin, transglutaminase, and filaggrin are labeled. Proliferating basal cells are localized by labeling with a Ki67/Mib1 antibody. Nuclei were counterstained with bisbenzimide (blue). Epithelial border to the collagen gel is indicated by a dotted line. *Scale bar*: 100 μm.

100 ng per ml, KGF 10 ng per ml, TGF-α 1-ng per ml; R&D Systems, Wiesbaden, Germany) was replaced every 2 d. For cell counting fibroblasts were separated from the collagen by melting the gels in 4 × vol. PBS at 60°C. Cultures were either fixed according to a standardized protocol for routine histology or embedded in Tissue Tec OCT compound (Medim, Gießen, Germany) and frozen in liquid nitrogen vapor for cryosectioning. Cryosections at 4–6 μm were mounted on glass slides (Histobond, Medim) and fixed for 5 min in acetone.

Indirect immunofluorescence microscopy Cryosections were incubated with first antibodies against human keratin 1/10 (clone 8.60, Sigma), involucrin (clone SY5, Sigma), transglutaminase (Cell Systems, St Katharinen, Germany), filaggrin (Cell Systems) or Ki67/Mib1 (Dianova, Hamburg, Germany) overnight at 4°C and after washings for 1 h at room temperature with fluorochrome-conjugated secondary antibodies (Dianova) containing additionally 0.5 μg bisbenzimide per ml (Hoechst No. 33258) DNA dye for total nuclear staining (Stark *et al*, 1999). Specimens were embedded in Mowiol (Medim) and examined on a Leica microscope (Leitz DMRBE, Beksheim, Germany) equipped with epifluorescence optics.

Protein determination by ELISA Mouse and human IL-1α in the supernatant of organotypic cultures were measured by enzyme-linked immuno-sorbent assays (ELISA) (R&D Systems). Protein amounts in aliquots of 2-d-conditioned culture medium were measured and given as pg per ml with standard deviations of data derived from duplicate measurements from two independent experiments.

RESULTS AND DISCUSSION

In homologous as well as heterologous organotypic cocultures, the latter with MEF and human keratinocytes, a typical epidermal tissue is formed within 1 wk (**Fig 1**). Both the epithelial architecture and the expression of characteristic differentiation markers such as Keratin 1/10 (data not shown but see Szabowski *et al*, 2000), involucrin, transglutaminase, filaggrin (**Fig 1**), and loricrin (data not shown) are comparably induced by HDF and MEF. No major differences in cell proliferation within the basal layer of the epithelium can be seen whether mouse or human fibroblasts have been used (Mib1 staining). This is probably due to the homology of growth factors of both species, as also observed in two-dimensional feeder-layer cultures (Rheinwald and Green, 1975).

Recently we have demonstrated a double paracrine growth regulatory pathway between fibroblasts and keratinocytes that involves upregulation and release of IL-1 in keratinocytes, which then stimulates KGF expression in cocultured fibroblasts (Maas-Szabowski *et al*, 1999, 2000; see **Fig 4**). The identification of KGF as a product of fibroblasts was uncomplicated because KGF is produced by mesenchymal cells only, whereas the receptor is exclusively found in epithelial cells (Finch *et al*, 1989; Werner, 1998). When components are produced by both cell types, the heterologous coculture system provides a significant advantage for the identification of the origin of such regulatory factors released into the culture medium. More importantly the heterologous skin equivalent has the unique advantage that genetically modified embryonic fibroblasts from knockout or transgenic mice can be functionally analyzed. This model is of particular benefit when the

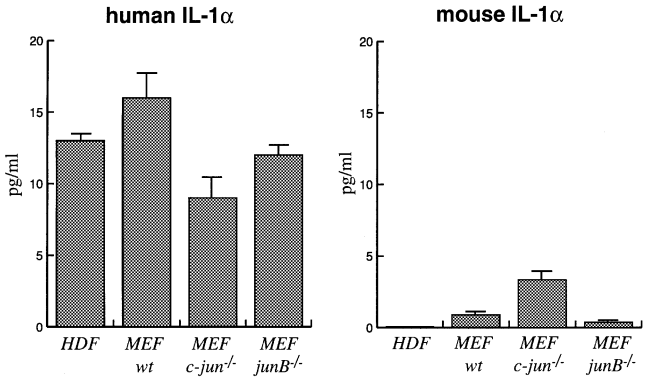

Figure 2. Quantitation of human and mouse IL-1α secreted into the medium in heterologous organotypic cocultures. Concentrations of human and mouse IL-1α in supernatants of 4 d cocultures with human fibroblasts (HDF), mouse (MEF) wild-type (wt), *c-jun*$^{-/-}$, and *junB*$^{-/-}$ fibroblasts were determined in aliquots of 2-d-conditioned media by ELISA and calculated as pg per ml. Bars represent means with standard deviation of duplicate measurements performed in two independent experiments.

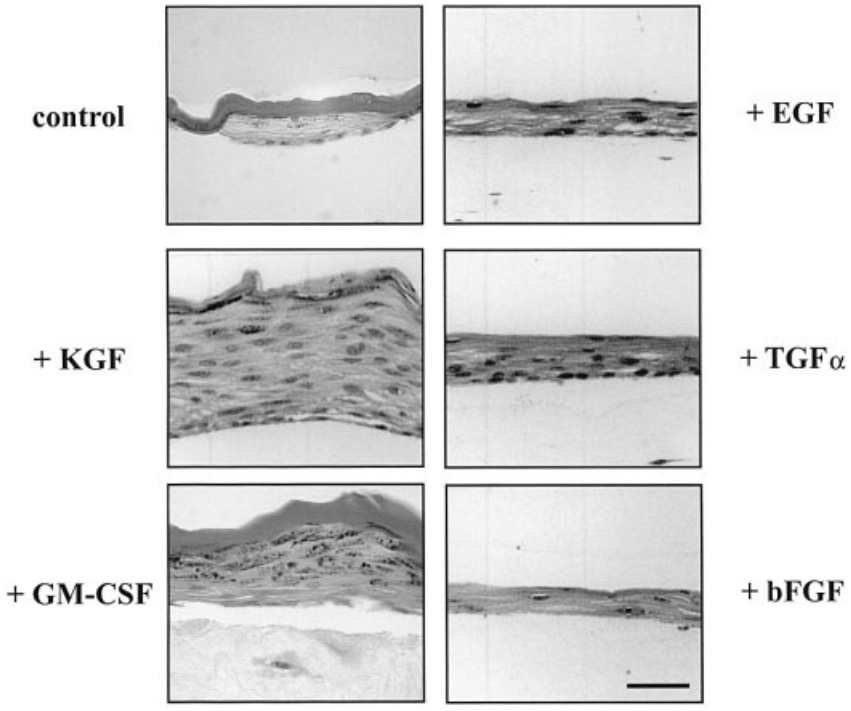

Figure 3. Epithelial tissue formation is differentially modulated by growth factors. Epidermal tissue morphology of 7 d organotypic cocultures containing *c-jun*-deficient fibroblasts in normal growth medium (control) or after addition of KGF (10 ng per ml), GM-CSF (100 ng per ml), EGF (1 ng per ml), TGF-α (1 ng per ml), and bFGF (10 ng per ml) (hematoxylin and eosin staining; *scale bar*: 100 μm).

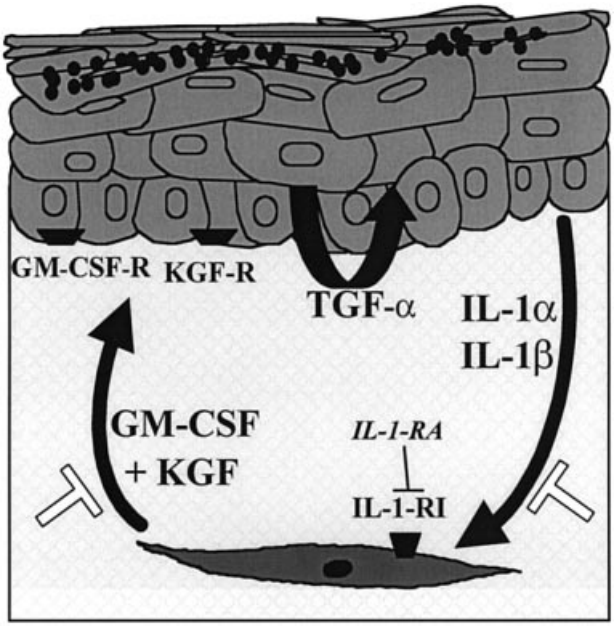

Figure 4. Schematic illustration of the mesenchymal-epithelial cross-talk in organotypic skin cultures. Inhibition of each part significantly decreased keratinocyte growth and differentiation.

mutant animals exhibit an embryonic lethal phenotype that does not allow functional analysis of the adult animal.

To extend our knowledge on the dermal–epidermal interplay we were particularly interested in studying signal transduction of IL-1 in cocultured fibroblasts to identify further downstream regulated genes that might be of importance in keratinocyte growth regulation. Several genes are regulated by IL-1 via members of the transcription factor family AP-1, namely *c-Jun* and *JunB* (Szabowski *et al*, 2000). Loss of function approaches led to the identification of specific functions of *c-Jun* and *JunB* in cell proliferation, differentiation, and apoptosis (Hilberg *et al*, 1993; Johnson *et al*, 1993; Eferl *et al*, 1999; Schorpp-Kistner *et al*, 1999; Wisdom *et al*, 1999; Kolbus *et al*, 2000). Recently 3T3-type cell lines were established from primary embryonic *c-jun*$^{-/-}$ (Schreiber *et al*, 1999) and *junB*$^{-/-}$ fibroblasts (S. Andrecht, A. Kolbus, P. Angel and M. Schorpp-Kistner, unpublished; Szabowski *et al*, 2000). As feeder cells for keratinocytes these Jun-defective fibroblasts behave like normal cells, in that plating efficiency of keratinocytes were comparable with wild-type and mutant feeder-fibroblasts (data not shown).

When embedded in the collagen matrix of organotypic cocultures human, mouse wild-type, and *junB*$^{-/-}$ fibroblasts show the same elongated cell phenotype and horizontal orientation, whereas *c-jun*$^{-/-}$ cells remain more rounded shaped. This is possibly due to the reduced expression of matrix metalloproteinases such as stromelysin (MMP-3) and interstitial collagenase (MMP-13) in *c-jun*$^{-/-}$ fibroblasts (Kolbus *et al*, 2000). Irrespective of the morphology, all fibroblasts remained vital through 10 d in the collagen gel as determined by MTT test and by cell number (data not shown).

The epithelia of organotypic cocultures containing *c-jun- and junB*-deficient fibroblasts, respectively, however, exhibited remarkably different phenotypes (**Fig 1**). Lack of c-Jun in fibroblasts resulted in a decreased keratinocyte proliferation and differentiation. In contrast, *junB*$^{-/-}$ fibroblasts induced keratinocyte hyperproliferation and altered differentiation (**Fig 1**). The hyperplastic epithelium exhibits a striking enlargement of the stratum granulosum and, accordingly, filaggrin labeling and increased cell layers labeled for loricrin (not shown, but see Szabowski *et al*, 2000). These differences in the epidermal phenotype were caused by differential amounts of mouse-cell derived KGF and GM-CSF, the latter not known so far as a keratinocyte growth factor. Organotypic cocultures containing *c-jun*$^{-/-}$ fibroblasts exhibit dramatically reduced levels of both growth factors as compared with wild-type cells, whereas they were extensively increased in those containing *junB*$^{-/-}$ fibroblasts (data not shown, see Szabowski *et al*, 2000). Rescue experiments performed by the addition of the respective cytokines or their neutralization by specific antibodies revealed the functional significance of both factors for epithelial tissue restoration (Szabowski *et al*, 2000).

The differential expression of KGF and GM-CSF by *c-jun*$^{-/-}$ and *junB*$^{-/-}$ fibroblasts, respectively, was not due to significantly altered levels of the major inducer IL-1 in the culture medium (**Fig 2**). Taking advantage of the heterologous coculture system we could substantiate earlier RNA data (Maas-Szabowski *et al*, 2000) and identify the cocultured keratinocytes as main producers of IL-1 by species specific ELISA assays (**Fig 2**). Moreover, *c-jun*$^{-/-}$ fibroblasts were refractory for the IL-1-induced KGF and GM-CSF expression (Szabowski *et al*, 2000).

Finally, such organotypic cocultures containing *c-jun*-deficient fibroblasts resulting in minimal keratinocyte growth are an excellent tool to identify further factors involved in keratinocyte growth and/or differentiation. Interestingly both KGF and GM-CSF were unable to substitute for fibroblasts and provide keratinocyte growth in their absence, indicating that further factors are required (data not shown). Among those TGF-α and EGF, both acting via the EGF-receptor, as well as bFGF, a fibroblast-derived mediator (Luger and Schwarz, 1990; Schröder, 1995) supported epithelial formation in *c-jun*$^{-/-}$ cocultures only to a minor extend (TGF-α and EGF) or not at all (bFGF) (**Fig 3**). Only addition of KGF and GM-CSF, respectively, resulted in a significant restoration of the epidermis, while optimal results were obtained with both factors simultaneously (Szabowski *et al*, 2000).

These results now allow to complement the previously described regulatory mechanisms in that in fibroblasts keratinocyte-released IL-1 induces the expression of the two factors KGF and GM-CSF via AP-1. Together with the autocrine acting TGF-α they regulate keratinocyte proliferation and differentiation in a well-orchestrated interplay (**Fig 4**). Whether TGF-α is constitutively expressed in keratinocytes or induced by KGF (Dlugosz *et al*, 1994) is not fully understood. Further regulatory mechanisms may be involved as well.

Thus, the heterologous organotypic coculture system using genetically modified mouse fibroblasts is a powerful tool to study functional complementation of deficiencies in the stromal compartment of engineered tissues *in vitro*. This model is a substantial improvement of our means to identify additional players in the molecular mechanisms of epithelial–mesenchymal cell interactions. This assay may be similarly advantageous to elucidate novel drug targets, such as low molecular weight components to modify keratinocyte growth and differentiation. More specifically, this model may serve as a basis for the dissection of disease mechanisms and for the design of novel mechanism-based therapeutic strategies for skin diseases such as psoriasis, dermatofibrosis, epithelial tumors, or deficiencies in wound healing.

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