Bone Marrow Stromal Cells, Preadipocytes, and Dermal Fibroblasts Promote Epidermal Regeneration in Their Distinctive Fashions

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INTRODUCTION

Mesenchymal-epithelial interaction plays an essential role in organogenesis and tissue regeneration at both embryonic and adult stages (Saunders et al., 1957; Gilbert, 2003). In adult tissue regeneration, which contributes to the maintenance of tissue homeostasis and to its repair against injury by various agents, tissue-localized mesenchymal cell types actively participate under conditions of mesenchymal-epithelial cell communication (Rheinwald and Green, 1975; Depuy et al., 2002; Ootani et al., 2003). Recently, bone marrow mesenchymal cell types have also been shown to infiltrate into impaired tissues and to be involved in tissue regeneration and repair of certain organs (Petersen et al., 1999; Krause et al., 2001; Mitaka, 2001).

Wound healing is a promising model for studying the mechanisms of tissue regeneration of various organs. This healing process demonstrates dynamic regenerative processes consisting of inflammation, angiogenesis, tissue remodeling, and scarring (Cotran et al., 1999). In wound healing of the skin, epidermal regeneration is a critical event for reorganizing normal cutaneous structure (Tomlinson and Ferguson, 2003). Dermal fibroblasts play a crucial role in epidermal reepithelialization through their growth and cytokine and extracellular matrix production (Green and Thomas, 1978; el-Ghalbzouri et al., 2002). We have shown that a mesenchymal cell type of mature adipocytes in the subcutis promotes the reorganization of the epidermal layer together with keratinocyte growth and differentiation (Sugihara et al., 1991; Sugihara et al., 2001). However, whether subcutaneous adipose tissue-localized preadipocytes and BMSCs are able to effect epidermal regenerations remains to be elucidated. In addition, it is still obscure whether BMSCs, preadipocytes, and fibroblasts, with their mesenchymal cell type-specific characters, induce different character to the epidermal regeneration of keratinocytes.

To clarify these important issues, we investigated the effects of BMSCs, subcutaneous preadipocytes, and dermal fibroblasts on epidermal regeneration of keratinocytes, using a cutaneous reconstruction model, in which three mesenchymal cell types were cocultured with keratinocytes under conditions with or without direct epithelial-mesenchymal contact. In the culture assemblies, characteristics of the epidermal structures together with keratinocyte growth, apoptosis, and differentiation were analyzed by histochemistry, morphometry, and immunohistochemistry. We also examined the expressions of IL-1α and c-Jun that are critical for mesenchymal-epithelial cross-talk in the skin (Maas-Szabowski et al., 2000; Ng et al., 2000; Szabowski et al., 2000; Angel et al., 2001; Li et al., 2003). To estimate the factors responsible for the epidermal regeneration under mesenchymal cell type-keratinocyte interactions, we also studied their gene expressions, using DNA microarray analysis.
MATERIALS AND METHODS

Preparation of Keratinocytes and Mesenchymal Stromal Cell Types

All procedures involving animal materials were performed in accordance with the regulations laid down by the ethical guidelines of Saga University. Keratinocytes were isolated from newborn Wistar rats (Charles River Japan, Yawata, Japan), as previously described (Sugihara et al., 2001). Briefly, skin with the epidermis and back and abdominal keratinocytes were scraped from the tibia of newborn rats and cultured on a collagen-coated dish in complete medium. Using this method, the keratinocytes were exposed to air, xed with 5% formalin, routinely processed, and stained with hematoxylin and eosin (H&E). In these stained sections we could easily identify the basal, prickle cell, granular and corniﬁcal layers of newborn rat skin.

To characterize the direct or indirect mesenchymal interaction in the skin (Szabowski et al., 2000), mouse monoclonal IL-1α and c-Jun antibodies, and rabbit polyclonal KGF antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To estimate the differentiating properties of keratinocytes, mouse monoclonal cytokeratin 10 (CK 10, a marker of suprabasal keratinocytes (Ivanyi et al., 1989) antibody (Dako), and mouse monoclonal cytokeratin 14 (CK 14, a marker of basal keratinocytes) (Dako) were used. To examine the expression of laminin and type IV collagen in culture assembly, using mouse monoclonal antibodies (Dako). Immunohistochemistry was carried out on deparafﬁnized sections by an avidin–biotin complex immunoperoxidase method, as described previously (Toda et al., 1999; Aoki et al., 2003). As a positive control, rat or human skin was used in an appropriate way for immunohistochemistry. As a negative control, phosphate-buffered saline or normal mouse and rabbit IgGs was used instead of each primary antibody. To avoid the possibility of nonspeciﬁc expression of IL-1α, c-Jun, and KGF antibodies (1 μg/ml neutralized with 1:10,000, and 10 μg/ml), respectively, were used. These results were always negative.

Cell Proliferation

Cell proliferation was examined by immunohistochemistry with mouse monoclonal proliferating cell nuclear antigen (PCNA) antibody (Dako), as previously described (Wilkins et al., 1992). A total of 1000 cells were cultured and the percentage of PCNA-positive nuclei was calculated. We also examined PCNA expression in newborn Wistar rat skin.

Apoptosis

Apoptosis was detected by immunohistochemistry with mouse monoclonal single-stranded DNA (ssDNA) antibody (Dako), as previously described (Sturmie et al., 1999). A total of 1000 cells were counted and the percentage of ssDNA-positive nuclei was calculated. We also examined ssDNA expression in newborn Wistar rat skin.

Microarray Analysis

To elucidate the gene expression of keratinocytes and mesenchymal cell types during epidermal regeneration, we carried out DNA microarray analysis, using a mesenchymal cell type-speciﬁc separating condition that did not permit the contamination of the mesenchymal cell type- and keratinocyte-derived mRNAs. The materials were obtained from three independent experiments. The gene expression of keratinocytes with mesenchymal supports was compared with that of keratinocytes, mesenchymal cell types from keratinocytes, mouse monoclonal IL-1α, c-Jun, and KGF antibodies (1 μg/ml neutralized with 1:10,000, and 10 μg/ml), respectively, were used. These results were always negative.

RNA Isolation and Reverse Transcriptase PCR

Total RNA was extracted using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Equal amounts of RNA (5 μg) were used in the reverse transcriptase (RT) reaction, using Random Hexamers as the primer, to generate ﬁrst-strand cDNA and SuperScrip reverse transcriptase (50 U/mL; Invitrogen Life Technologies, Frederick, MD), by following the manufacturer’s instructions. The conditions for each PCR were determined in an preliminary experiments and optimized for each set of primers. Expression levels of the IL-1α, IL-6 and TNF-α were examined. Expression levels of the IL-1α were determined with the following speciﬁc primers (5’ to 3’): CCCTTGAGGCCTGGAGGAAAGAACATC and CACATGCATGATGCAGTGTA; primers for KGF (5’ to 3’): GTACCAGTCAACCTAACGCTC and ACACGGCATATCCAGACCTCA; and speciﬁc primers for the c-Jun (5’ to 3’): CGCGCCGAGCCCGCAAACTAC and CACATGCATGATGCAGTGTA.
To amplify the GAPDH, the following primers (5' to 3') were used: TCCCTCAAGATGTGCAAGCGGTGTCTTA and TAGTGGTGATGTGCCCATTG. To amplify the GAPDH, the following primers (5’ to 3’) were used: TCCCTCAAGATGTGCAAGCGGTGTCTTA and TAGTGGTGATGTGCCCATTG. To amplify the GAPDH, the following primers (5’ to 3’) were used: TCCCTCAAGATGTGCAAGCGGTGTCTTA and TAGTGGTGATGTGCCCATTG.

The amplification condition was one cycle at 94°C for 4 min. Then for IL-1α, 37 cycles of denaturation (94°C for 1 min), annealing (54°C for 1 min), and extension (72°C for 1 min) with a final extension of 72°C for 7 min were used; the differences among the different amplifications were with regard to the number of cycles so that for c-Jun, there were 40 cycles, for KGF there were 36 cycles, and for GAPDH there were 34 cycles. The resulting PCR products were separated by electrophoresis on a 2.0% (wt/vol) agarose gel in TBE (90 mM Tris/90 mM boric acid/2 mM EDTA, pH 8.0) and stained with ethidium bromide. The expected sizes for the PCR products were IL-1α (941 base pairs), KGF (570 base pairs), c-Jun (486 base pairs), and GAPDH (308 base pairs). The density of each band was quantified using NIH Image software (http://rsb.info.nih.gov/nih-image/). We determined the relative gene expression by dividing the densitometric value of the mRNA RT-PCR product by that of the GAPDH product. The final ratio was obtained by dividing the value of treatment group by that of the control group.

Figure 1. Structures of regenerative epidermis caused by keratinocytes in a culture with (A–C) or without (D) mesenchymal-epithelial interaction. BMSCs (A), preadipocytes (B), and fibroblasts (C) promote the stratification of keratinocytes and result in formation of epidermal layers made up of basal, prickle cell, granular and cornified layers, whereas keratinocytes alone (D) form only a thin epidermal layer. (A) BMSCs cause keratinocytes to reorganize the rete ridge-like structure. (B) Preadipocytes induce an epidermal ridge-like structure in the epidermal layer. (C) Fibroblasts induce the greatest thickness of granular and cornified layers in regenerative epidermis. Left side: schematic illustration; right side: H&E staining. Scale bar: 50 μm (A–D).

Statistical Analysis

The data obtained through five to seven independent experiments were analyzed by Schelte test. Values represented the mean ± SD. p < 0.05 was considered significant.
RESULTS

**BMSCs, Preadipocytes, and Fibroblasts Distinctively Promote Epidermal Regeneration**

BMSCs, subcutaneous preadipocytes and dermal fibroblasts clearly promoted the stratification of keratinocytes, resulting in the formation of an epidermal layer consisting of basal, prickle cell, granular and cornified layers (Figure 1, A–C). In contrast, keratinocytes without these mesenchymal supports formed only a thin epidermal layer (Figure 1D) underwent apoptosis, and disappeared in the culture assembly after 14 d. Dermal fibroblasts, among the mesenchymal cell types, caused keratinocytes to reorganize the greatest thickness of prickle cell, granular and cornified layers (Figure 1C). Only BMSCs made it possible for keratinocytes to reconstruct a rete ridge-like structure in a basal layer (Figure 1A), whereas preadipocytes and fibroblasts could not. Preadipocytes induced an epidermal ridge-like structure in the regenerative epidermis (Figure 1B), whereas the others did not. CK 14, which displayed locally in basal layer of the skin in vivo (Figure 2A), was expressed in the basal to granular layer of the epidermis reorganized by keratinocytes with mesenchymal support (Figure 2, C–H). In normal rat skin (A), only basal cells express CK 14. However CK 14 is expressed in the basal to granular layer of epidermis regenerated by keratinocytes with BMSC (C), preadipocyte (E), or fibroblast (G) support. CK 10 is expressed in the suprabasal layer of the epidermis reorganized by keratinocytes with BMSC (D), preadipocyte (F), or fibroblast (H) support, as well as normal skin (B). No mesenchymal cell types display CK14 and CK 10. Scale bar: 50 μm (A–H).

**Mesenchymal Cell Types Promote Keratinocyte Growth**

Effects of BMSCs, preadipocytes and fibroblasts on keratinocyte proliferation were examined by immunohistochemis-
try for PCNA at 7 d in culture. PCNA-positive cells (insets of Figure 3) were limited within the basal layer in all conditions. The PCNA-labeling index of keratinocytes with each of three mesenchymal cell types was higher in a cell-type-independent manner than that of keratinocytes without mesenchymal support (Figure 3). The index of keratinocytes with mesenchymal support in vitro was similar to that of keratinocytes in vivo (Figure 3). There were no differences in the indices of each of the mesenchymal cell types among the all conditions.

**BMSCs and Preadipocytes Inhibit Keratinocyte Apoptosis**

Effects of BMSCs, preadipocytes and fibroblasts on keratinocyte apoptosis were determined by immunohistochemistry for ssDNA antibody at 7 d of culture (insets of Figure 4). The ssDNA-labeling index of keratinocytes with BMSCs was significantly the lowest, followed in order by that of keratinocytes with preadipocytes and then fibroblasts (Figure 4). The index of keratinocytes without any mesenchymal support was similar to that of keratinocytes with fibroblasts (Figure 4). These results suggest that the cellular turnover rate of keratinocytes with fibroblasts is higher than that of keratinocytes with BMSCs or preadipocytes, because there was no difference in growth of keratinocytes with each of three mesenchymal cell types. The index of keratinocytes in vivo was similar to that of keratinocytes with preadipocytes or fibroblasts (Figure 4). There were no differences in the ssDNA-positive rates of the mesenchymal cell types under mesenchymal-epithelial interaction.

**Immunohistochemical Expression of IL-1α, c-Jun, and KGF in Mesenchymal-Epithelial Interaction**

In this study, we investigated the expression of IL-1α, c-Jun, and KGF proteins, which are all critical for mesenchymal-epithelial cross-talk in the skin (Ng et al., 2000; Szabowski et al., 2000; Angel et al., 2001; Li et al., 2003). BMSCs and fibroblasts promoted a higher level of expression of IL-1α in keratinocytes than preadipocytes or keratinocytes alone. Fibroblasts had the highest IL-1α expression of mesenchymal cell types, followed in order by BMSCs and preadipocytes. IL-1α expression of keratinocytes in vivo was similar to that of keratinocytes with preadipocytes or of keratinocytes alone. Both fibroblasts in vivo and in vitro displayed IL-1α...
similarly (Figure 5, A, D, G, J, and M). c-Jun expression of keratinocytes with mesenchymal support was higher than that of keratinocytes alone. Fibroblasts had the greatest display of c-Jun, followed in order by BMSCs and preadipocytes. c-Jun expression of keratinocytes in vivo expressed more prominently than the three mesenchymal cell types. c-Jun expression of keratinocytes in vivo (M) was similar to that of keratinocytes in vitro (K). Both fibroblasts in vivo (N) and in vitro expressed c-Jun similarly. Only BMSCs (C) and preadipocytes (F) located near the regenerative epidermis expressed KGF, whereas it is not detected in any keratinocytes with (C, F, and I) or without mesenchymal support (L) and fibroblasts (I). In vivo, KGF (O) was detected in hair bulb (arrows) and external root sheath (arrowheads) of hair follicles, and dermal stroma, although its nonspecific expression was observed in cornified layer. Scale bar: 50 µm (A–L).

Figure 5. Immunohistochemical expression of IL-1α, c-Jun, and KGF in vitro and in vivo. Keratinocytes alone (J) as well as the cells with preadipocytes (D) express IL-1α weakly. Keratinocytes with BMSCs (A) as well as the cells with fibroblasts (G) strongly display IL-1α. In IL-1α expression of mesenchymal cell types, though weakly, fibroblasts (G) show the highest, followed in order by BMSCs (A) and preadipocytes (D). Keratinocytes among all conditions express IL-1α more prominently than the three mesenchymal cell types. IL-1α expression of keratinocytes in vivo (M) was similar to that of keratinocytes with preadipocytes (D) or of keratinocytes alone (J). Both fibroblasts in vivo (M) and in vitro (G) displayed IL-1α similarly. c-Jun expression of keratinocytes with mesenchymal support (B, E, and H) was higher than that of keratinocytes alone (K). In mesenchymal cell types, c-Jun expressions of fibroblasts (H) were the greatest, followed in order by BMSCs (B) and preadipocytes (E). Keratinocytes express c-Jun more prominently among all conditions than the three mesenchymal cell types. c-Jun expression of keratinocytes in vivo (N) was similar to that of keratinocytes alone in vitro (K). Both fibroblasts in vivo (N) and in vitro (H) expressed c-Jun similarly. Only BMSCs (C) and preadipocytes (F) located near the regenerative epidermis express KGF, whereas it is not detected in any keratinocytes with (C, F, and I) or without mesenchymal support (L) and fibroblasts (I). In vivo, KGF (O) was detected in hair bulb (arrows) and external root sheath (arrowheads) of hair follicles, and dermal stroma, although its nonspecific expression was observed in cornified layer. Scale bar: 50 µm (A–L).
Effects of Indirect Contact Between Mesenchymal Cell Types and Keratinocytes on Epidermal Morphogenesis

To determine whether mesenchymal-epithelial interaction is mediated by a direct cell-cell contact, keratinocytes and each of the mesenchymal cell types were cocultured under conditions that did not allow their direct contact. In these conditions, BMSCs and preadipocytes did not induce keratinocytes to organize rete ridge-like and epidermal ridge-like structures, respectively (Figure 6, A, C, E, and G). However, the other phenomena described above were clearly replicated even under conditions of indirect contact between keratinocytes and the mesenchymal cell types. These results indicate that direct contact between keratinocytes and BMSCs or preadipocytes are required for the morphogenesis of rete ridge-like and epidermal ridge-like structures, respectively. In addition, we did not detect tenasin in any epidermal cells, mesenchymal cell types and stroma under direct or indirect epithelial-mesenchymal contact. Interestingly, BMSCs and preadipocytes located near the epidermal layer, expressed α-SMA under direct mesenchymal-epithelial contact, although BMSCs and preadipocytes that were distantly located from the epidermal layer did not express α-SMA (Figure 6, B and F). BMSCs and preadipocytes did not express α-SMA under indirect mesenchymal-epithelial contact (Figure 6, D and H). Some fibroblasts can express α-SMA sporadically under both direct and indirect mesenchymal-epithelial contact.

Gene Expression by Microarray Analysis under Mesenchymal-Epithelial Interaction

We studied the gene expression of keratinocytes and mesenchymal cell types with or without mesenchymal-epithelial interaction under mesenchymal cell type-keratinocyte separating conditions, using DNA microarray analysis. Tables 1 and 2 summarize the expression of the various genes that are suggested to be critical for mesenchymal-epithelial cross-talk in the skin. Asterisks in Table 1 show the ratios of more than twofold decreased regulation of the following genes, compared with keratinocyte control: IL-1α in keratinocytes with preadipocytes, IL-1β in keratinocytes with preadipocytes and fibroblasts, JunB in keratinocytes with fibroblasts, and KGF in keratinocytes with preadipocytes. Asterisks of Table 2 indicate the ratios of more than twofold increased regulation of the following genes, compared with fibroblast control: IL-1α and IL-1β in BMSCs with keratinocytes, KGF in BMSCs, and preadipocytes and fibroblasts with keratinocytes. Tables 3 and 4 summarize the levels of expression of the various genes in keratinocytes and mesenchymal cell types, respectively, with or without mesenchymal-epithelial interaction.

Table 1. Cutaneous mesenchymal-epithelial cross-talk gene expression of keratinocytes with or without mesenchymal support

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank no.</th>
<th>Keratinocyte control</th>
<th>With BMSCs</th>
<th>With preadipocytes</th>
<th>With fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>D00403</td>
<td>1.00</td>
<td>0.62</td>
<td>0.32</td>
<td>0.64</td>
</tr>
<tr>
<td>IL-1β</td>
<td>M98820</td>
<td>1.00</td>
<td>0.54</td>
<td>0.25</td>
<td>0.57</td>
</tr>
<tr>
<td>c-Jun</td>
<td>X17163</td>
<td>1.00</td>
<td>1.21</td>
<td>0.91</td>
<td>0.53</td>
</tr>
<tr>
<td>JunB</td>
<td>X54656</td>
<td>1.00</td>
<td>0.79</td>
<td>0.86</td>
<td>0.46</td>
</tr>
<tr>
<td>KGF</td>
<td>X56551</td>
<td>1.00</td>
<td>0.94</td>
<td>0.16</td>
<td>0.53</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>U00620</td>
<td>1.00</td>
<td>0.75</td>
<td>0.60</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Values represent original ratios compared to the control keratinocyte gene expression as 1. Asterisks indicate ratios of more than twofold regulation.
We have shown that BMSCs, subcutaneous preadipocytes, and dermal fibroblasts with keratinocytes cause them to organize the rete ridge- and epidermal ridge-like structures, respectively. This suggests that BMSCs and preadipocytes may play a more vital role than fibroblasts and mature adipocytes in epidermal integration during wound healing. In addition, we have not shown the effects of mixed subpopulations of mesenchymal cell types on epidermal regeneration. To estimate the mechanisms involved in the regeneration, forthcoming studies with different subpopulations of adipose tissue and bone marrow would be helpful.

In this study, keratinocytes with BMSCs, preadipocytes and fibroblasts as well as keratinocytes in vivo expressed CK 10 in suprabasal layer, but not in basal layer. However, CK 14 was displayed in both basal and suprabasal keratinocytes of regenerative epidermis with mesenchymal support, whereas it is expressed only in basal cells in vivo. This suggests that suprabasal keratinocytes in reconstructed epidermis may retain an immature property of basal cells. To further examine the differentiating properties of the epidermal layer, we preliminarily tried to detect cytokeratins 5/8 and 19, and involucrin. But we did not detect these molecules in both reconstructed epidermis and natural skin. To address this issue, further studies are needed.

In our study, the growth, apoptosis, and IL-1α/c-Jun pathway of keratinocytes showed no change under conditions with or without direct contact between keratino-

### Table 2. Cutaneous mesenchymal-epithelial cross-talk gene expression of mesenchymal cell types with or without keratinocyte support

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank no.</th>
<th>Fibroblast control</th>
<th>BMSCs</th>
<th>Preadipocytes</th>
<th>Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>D00403</td>
<td>1.00</td>
<td>*2.69</td>
<td>1.18</td>
<td>1.16</td>
</tr>
<tr>
<td>IL-1β</td>
<td>M98820</td>
<td>1.00</td>
<td>*2.17</td>
<td>1.19</td>
<td>1.35</td>
</tr>
<tr>
<td>c-Jun</td>
<td>X17163</td>
<td>1.00</td>
<td>1.49</td>
<td>0.75</td>
<td>1.07</td>
</tr>
<tr>
<td>JunB</td>
<td>X54686</td>
<td>1.00</td>
<td>1.28</td>
<td>0.65</td>
<td>0.69</td>
</tr>
<tr>
<td>KGF</td>
<td>X65551</td>
<td>1.00</td>
<td>*4.21</td>
<td>*2.18</td>
<td>*2.00</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>U00620</td>
<td>1.00</td>
<td>1.81</td>
<td>1.11</td>
<td>1.47</td>
</tr>
</tbody>
</table>

Values represent original ratios compared to the control fibroblast gene expression as 1. Asterisks indicate ratios of more than twofold regulation.

### Table 3. Significantly regulated genes in keratinocytes with or without mesenchymal support

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank no.</th>
<th>Keratinocyte control</th>
<th>With BMSCs</th>
<th>With preadipocytes</th>
<th>With fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax-alpha</td>
<td>U49729</td>
<td>1.00</td>
<td>5.47</td>
<td>7.03</td>
<td>3.11</td>
</tr>
<tr>
<td>bcl-2</td>
<td>L14680</td>
<td>1.00</td>
<td>11.46</td>
<td>74.23</td>
<td>4.75</td>
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<td>c-fms</td>
<td>X61479</td>
<td>1.00</td>
<td>2.41</td>
<td>9.68</td>
<td>2.03</td>
</tr>
<tr>
<td>GIRQ4</td>
<td>L35771</td>
<td>1.00</td>
<td>7.49</td>
<td>19.01</td>
<td>20.07</td>
</tr>
<tr>
<td>Kv3.2</td>
<td>M84203</td>
<td>1.00</td>
<td>0.37</td>
<td>0.42</td>
<td>0.35</td>
</tr>
<tr>
<td>PRKAR1A</td>
<td>M17086</td>
<td>1.00</td>
<td>2.19</td>
<td>4.42</td>
<td>3.38</td>
</tr>
<tr>
<td>SHPS-1</td>
<td>D85183</td>
<td>1.00</td>
<td>24.62</td>
<td>15.69</td>
<td>10.84</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>M25890</td>
<td>1.00</td>
<td>0.18</td>
<td>2.92</td>
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<td>TXR2</td>
<td>D21158</td>
<td>1.00</td>
<td>3.32</td>
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<td>VSNL2</td>
<td>D13125</td>
<td>1.00</td>
<td>2.85</td>
<td>3.39</td>
<td>8.03</td>
</tr>
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</table>

Values represent original ratios compared to the control keratinocyte gene expression as 1. Abbreviations: 1) Bax-alpha, Bcl-2-associated X protein membrane isoform alpha; 2) bcl-2, B-cell leukemia/lymphoma protein 2; 3) c-fms, macrophage colony-stimulating factor I receptor; 4) GIRQ4, G protein-activated inward rectifier potassium channel 4; 5) Kv3.2, voltage-gated potassium channel protein 3.2; 6) PRKAR1A, cAMP-dependent protein kinase type I alpha regulatory subunit; 7) SHPS-1, receptor-like protein with SH2 binding site; 8) TXR 2, thromboxane A2 receptor; and 9) VSNL2, neural visinin-like protein 2.
cytes and each mesenchymal cell types. This suggests that autocrine and paracrine pathways regulate these events during epidermal regeneration in a direct cell-cell contact-independent manner. In contrast, BMSCs and preadipocytes cause keratinocytes to organize the rete ridge- and epidermal ridge-like structures, respectively. However, this only occurred in conditions with direct cell-cell contact, whereas fibroblasts failed to induce these structures.

It seems likely that BMSCs and preadipocytes integrate these skin-specific structures through their direct cell-cell contact-dependent mechanisms, which are different from IL-1α/c-Jun pathway. In addition, BMSCs and preadipocytes which were located near the epidermal layer expressed α-SMA, suggesting that they regain myofibroblastic differentiation only under their direct contact with keratinocytes. The myofibroblastic differentiation of BMSCs and preadipocytes may contribute to wound contraction. Finally, to estimate the transdifferentiation of mesenchymal cell types into keratinocytes, we carried out the following preliminary study, using a PKH 2 fluorescent staining kit (Zynaxis Cell Science, Malvern, PA), as previously described (Toda et al., 1993). When PHK 2 dye-labeled mesenchymal cell types and -nonlabeled keratinocytes were cocultured, we did not detect the dye-labeled cells in the regenerative epidermis. It seems unlikely that mesenchymal cell types may transdifferentiate into keratinocytes. However, the issue is crucial, and thus we will study this process, using in vitro and in vivo systems.

Mesenchymal-epithelial cross-talk in skin is controlled by various molecules, including IL-1α, IL-1β, c-Jun, JunB, and KGF (Maas-Szabowski et al., 2000; Ng et al., 2000; Szabowski et al., 2000; Angel et al., 2003). In this study, fibroblasts and BMSCs promote IL-1α protein expression of keratinocytes more prominently than preadipocytes. Fibroblasts show the highest c-Jun protein expression in mesenchymal cell types with keratinocyte support, followed in order by BMSCs and preadipocytes. This suggests that fibroblasts, BMSCs, and preadipocytes, in that order, may be involved in the mesenchymal-epithelial cross-talk in skin. In our study, KGF protein was expressed in BMSCs and preadipocytes only under their direct contact with keratinocytes, suggesting that BMSCs and preadipocytes may be involved in KGF production during epidermal regeneration. Furthermore, the degree of IL-1α, c-Jun, and KGF protein expression in keratinocytes and mesenchymal cell types with or without their interaction was not parallel to that of these mRNA expressions by microarray and RT-PCR analyses. We have not clarified the reasons for this discrepancy, and it is conceivable that the pathway of IL-1α, c-Jun, and KGF protein production from their mRNA expression may be complexly regulated by unknown factors. This idea is supported by recent evidence that the discrepancy between proteome and transcriptional regulation, apart from different translation efficiency, indicates a changed turnover.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank no.</th>
<th>Fibroblast control</th>
<th>BMSCs</th>
<th>Preadipocytes</th>
<th>Fibroblasts</th>
</tr>
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<tbody>
<tr>
<td>11DH</td>
<td>J05107</td>
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<td>0.36</td>
<td>0.29</td>
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<td>beta-ARK1</td>
<td>M87854</td>
<td>1.00</td>
<td>0.23</td>
<td>0.29</td>
<td>0.33</td>
</tr>
<tr>
<td>Ekh3</td>
<td>U21954</td>
<td>1.00</td>
<td>0.34</td>
<td>0.49</td>
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<tr>
<td>IGFBP5</td>
<td>M62781</td>
<td>1.00</td>
<td>0.09</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Pep T2</td>
<td>D63149</td>
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<td>PYY</td>
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<td>0.10</td>
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<td>VAMP-2</td>
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<td>3.71</td>
<td>8.84</td>
<td>2.84</td>
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</table>

Values represent original ratios compared to the control fibroblast gene expression as 1. Abbreviation: 1) 11-DH, corticosteroid 11-beta-dehydrogenase isozyme 1; 2) beta-ARK1, beta-adrenergic receptor kinase 1; 3) Ekh 3, ephrin type-A receptor 7; 4) IGFBP5, insulin-like growth factor binding protein 5; 5) Pep T2, kidney oligopeptide transporter; 6) RET1, RET ligand 1; 7) PYY, peptide YY; 8) VAMP 2, vesicle-associated membrane protein 2.
rate of proteins in different conditions (Ohlmeier et al., 2004).

In this study, there was a discrepancy between microarray- and RT-PCR-derived data in keratinocytes and mesenchymal cell types. In microarray the housekeeping genes GAPDH, cytoplasmic beta actin and 40S ribosomal protein S29 were used as a control, whereas only GAPDH was used in RT-PCR. Therefore, different selection of the housekeeping genes may be responsible for the discrepancy above. It is also conceivable that the discrepancy may depend on the labeling efficiency of cDNAs with fluorescence dyes in microarray. Although the precise reason remains unclear, our results support the recent facts that there may be a discrepancy among the data of gene expressions by DNA microarray, RT-PCR, etc. (Eriksson et al., 2003). Thus, we think that one should estimate the validity of the microarray-based gene expressions in our study.

BMSCs and preadipocytes may not be the major cell types of normal skin. However, as we described, BMSCs and preadipocytes promote epidermal regeneration. Recent studies (Kawamoto et al., 2002; Sata et al., 2002; Kim et al., 2003; Korbling and Estrov, 2003) have shown that marrow-derived endothelial progenitor cells exist in adult circulation and that the progenitor cells differentiate into endothelial cells after homing to neovascularization sites. Preadipocytes develop from mature adipocytes under adipocyte-endothelial cell interaction (Aoki et al., 2003). Taken together, these results suggest that homing BMSCs and preadipocytes may contribute to epidermal regeneration in wound healing and to homeostasis of skin structure.

In conclusion, we have shown that not only skin-localized fibroblasts and preadipocytes, but also BMSCs promote epidermal regeneration. We have also shown that direct contact between keratinocytes and BMSCs or preadipocytes is required for the skin-specific morphogenesis. This suggests that BMSCs and preadipocytes may be applicable to cellular therapy for skin injuries.

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