Overexpression of Bone Morphogenetic Protein-6 (BMP-6) in the Epidermis of Transgenic Mice: Inhibition or Stimulation of Proliferation Depending on the Pattern of Transgene Expression and Formation of Psoriatic Lesions

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Abstract. Bone morphogenetic protein-6 (BMP-6) belongs to the family of TGF-β–related growth factors. In the developing epidermis, expression of BMP-6 coincides with the onset of stratification. Expression persists perinatally but declines after day 6 postpartum, although it can still be detected in adult skin by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. We constitutively overexpressed BMP-6 in suprabasal layers of interfollicular epidermis in transgenic mice using a keratin 10 promoter. All mice expressing the transgene developed abnormalities in the skin, indicating an active transgene-derived factor. Depending on the pattern of transgene expression, the effects on proliferation and differentiation were completely opposite. Strong and uniform expression of the BMP-6 transgene resulted in severe repression of cell proliferation in embryonic and perinatal epidermis but had marginal effects on differentiation. Weaker and patchy expression of the transgene evoked strong hyperproliferation and parakeratosis in adult epidermis and severe perturbations of the usual pattern of differentiation. These perturbations included changes in the expression of keratins and integrins. Together with an inflammatory infiltrate both in the dermis and in the epidermis, these aspects present all typical histological and biochemical hallmarks of a human skin disease: psoriasis.

BONE morphogenic protein-6 (BMP-6)[1] is a member of the TGF-β superfamily of growth factors (Lyons et al., 1989*a*; Lyons et al., 1991). These signalling molecules are produced as precursors that are proteolytically cleaved to yield the carboxy-terminal active peptide. The mature factor binds to specific cell surface receptors on the target cells and exerts multiple biological activities in vitro and in vivo (Sato et al., 1990; Massague et al., 1991; Drozdoff et al., 1994; Gitelman et al., 1994, 1995; Perides et al., 1994; Hughes et al., 1995). Expression of BMP-6 in mammals was demonstrated in various cell types of the nervous system, smooth muscle cells, growth plate chondrocytes, neonatal bronchiolar epithelium, cornea, and epidermis (Lyons et al., 1989*b*; Wall et al., 1993; Schluesener and Meyermann, 1994; Carey and Liu, 1995; Schluesener and Meyermann, 1995; Schluesener et al., 1995; Tomizawa et al., 1995). In murine epidermis, expression of BMP-6 is first seen after day 15 postcoitum and accompanies the acquisition of a multilayered structure of this organ. BMP-6 synthesis is confined to postmitotic keratinocytes undergoing terminal differentiation in the suprabasal compartment of interfollicular epidermis. Expression persists perinatally and declines to low levels in adult skin after day 6 postpartum (Lyons et al., 1989*b*; Wall et al., 1993).

Proliferation and differentiation in the epidermis is tightly controlled in order to maintain homeostasis. Cell division is usually restricted to the basal layer, whereas cells in the suprabasal layers are quiescent and in the process of terminal differentiation. This process can be followed by a switch in expression of marker keratins from the basal cell specific keratins 5 and 14 to the suprabasal cell specific keratins 1 and 10. (Fuchs and Green, 1980; Tseng et al., 1982; Woodcock-Mitchell et al., 1982; Nelson and Sun, 1983; Skerrow and Skerrow, 1983). Perturbations in the distribution of proliferating and differentiating cells and the usual pattern of terminal differentiation are seen in many pathological skin conditions of humans, most notably in psoriasis. Psoriatic skin is characterized by epidermal thickening and the presence of nucleated cells in the

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1. *Abbreviations used in this paper*: BMP-6, bone morphogenetic protein-6; PCNA, proliferating cell-associated nuclear antigen; RT-PCR, reverse transcriptase-polymerase chain reaction.

stratum corneum, reflecting the increased proliferative potential of suprabasal cells (Weinstein and VanScott, 1965; Lavker and Sun, 1983; Leigh et al., 1985). In addition to parahyperkeratosis, a change in the expression of marker keratins is observed: suprabasal localization of the basal type keratin 14 including the outermost layers of the stratum corneum, partial repression of the differentiation associated keratins 1 and 10, and interfollicular induction of the hyperproliferative marker keratins 6 and 16 (Weiss et al., 1984; Stoler et al., 1988). Similarly, the expression domains of some integrin subunits are expanded from basal into suprabasal layers in psoriatic lesions (for review see Watt and Hertle, 1994). Integrins could be directly involved in lesion formation because ectopic integrin expression as well as a hypomorphic mutation in the leukocyte integrin subunit β_2/CD18 induce plaques in mice very similar to human psoriatic lesions (Lever and Schaumburg-Lever, 1975; Wade and Finan, 1990; Carroll et al., 1995; Bullard et al., 1996).

Alterations in the expression patterns for several growth factors, growth factor receptors, and cytokines have been observed in psoriatic skin including TGF-α and TGF-β (Elder et al., 1989; Kane et al., 1990). However, transgenic mice with epidermal changes in growth factor expression including TGF-α and TGF-β so far failed to develop psoriatic lesions (Vassar et al., 1991; Dominey et al., 1993; Sellheyer et al., 1993; Cui et al., 1995). The TGF-β–related factor BMP-6 appears in differentiating keratinocytes before the expression of the differentiation markers, keratins 1 and 10, and has so far been demonstrated to inhibit keratinocyte proliferation only in vitro (Drozdoff et al., 1994). Possible functions in triggering terminal differentiation, however, or possible roles in disturbances of epidermal homeostasis remained uncertain.

To analyze functions of BMP-6 in controlling proliferation and differentiation of epidermal cells in vivo and to evaluate the possible role of this TGF-β–related molecule in the etiology of psoriatic skin diseases, we generated transgenic mice overexpressing BMP-6 in suprabasal layers of interfollicular epidermis using a keratin 10 promoter driven transgenic construct (Werner et al., 1993). The mitotic response of the keratinocytes to BMP-6 in vivo was positive or negative depending on the pattern of transgene expression. In addition, animals of two independent lines developed lesions with all characteristics of human psoriasis.

Materials and Methods

Construction of the Expression Vector and Generation of Transgenic Mice

The keratin 10–based expression vector and a human cDNA coding for BMP-6 had been described before (Wall et al., 1993; Werner et al., 1993; Drozdoff et al., 1994). The cDNA was inserted into the SmaI site between the beta-globin intron and the polyadenylation sequence from the human gene for growth hormone. The expression cassette was excised by KpnI digestion, gel purified, and used for pronuclear microinjection of fertilized eggs of strain FVB/N essentially as described (Hogan et al., 1986). Offsprings were biopsied at the ears or tails. After DNA extraction, PCR-analysis was performed using primers derived from the human BMP-6 cDNA (5′-ctt ggt tca cct tat gaa ccc c-3′) and from the poly(A) addition signal region from the human gene for growth hormone (5′-taa tcc cag caa ttt ggg agg c-3′). Subsequently, the samples were subjected to agarose gel electrophoresis to visualize the diagnostic fragment of size 480 bp.

In Situ Hybridization and Reverse Transcriptase–Polymerase Chain Reaction Analysis

In situ hybridization experiments were performed under high stringency conditions essentially as described (Jones et al., 1991) with the following modification. Fresh tissue samples were fixed in methanol/dimethylsulfoxide (4:1), dehydrated in ethanol and xylene, and embedded in paraffin for sectioning. After rehydration, the sections were refixed with 4% paraformaldehyde and hybridized to a ^{35}S-labeled cRNA probe complementary to the sequence preceding the human growth hormone polyadenylation signal (Werner et al., 1993).

Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis was performed using DNAse I–treated pools of total RNA from different tissues. Reverse transcription was accomplished using the RT-PCR kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. PCR was performed using vent (exo-) polymerase (New England Biolabs, Beverly, MA) with 64°C annealing temperature and 35 cycles. Primer B6 (AGACCTGGGATGGCAGG) is derived from a part of the mature region of BMP-6 that is identical in mouse and man (position 1473–1489 in the murine cDNA, Gitelman et al., 1994; and position 1277–1293 in the human cDNA, Celeste et al., 1990). The first reverse primer aB6 (ACCATCCCGCTTCGCTGTGC) was derived from the 3′-untranslated sequence of the murine BMP-6 cDNA (position 1855–1836; Gitelman et al., 1994), and the second reverse primer pA (GGAGTGGCAACTTCCAGG) was derived from the region preceding the polyadenylation sequence of the human gene for growth hormone (Werner et al., 1993). Thus, the primer pair B6/aB6 specifically amplifies murine BMP-6 sequences yielding a 363-bp diagnostic band, and the primer pair B6/pA specifically recognizes transgene-derived sequences yielding a 390-bp diagnostic fragment. In both cases, the amplified sequences encompass nearly the entire parts of the mRNAs coding for the mature region of the growth factor. Primers for amplification of a 403-bp β-actin cDNA fragment were act1 (GTGGGCCGCTCTAGGCACCA) and act2 (TAGCCCTCGTAGATGGGCACAG).

Histology and Ultrastructural Analysis

Hematoxilin, eosin, and chloroacetate esterase staining were performed according to standard protocols. For transmission electron microscopy, tissues were fixed in 2.5% glutaraldehyde (pH 7.2) overnight, postfixed in 1% OsO_4 for 2 h, and dehydrated in graded ethanol. After carrying the sections through propylene oxide as an intermedium, the tissues were embedded in agar 100 resin (PLANO, Wetzlar, Germany) and submitted to polymerization at 60°C for 24 h. Semithin and 80-nm ultrathin sections were cut with an ultramicrotome ultracut (Reichert Jung, Vienna, Austria) and ultrathin sections were placed onto slit foils. Ultrastructural analysis and photomicroscopy was performed with a transmisssion electron microscope (model EM 410; Philips, Eindhoven, Netherlands).

For scanning electron microscopy tissue, specimens were fixed and postfixed as described above. After dehydration in graded acetone and critical point drying (CPD 030; Balzers, S.P.A., Nordenstadt, Germany), specimens were placed onto Leit C–covered metal plates (Neubauer, Münster, Germany), sputtered with gold in a coating unit (model E5100; Polaron Instruments, Inc., Munich, Germany), and analyzed under a scanning electron microscope (model DSM962; Carl Zeiss Inc., Oberkochen, Germany).

Immunostaining

Fresh tissue samples were fixed in methanol/dimethylsulfoxide (4:1), embedded in paraffin, and sectioned. Frozen sections for antiintegrin immunostaining were fixed in acetone. For double-label immunostaining with monoclonal antibodies specific for keratin 14 and keratins 1/10 (clone CKB1 and clone 8.6 from Sigma Immunochemicals, Deisenhofen, Germany) the rehydrated paraffin sections were pretreated with pronase. Antibody CKB1 (antikeratin 14) was used at a 1/200 dilution and antibody 8.6 (antikeratins 1/10) at a 1/400 dilution. Fluorochrome-labeled secondary antibodies (Nos. 115-165-071 and 115-015-075; Dianova, Hamburg, Germany) were used at a 1/600 dilution. The rat monoclonal antibody specific for the α_6 integrin subunit (GoH3 from Progen, Heidelberg, Germany; No. 10709) was used at a 1/2 dilution and the fluorochrome labeled secondary antibody (No. 212-166-082; Dianova) was used at a 1/800 dilution. The affinity purified polyclonal anti–BMP-6 antibody (Wall et al., 1993) was used at a 1/200 dilution. A polyclonal antiserum raised against a carboxy-terminal peptide of mouse keratin 6 as described (Roop et al.,

1984) was used at a 1/5,000 dilution. The staining procedures have been described previously (for references see Wall et al., 1993).

For anti-proliferating cell-associated nuclear antigen (PCNA) staining, 5 μm paraffin sections were incubated with 1% hydrogensuperoxide in methanol after dewaxing and refixed with 4% paraformaldehyde after rehydration. The primary antibody (clone PC10, No. 32551A; PharMingen, Hamburg, Germany; Garcia et al., 1989) was used at a 1/100 dilution and binding was visualized using the StreptAB-Complex/HRP-Duett System, according to the manufacturers instructions (No. K492; DAKO, Hamburg, Germany).

BrdU Labeling Experiments

These experiments were performed using the Cell Proliferation Kit (No. RPN20; Amersham Corp., Braunschweig, Germany) according to the manufacturer's instructions with the following modifications: age matched transgenic animals and normal controls were sacrificed after 3 h of labeling, and tissue samples were fixed with methanol/dimethylsulfoxide (4:1), embedded in paraffin, and sectioned. Rehydrated paraffin sections were then refixed with 4% paraformaldehyde and treated with pronase before incubation with the primary antibody–nuclease mix.

Results

Phenotypes of Transgenic Mice

Transgenic founders carrying the BMP-6 expression vector (Fig. 1) were identified by PCR analysis and fell into three classes: severely affected founders were born with a tightly stretched skin with a shiny and pergament like appearance lacking dermatoglyphic patterning (Fig. 2 *a*). All three founder animals of this type were alive and feeding the day after birth. They were sacrificed immediately. Four moderately affected transgenic founders and offspring from two of those (lines VI-6-1 and VI-6-32) looked normal at birth but developed a shiny and flaky skin, starting six days after birth, which could be best observed in pigmented skin of transgenic FVB/N X C57/Bl6 F1 pups (Fig. 2 *b*). 2–3 wk after birth, these mice developed lesions characterized by inflammatory processes. These lesions could first be observed on the markedly thickened ears (Fig. 2, *c* and *d*) but also affected other body sites including dorsal and lateral skin. The condition worsened with age eventually encompassing the entire skin and was accompanied by a progressive hair loss, flaky skin, and abnormalities of the nails (Fig. 2, *c–e*). Some animals of lines VI-6-1 and VI-6-32 showed a growth retardation when compared to nontransgenic littermates (Fig. 2 *b*). This variable growth retardation was most notably visible in large litters. Removal of nontransgenic littermates resulted in lean but approximately normally sized transgenic adults (Fig. 2 *c*), indicating a reduced competitivity of these transgenic pups rather than a physiological problem in food uptake or processing. This assumption is strengthened by the absence of histological abnormalities in oral mucosa or stomach epithelia in these transgenics (data not shown). In addition, full thickness grafts of transgenic dorsal skin onto nontransgenic recipients remained hyperkeratotic, proving a primary epidermal defect (data not shown). Two additional transgenic founder mice (VI-6-19 and VI-6-21) and their transgenic offspring showed no abnormalities.

Expression of BMP-6 in Skin and Other Epithelia

Transgene expression was analyzed by in situ hybridization experiments using a riboprobe derived from the region preceding the polyadenylation signal in the human gene for growth hormone. Severely affected neonatal founders showed a strong and uniform pattern of transgene expression in interfollicular epidermis (Fig. 3, *a* and *a'*). Higher power magnification demonstrated strong accumulation of transgene-derived RNA in the suprabasal layers and exclusion from the basal layers (Fig. 3, *b* and *b'*). By contrast, neonates of the moderately affected line VI-6-1 displayed a much weaker accumulation of transgene derived RNA in the epidermis. In addition to generally being weak, the pattern of expression was patchy but still properly confined to the suprabasal layers (Fig. 3, *c* and *c'*).

Endogenous and transgene-dependent expression of BMP-6 were compared in skin, oral mucosa, and stomach of adult mice by RT-PCR analysis. The specificity of the primer pair B6/aB6 for murine BMP-6 sequences and B6/pA for transgene-derived sequences was verified using the transgene construct and a murine BMP-6 cDNA as templates. Starting from equimolar amounts of templates, amplification with the two primer pairs resulted in comparable yields of the diagnostic bands in the combinations B6/aB6+murine BMP-6 cDNA and B6/pA+transgene construct and no product in the heterologous combinations (data not shown). While endogenous murine BMP-6 RNA is detected in skin, tongue, and stomach of normal mice and mice of lines VI-6-1 and VI-6-32, transgene-derived human BMP-6 RNA is detectable in the skin of transgenic mice of both lines and in oral mucosa of line VI-6-32, but not in stomach (Fig. 4). The yield of amplified product from skin using the B6/pA primer pair always exceeded

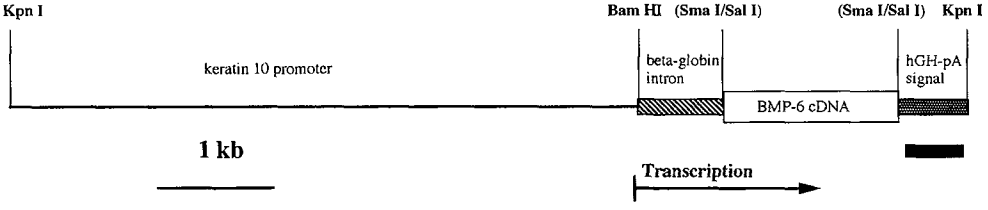

Figure 1. BMP-6 expression vector. A human cDNA for BMP-6 was brought under the control of the bovine keratin 10 promoter. A beta-globin intron and a polyadenylation sequence derived from the human gene for growth hormone were included. The black rectangle represents the region of the construct which was used as a probe for in situ hybridization analysis.

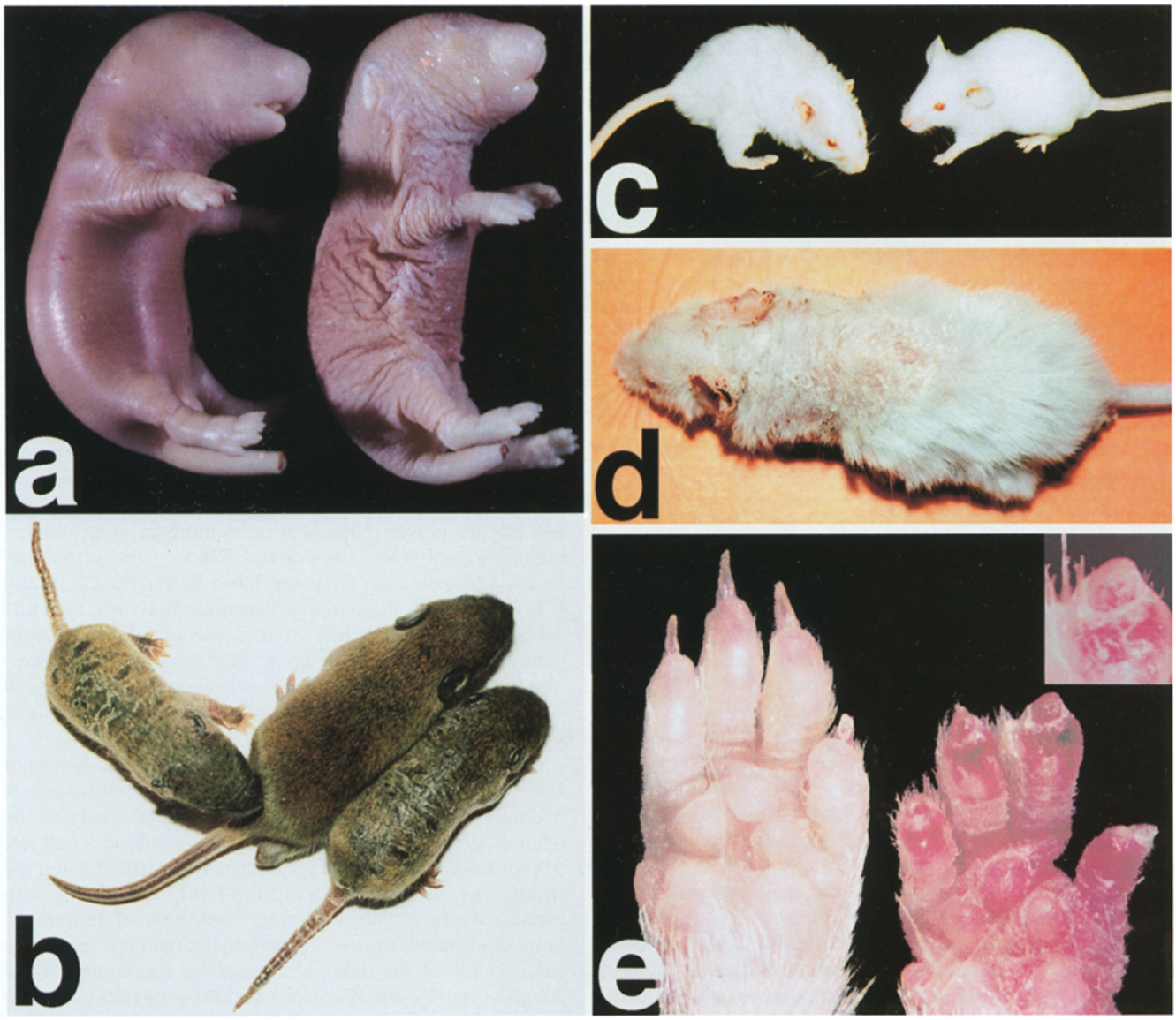

Figure 2. Phenotypes of BMP-6 transgenic mice. (*a*) Severely affected transgenic neonate (*left*) and control littermate (*right*). Note the absence of dermatoglyphic patterning and the tightly stretched skin of the transgenic founder. (*b*) Moderately affected 7-d-old mice of line VI-6-1 (*left, right*) and control littermate (*middle*). The skin is shiny and flaky and eruption of hair shafts is delayed. (*c*) 8-wk-old mouse of line VI-6-1 (*left*) and normal control mouse (*right*). (*d*) 5-mo-old mouse of line VI-6-1 with fully developed lesions and thickened ear lobes. (*e*) Paws of normal (*left*) and transgenic mice (*right*) of line VI-6-32. Note the swelling of the palms of the paws and toes as well as the abnormal nails of transgenic mice (*e*, *inset*).

the yield obtained with the B6/aB6 primer pair, indicating an overexpression of exogenous BMP-6 over endogenous BMP-6 in transgenic skin. In addition, the ratio of endogenous BMP-6 signal to β-actin signal in skin is comparable between transgenic lines and normal mice, indicating that there is no induction or repression of endogenous BMP-6 on the RNA level by transgene derived-factor (Fig. 4).

Expression of BMP-6 was also analyzed on the protein level in normal and transgenic animals (Fig. 5). While there is strong and generalized expression of BMP-6 in interfollicular epidermis of normal neonates on the protein level (Fig. 5 *a*), there is only a very faint immunostaining in normal adult epidermis after prolonged incubation in the substrate solution (Fig. 5 *b*; sections in *a*, *c*, and *d* developed for 30 min in the substrate solution, the section in *b* for 4 h). In adult animals of the moderately affected transgenic lines VI-6-1 and VI-6-32, however, BMP-6 protein was synthesized in a mosaic pattern both in interfollicular epidermis and in the infundibula of hair follicles (Fig. 5, *c* and *d*). There is a direct correlation between BMP-6 expression and acanthosis. While regions around strongly staining cells are markedly thickened, adjacent regions with weaker or no staining show no pronounced thickening (Fig. 5 *c*). Because of the resemblance of protein distribution and relative amount of transgene-derived RNA as well as the absence of endogenous BMP-6 mRNA induction, the majority of BMP-6 in adult epidermis obtained from mice of lines VI-6-1 and VI-6-32 is most likely de-

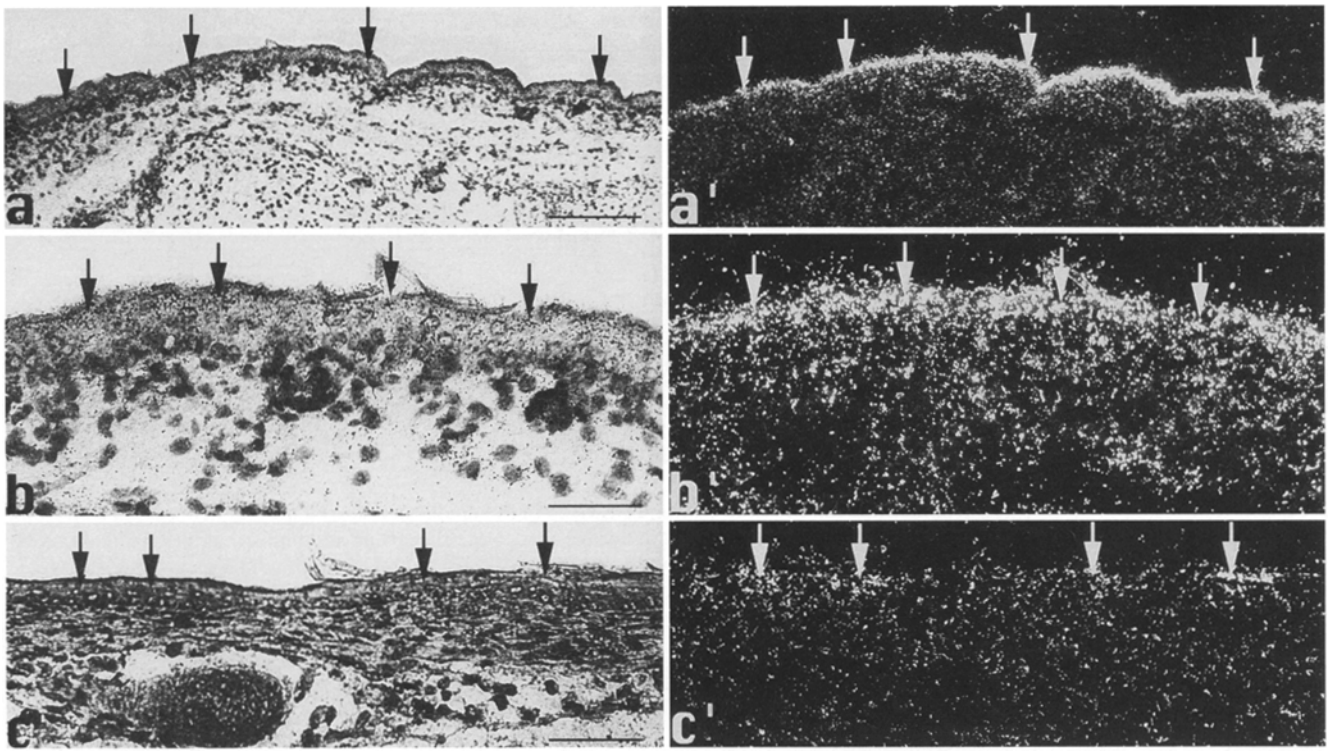

Figure 3. Expression of transgene-derived RNA. Brightfield (*a–c*) and corresponding darkfield (*a'–c'*) photomicrographs of in situ hybridization experiments on skin sections (*a–b'*) from severely affected neonatal transgenic founders and (*c* and *c'*) from a neonate of the moderately affected line VI-6-1. Note the strong and uniform labeling in the epidermis of the severely affected founder and the weak and patchy labeling in the neonate from a moderately affected line using a transgene-specific probe derived from the region preceding the polyadenylation signal in the human gene for growth hormone. Arrows point to labeling in interfollicular epidermis. Bars: (*a*) 200 μm; (*b* and *c*) 50 μm.

rived from the human transgene (Fig. 3, *c* and *c'*, Fig. 4 and Fig. 5, *c* and *d*). Skin sections from unaffected adults of lines VI-6-19 and VI-6-21 showed no difference in staining for BMP-6 when compared to adult skin from nontransgenic controls, suggesting an inactive transgene (data not shown).

Histology of Transgenic Skin

There were no apparent differences in epidermal thickness and organization between the class of severely affected neonatal transgenic founders and control littermates (data not shown). However, skin from adult mice of lines VI-6-1 and VI-6-32 showed distinct features absent in normal skin (Fig. 6). The epidermis of 3-wk-old transgenics exhibited marked acanthosis and pronounced parahyperkeratosis with diffuse manifestation in line VI-6-1 and a less severe and more focal pattern in line VI-6-32. In the dermis of these transgenics, we observed an ongoing inflammatory process with considerable cellular infiltration in line VI-6-1 and weaker inflammation in line VI-6-32 (Fig. 6, *a* and *b*). Neutrophil-laden pustules in the stratum corneum comparable to Munro microabscesses of human psoriatic lesions were present (Fig. 6 *c*). Ultrastructural analysis revealed a higher degree of capillarization in the dermis of transgenics and a reduction in number and size of hair follicles (Fig. 7, *a* and *b*). While there are plenty of large guard hair follicles ranging down to the dermal–subdermal border in normal mice, this follicle type is missing completely in mice of line VI-6-1 and is reduced in numbers in mice of line VI-6-32. The dermal papilla cells are atrophic and there is strong hyperplasia in the upper part of the outer root sheath. Acanthosis in transgenics is due to multiple layers of nucleated cells, and there are structural differences in the stratum corneum between normal and transgenic mice (Fig. 7, *a* and *b*). The regular shape and smooth surface of a normal skin flake is in sharp contrast to the highly irregular shapes of transgenic skin flakes

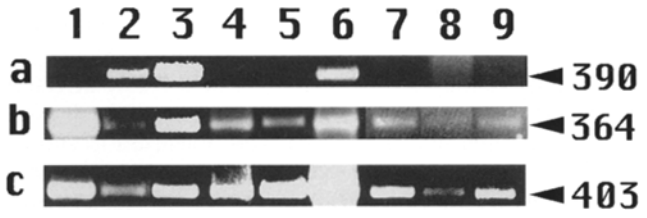

Figure 4. Agarose gel electrophoresis of RT-PCR assays using RNA from skin (lanes *1–3*), oral mucosa (lanes *4–6*), and stomach (lanes *7–9*) of normal adult mice (lanes *1*, *4*, and *7*), adult mice of lines VI-6-1 (lanes *2*, *5*, and *8*), and VI-6-32 (lanes *3*, *6*, and *9*). The primer pairs used were (*a*) B6/pA, which detect the transgene derived transcript, (*b*) B6/B6a, which detect endogenous BMP-6 transcripts, and (*c*) act1/act2, which detect β-actin transcripts. The sizes of the diagnostic bands are indicated on the right side in bp.

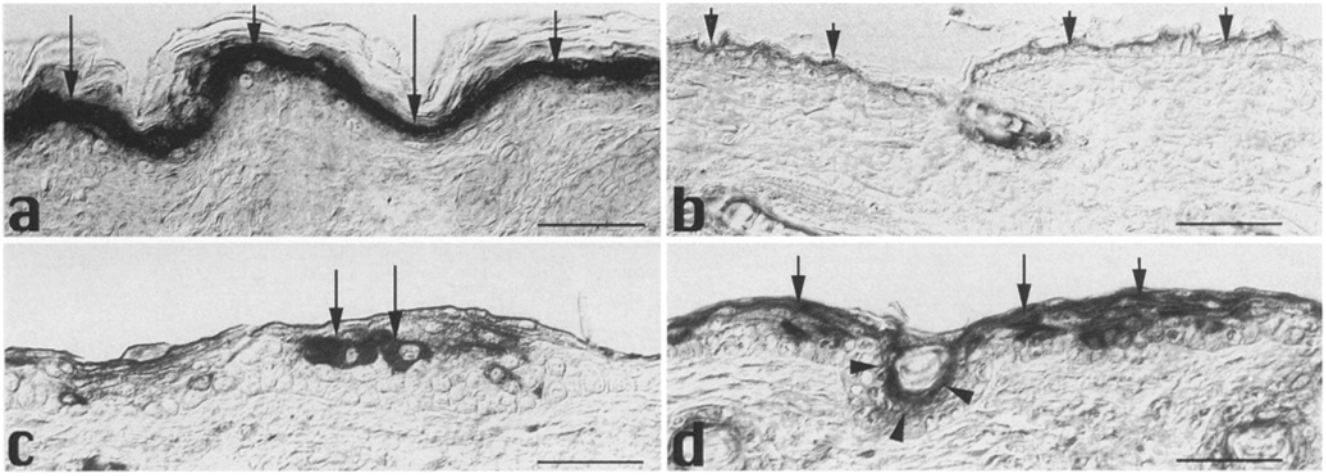

Figure 5. Expression of BMP-6 in the epidermis. Immunolocalization of BMP-6 in the epidermis of (*a*) a normal neonate, (*b*) a normal adult, (*c*) a transgenic adult of line VI-6-1, and (*d*) a transgenic adult of line VI-6-32. Note the strong and uniform interfollicular expression in normal neonates (*a*) and the very weak staining in normal adults (*b*). Patchy expression is seen in adults from both transgenic lines (*c* and *d*). The arrows denote expression in interfollicular epidermis, arrowheads denote expression in infundibula of hair follicles. Bar, 50 μm.

(Fig. 7, *c* and *d*). In addition, transgenic skin flakes display a multitude of breaks and ridges on the surface resulting in fragility.

Expression of Integrin Subunits and Marker Keratins

Expression of the α_6-integrin subunit was analyzed in the skin of normal adults and moderately affected adults of line VI-6-32 (Fig. 8). Immunostaining for this integrin subunit in normal adult epidermis was largely confined to the basal layer with the strongest reactivity towards the basal lamina and weaker staining in the lateral domains (Fig. 8, *a* and *a'*). In acanthotic epidermis from moderately affected transgenic adults, there was generally stronger staining for α_6-integrin in the basal layer of the epidermis and additional staining in suprabasal cells of interfollicular epidermis. The intensity of the staining both in basal and suprabasal cells correlated with the degree of epidermal thickening (Fig. 8, *b* and *b'*).

Expression of the basal type keratin 14, the suprabasal differentiation markers keratins 1 and 10 and the hyperproliferative marker keratin 6 were analyzed in severely affected neonatal founders and in moderately affected adults of lines VI-6-1 and VI-6-32. There were no apparent aberrations in the patterns of expression of keratins 1, 10, and 14 in severely affected neonatal transgenic founders when compared to normal neonates. Keratin 14 was predominantly expressed in the basal layer and keratins 1 and 10 were expressed in suprabasal layers (data not shown). Moderately affected adults of lines VI-6-1 and VI-6-32, however, displayed aberrant expression of keratins 1, 10, and 14 (Fig. 9). Unusually thickened epidermis showed expansion of the keratin 14 expression domain from the basal compartment into suprabasal layers including the stratum corneum. In parallel, there was focal repression of the differentiation specific keratins 1 and 10 (Fig. 9, *a*– *b''*). Expression of the stress keratin 6 was similar in the epidermis of severely affected transgenic founders and normal neonates. Keratin 6 expression was largely restricted to the hair follicles and only very rarely small patches of suprabasal interfollicular cells expressed this keratin (Fig. 9, *c* and *d*). In contrast to these findings and also to the expression pattern in normal adult epidermis, moderately affected adults of lines VI-6-1 and VI-6-32 showed a strong interfollicular induction of keratin 6 in acanthotic epidermis (Fig. 9, *e* and *f*).

Proliferation in Normal and Transgenic Skin

Proliferation in normal and transgenic skin was evaluated by two different techniques. BrdU incorporation analysis was used to compare the distribution of S phase nuclei in the epidermis of normal adults and adults of lines VI-6-1 and VI-6-32 (Fig. 10). Labeled nuclei were confined to the basal compartment in normal adult dorsal epidermis. By contrast, S phase nuclei were not only seen in abundance in the basal epidermal layer of moderately affected transgenic adults but also were plentiful in the suprabasal epidermal compartments (Fig. 10, *a* and *b*).

Proliferation in the skin of severely affected transgenic neonates was asessed by immunostaining for PCNA (Garcia et al., 1989). Epidermis from various body sites was analyzed and while there was always specific nuclear staining in basal interfollicular cells in normal epidermis, there was hardly any staining in the epidermis of severely affected transgenic neonates, and the few stained cells often showed aberrant cytoplasmic staining or were localized to suprabasal layers (Fig. 10, *c–f*). In summary, there is parahyperkeratosis with strong hyperproliferation in suprabasal epidermis of moderately affected adults of lines VI-6-1 and VI-6-32, and strong repression of epidermal cell proliferation in severely affected transgenic neonates.

Discussion

BMP-6 and Epidermal Cell Proliferation

The effect of BMP-6 on keratinocyte proliferation has been

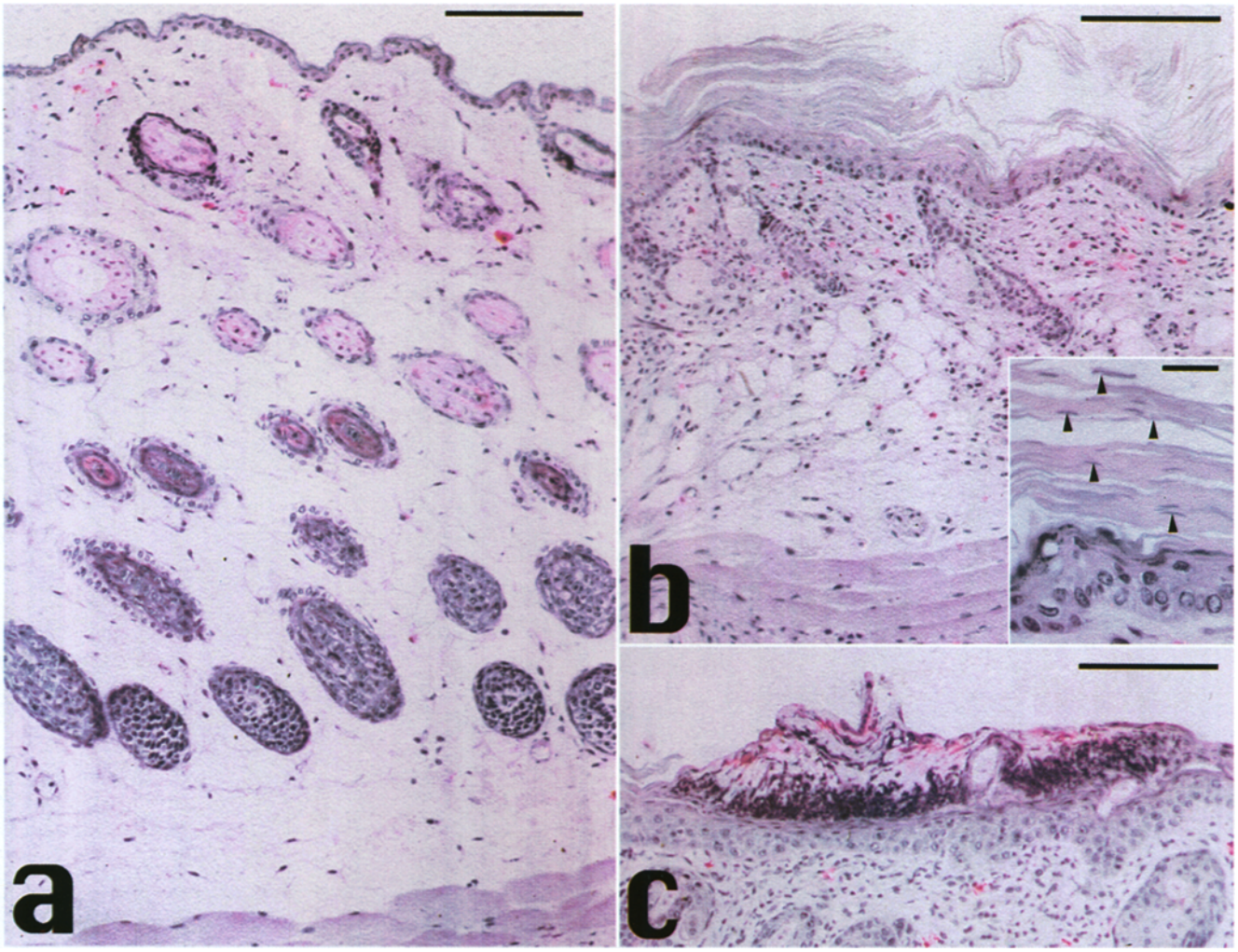

Figure 6. Histology of transgenic skin. Hematoxylin/eosin and chloroacetate esterase-stained dorsal skin sections (*a*) from a 3-wk-old normal and (*b*) from a 3-wk-old transgenic mouse of line VI-6-1. There is acanthosis and hyperkeratosis in the transgenic skin as well as dermal infiltration. The inset in *b* demonstrates parakeratosis in the epidermis from a 3-wk-old transgenic mouse of line VI-6-32. Note the remnants of nuclei present in the stratum corneum (*arrowheads*). (*c*) Skin section from an adult of line VI-6-32 showing a pustule comparable to Munro microabscesses. Bars: (*a–c*) 200 μm; (*Inset*) 40 μm.

shown to be strongly inhibitory in cultures transfected with a retroviral BMP-6 expression vector (Drozdoff et al., 1994). However, our study suggests a much broader spectrum of possible BMP-6 effects on keratinocyte proliferation in vivo: On one hand, strong and uniform overexpression of the BMP-6 transgene in suprabasal cells leads to a repression of basal cell proliferation. On the other hand, weak and mosaic expression of the BMP-6 transgene in interfollicular epidermis does not markedly affect neonatal skin but leads to parahyperkeratosis and hyperproliferation in suprabasal cells after day 6 postpartum. These dose-dependent alternative responses of keratinocytes to BMP-6 could be a means of controling cell proliferation and epidermal thickness in late gestation and newborn mice. By alternatively stimulating or repressing epidermal cell proliferation in a dose-dependent manner, BMP-6 could change epidermal thickness and the number of suprabasal cells, the source of BMP-6. Although this model is speculative and oversimplifies the mechanisms by which cell proliferation and thickness in the epidermis is controlled, such an autoregulatory circuit would keep these parameters within certain boundaries. There are striking similarities to the results obtained with two different transgenic lines expressing an activated form of TGF-β in the epidermis, which either strongly repressed neonatal keratinocyte proliferation (Sellheyer et al., 1993) or stimulated epidermal cell proliferation in undisturbed adult epidermis (Cui et al., 1995). Our results with the BMP-6 transgenics indicate that these differences could be due to the pattern and strength of the TGF-β transgene expression. Although the presence of the completely processed mature factor was demonstrated in none of these studies, including ours, we are certain that transgene derived active BMP-6 protein is synthesized because: (*a*) Both human and murine cDNAs for BMP-6 have been shown to give rise to bioactive protein in various transfected cells including keratinocytes (Wall et al., 1993; Drozdoff et al., 1994; Gitelman et al., 1995); (*b*) We demonstrate the presence of transcripts derived from the human BMP-6 cDNA region encoding the mature region in RT-PCR experiments; and

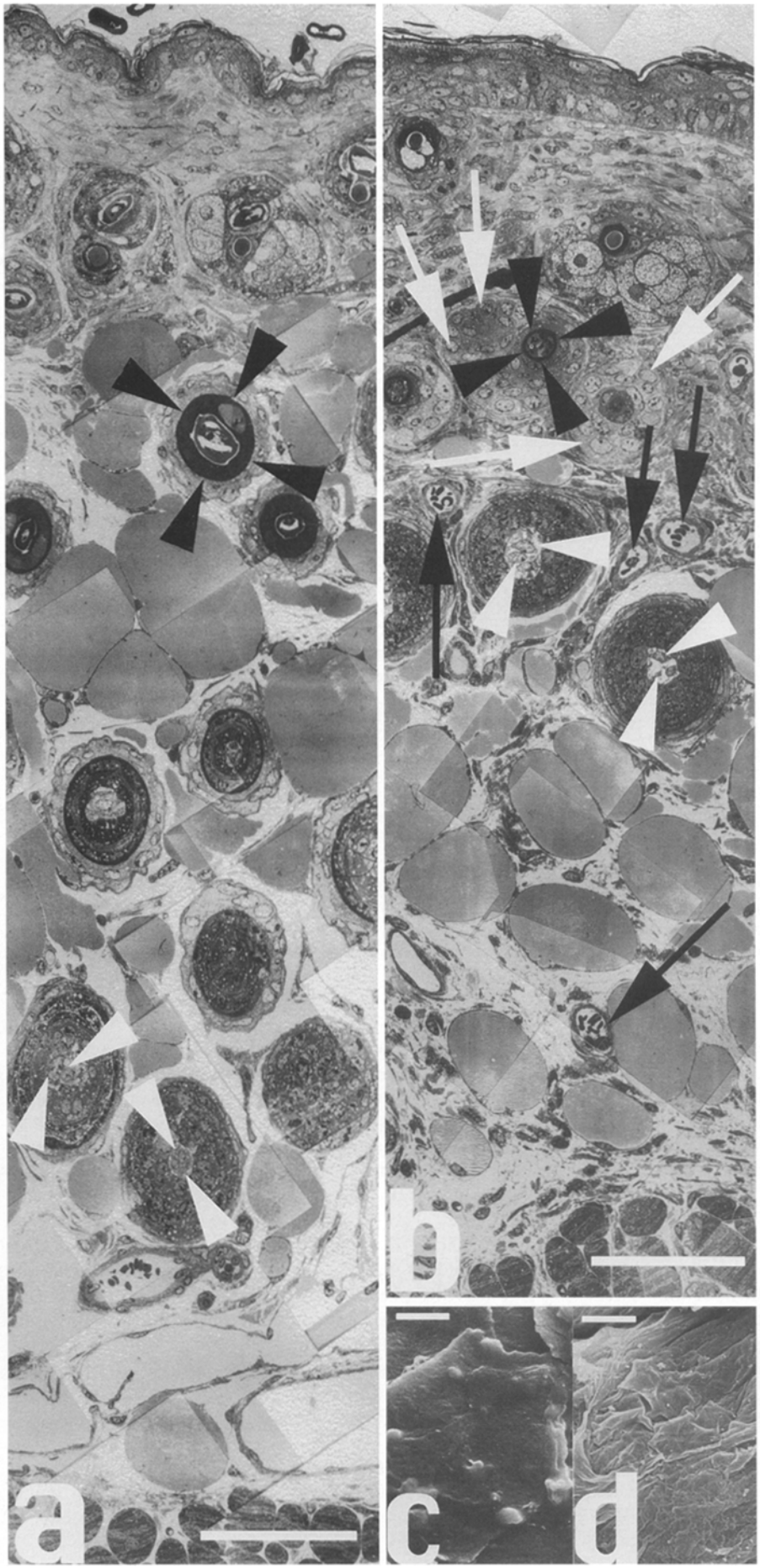

Figure 7. Ultrastructure of transgenic skin. Survey transmission electron photomicrograph of skin samples (*a*) from normal and (*b*) from transgenic mice of line VI-6-1. Note the reduced thickness of the dermis and the high degree of vascularization (*black arrows*) in the transgenic (*b*). White arrowheads point to (*a*) normal and (*b*) atrophic dermal papilla cells in the transgenics. In addition, the calibers of hair shafts (*black arrowheads*) are reduced, and there is hyperplasia in outer root sheath cells in these transgenics (*white arrows* in *b*). (*c* and *d*) Scanning electron microscopy of skin from (*c*) normal adults and (*d*) adults of line VI-6-1. Note the regular shape and smooth surface of normal skin flakes (*c*) as opposed to the highly irregular shapes and brittle surface of the transgenic flakes (*d*). Bars: (*a* and *b*) 420 μm; (*c* and *d*) 0.7 μm.

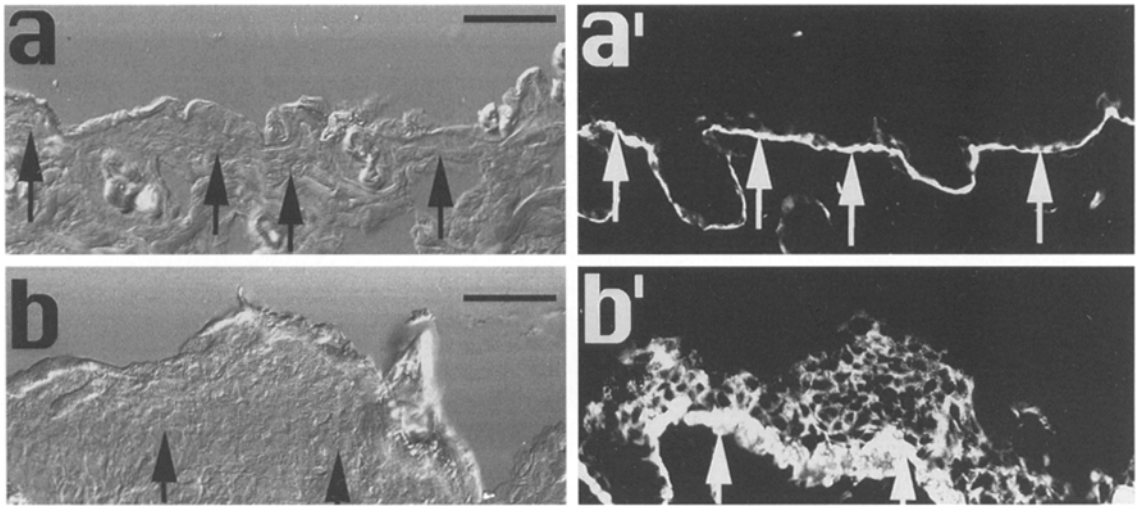

Figure 8. Distribution of the α_6-integrin subunit in skin. Differential interference contrast photomicrograph of dorsal skin sections from (*a*) normal adults and (*b*) transgenic adults of line VI-6-32. (*a'* and *b'*) Corresponding epifluorescence photomicrographs after staining with α_6-integrin–specific antibody GoH3. Note the confinement of α_6-integrin to basal cells in normal skin (*a'*) and the stronger basal and additional suprabasal staining in the epidermis of transgenic mice (*b'*). Bar, 50 μm.

(*c*) we demonstrate increased BMP-6 protein synthesis in adult transgenic versus normal epidermis without a visible induction of the endogenous gene as seen in the RT-PCR experiments. Unlike TGF-β, BMP-6 overexpression activates suprabasal cell proliferation, whereas overexpression of TGF-β stimulated basal cells (Cui et al., 1995). Also, BMP-6 derived from a native cDNA shows strong activity, implying that a substantial part of BMP-6 is properly processed and activated by keratinocytes in vivo.

Because neonatal cultured keratinocytes that resemble a basal cell rather than a suprabasal cell can be directly inhibited by BMP-6 (Drozdoff et al., 1994), we assume that in neonatal epidermis of severely affected founders, suprabasally synthesized BMP-6 directly represses basal cell proliferation in a paracrine fashion. In adult epidermis, transgene-derived BMP-6 may induce suprabasal cell proliferation either directly in an autocrine manner or indirectly in a paracrine manner by predisposing basal cells to continued proliferation after detachment from the basal lamina. However, since nothing is known about BMP-6 receptor distribution, this model remains speculative.

BMP-6 and Epidermal Differentiation

In vitro studies revealed that BMP-6 is switched on in cultured keratinocytes undergoing terminal differentiation before the appearance of the differentiation-specific keratins 1 and 10, making it an interesting candidate for triggering earliest events in this process (Drozdoff et al., 1994). A direct influence on the expression of differentiation markers, however, was not established in keratinocytes transfected with a BMP-6 expression vector (Drozdoff et al., 1994). Similarly, we do not see major changes of marker keratin expression in our severely affected transgenic neonates, which, like the transfected keratinocytes, display reduced epidermal cell proliferation. There is not even a generalized interfollicular induction of the stress keratin 6 as seen in comparable TGF-β transgenic mice with a similar repression of keratinocyte proliferation and skin development (Sellheyer et al., 1993). Therefore, strong overexpression of BMP-6 in neonatal epidermis does not markedly change the usual distribution of differentiation markers.

In parahyperkeratotic epidermis of moderately affected BMP-6 transgenics, we see major changes in keratin expression. BMP-6 inhibits the normal pathway of keratinocyte differentiation in adult epidermis and triggers an alternative pathway characterized by the persistence of keratin 14 in suprabasal layers, including the stratum corneum and the partial replacement of keratins 1 and 10 by keratin 6 and probably keratin 16.

BMP-6 and the Psoriatic Phenotype

It is well established that there are altered expression patterns for a variety of growth factors and their receptors in psoriatic skin as compared to normal skin including TGF-α, many cytokines, and TGF-β (Gottlieb et al., 1988; Elder et al., 1989; Kane et al., 1990; Krueger et al., 1990; Kupper, 1990; Nickoloff et al., 1991; Nanney et al., 1992). Overexpression of some of these factors, including TGF-β, in the epidermis of transgenic mice, however, did not evoke lesions that could be viewed as murine equivalents of human psoriatic lesions either for lack of hyperproliferation and parakeratosis or the absence of dermal infiltrates (Vassar and Fuchs, 1991; Cheng et al., 1992; Turksen et al., 1992; Blessing et al., 1993; Dominey et al., 1993; Guo et al., 1993; Sellheyer et al., 1993; Cui et al., 1995). With the exception of the thickening of the rete ridges that are absent in murine dorsal epidermis, the moderately affected transgenic mice overexpressing BMP-6 develop skin abnormalities with all characteristics of psoriasis in humans: parahyperkeratosis, changes in integrin and keratin expression, the abnormally formed nails, the brittle and fragile skin flakes, extensive dermal capillarization, the presence of dermal infiltrates, and lesions resembling Munro microabscesses (Lever and Schaumburg-Lever, 1975; Weiss et al., 1984;

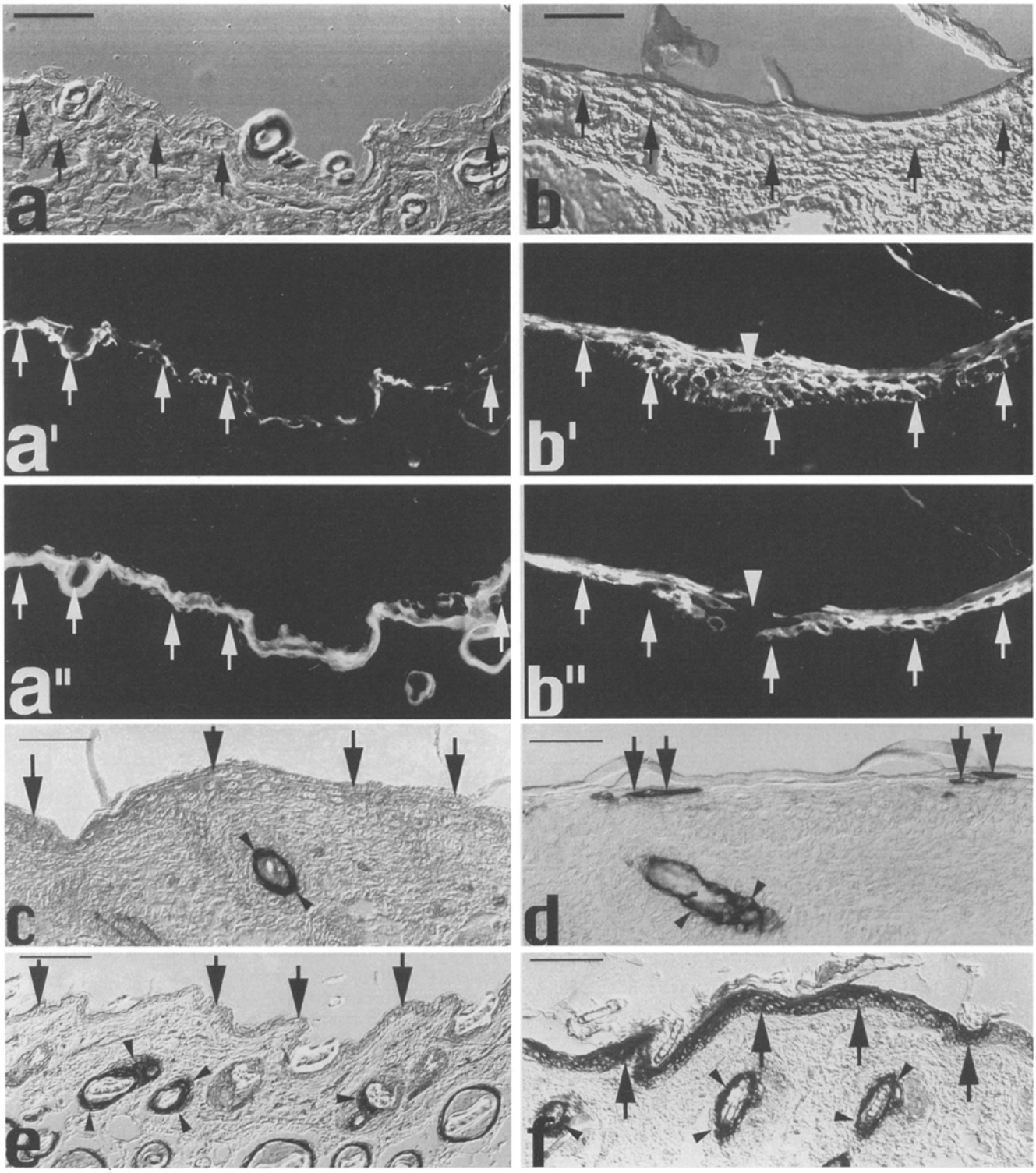

Figure 9. Distribution of keratin components in normal and transgenic skin. (*a* and *b*) Expression of keratins 1, 10, and 14 as shown by double label immunofluorescence staining. Differential interference contrast of 5-μm skin sections from (*a*) normal adults and (*b*) adults of line VI-6-32. Expression of keratin 14 is demonstrated by labeling with monoclonal antibody CKB1 (*a'* and *b'*), expression of keratins 1/10 is shown by labeling with monoclonal antibody 8.6 (*a''* and *b''*). Note the expansion of keratin 14 expression into suprabasal layers in thickened epidermis of transgenic adults (*b'*) and the focal repression of keratins 1 and 10 (*arrowhead in b''*). Arrows mark the basal layer of the epidermis. (*c–f*) Expression of keratin 6 as demonstrated by immunostaining using a polyclonal antibody on skin sections from (*c*) normal neonates, (*d*) severely affected neonatal founders, (*e*) normal adults, and (*f*) transgenic adults of line VI-6-1. Note the staining in the outer root sheaths of all specimen (*arrowheads*) and the absence of staining in interfollicular epidermis of normal mice (*arrows* in *c* and *e*). Weak and patchy staining is seen in epidermis from the severly affected founder (*arrows* in *d*), and strong interfollicular expression is seen in transgenic epidermis from adults of line VI-6-1 (*arrows* in *f*). Bars: (*a*, *b*, *e*, and *f*) 50 μm; (*c* and *d*) 100 μm.

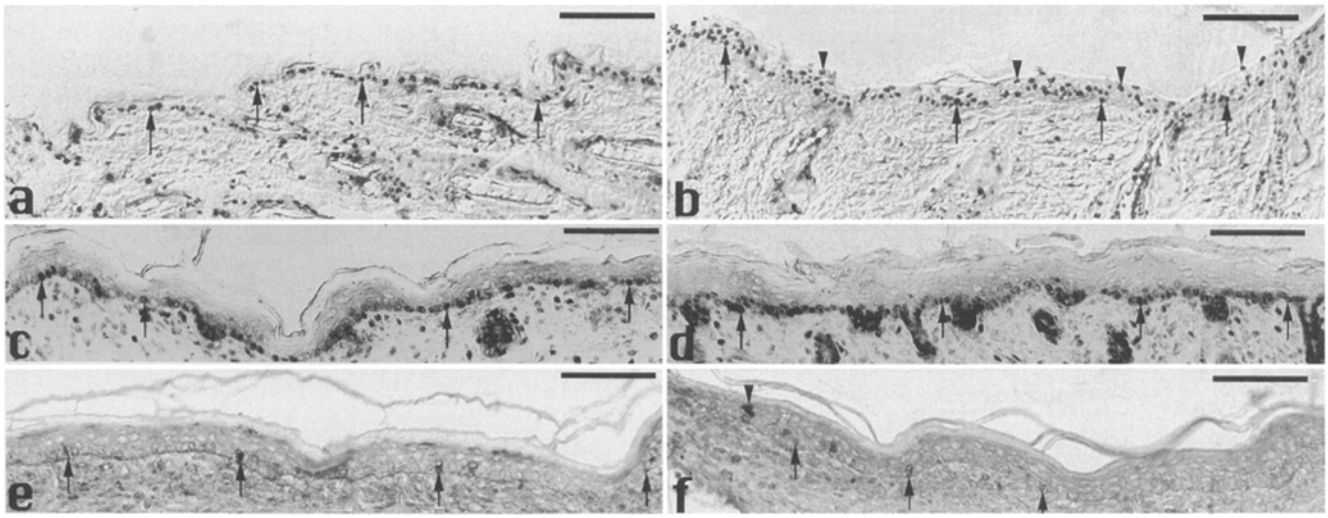

Figure 10. Proliferation in normal and transgenic epidermis. Anti-BrdU staining on sections of labeled skin from (*a*) normal adults and (*b*) a transgenic adult of line VI-6-32. In normal and transgenic skin, many labeled nuclei are found in the basal layer of interfollicular epidermis (*arrows* in *a* and *b*). In the transgenic adult, additional strong staining of many nuclei is seen in suprabasal layers (*arrowheads* in *b*). Anti-PCNA staining (PC10 antibody) of skin sections from (*c* and *d*) normal neonates and (*e* and *f*) severely affected transgenic founders from (*c* and *e*) the mid-dorsum and (*d* and *f*) facial regions (cheek). Note the abundant nuclear staining in basal interfollicular keratinocytes in normal skin from different body sites (*arrows* in *c* and *d*) and the rare and aberrant cytoplasmic staining in basal cells of transgenic epidermis (*arrows* in *e* and *f*) and suprabasal cells (*f*). Bars, 100 μm.

Stoler et al., 1988; Wade and Finan, 1990; Watt and Hertle, 1994). An important question is if the disturbances seen in the epidermis are a primary result of epidermal overexpression of BMP-6 or if BMP-6 expressed in skin or other epithelia first become systemic and then trigger responses that result in skin aberrations. There are several facts which speak against the indirect model and favor a model where the epidermal aberrations are a primary result of BMP-6 overexpression in the epidermis: (*a*) There is no induction of ectopic cartilage or bone formation which would be expected if BMP-6 diffused into the dermis (Wozney et al., 1988; Gitelman et al., 1994); (*b*) Unlike TGF-β, BMP-6 does not seem to achieve systemic levels or to exert long range effects. Even if transfected CHO cells producing BMP-6 are allowed to form massive solid tumors in mice, there are only local effects on cartilage induction, and no generalized systemic or epidermal changes have been reported (Gitelman et al., 1994); (*c*) Full thickness grafting experiments show that epidermal aberrations of the transgenic graft are maintained on a nontransgenic host; and (*d*) Acanthosis and hyperkeratosis coincide with the expression of BMP-6. In conclusion, this is strong evidence that the skin phenotype in our BMP-6–overexpressing mice is a direct result of epidermal BMP-6 expression and is not evoked by growth retardation or systemic levels of BMP-6. Dermal changes such as inflammation or dermal papilla atrophy are most likely secondary effects due to cytokines or growth factors produced by activated keratinocytes (Gottlieb et al., 1988; Elder et al., 1989; Kane et al., 1990; Krueger et al., 1990; Kupper, 1990; Nickoloff et al., 1991; Nanney et al., 1992). These findings together with our observation that epidermal aberrations precede inflammation in the transgenics suggest that psoriasiform dermatitis is primarily a disease of the keratinocyte and not of the immune system as has been debated (see Gottlieb et al., 1995 vs Carroll et al., 1995).

Changes in expression of quite unrelated molecules, however, are also able to evoke psoriasis-like lesions in animal models (Hammer et al., 1990; Carroll et al., 1995; Bullard et al., 1996). Transgenic mice with an altered integrin expression pattern in the epidermis and mice carrying a hypomorphic CD18 mutation are among those that closely reflect the human condition, again with the exception of the thickening of rete ridges (Carroll et al., 1995; Bullard et al., 1996). Therefore, distinct etiological agents belonging to as different categories as growth factors or cell adhesion molecules could be responsible for different types of psoriatic lesions in humans. This hypothesis is supported by the fact that there are distinct differences between the integrin-induced and the BMP-6–induced psoriasis models. The integrin transgenic mice show spontaneous episodes of remission resembling the human condition psoriasis vulgaris. By contrast, the BMP-6 transgenics never went into complete remission and suffered from extremely strong inflammatory processes resembling severe human erythrodermic types of psoriasis. On the other hand, growth factors of the TGF-β superfamily regulate integrin expression in keratinocytes (e.g., Zambruno et al., 1995). Similarly, BMP-6 overexpression in our transgenic mice evoked a change in α_6 integrin subunit expression which was also seen in the β_1 integrin subunit transgenic mice developing psoriatic lesions (Carroll et al., 1995). Thus, changes in growth factor activities appear to be upstream of the changes in expression of integrin cell adhesion molecules or cytoskeletal components like keratins during the process of psoriatic plaque formation. We are currently in the process of characterizing the epidermal expression pattern of BMP-6 in patients with psoriasis. Preliminary results suggest a strong but patchy expression in the epidermis of some of these patients. Therefore, BMP-6 is a prime candidate for a growth factor sponsoring the formation of psoriatic lesions in humans.

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