Epidermal Keratinocyte Self-Renewal Is Dependent upon Dermal Integrity

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The epidermis is a major site of self-renewal in which there is constant replacement by cell division in the basal layers of cells lost by desquamation in the superficial layers. Such a tissue is therefore likely to contain stem cells and in this study we have examined the role of the dermis in the maintenance of epidermal self-renewal. We have developed a mouse model to address the question of whether the maintenance of epidermal self-renewal is dependent, as in the hemopoietic system, upon a heterologous cell type. Intact epidermis separated from dermis at the dermo-epidermal junction or epidermis derived from disaggregated epidermal cells, can reconstitute a stratified squamous epithelium when grafted onto the lumbo-dermal fascia of the mouse or onto an experimentally induced granulation tissue bed. However, we have shown that, after grafting, the clonogenic capacity of the keratinocytes declines sharply and the colonies that are produced are incapable of self-renewal in vitro. Although initially hyperplastic, these epidermal grafts assume an atrophic appearance after 40–70 d and this may be related to the loss of self-renewal observed in vitro. With both experimental murine grafts and clinical grafts the failure of keratinocytes to self-renew can be alleviated, partially, by the presence of the dermis in full-thickness or split-thickness grafts, which implies that the dermis has a functional role in epidermal stem cell maintenance. The relevance of these observations to the clinical experience with cultured autologous keratinocyte sheets as wound dressings to patients is discussed. *J Invest Dermatol 99:422–430, 1991*

The epidermis is a tissue undergoing constant self-renewal; superficial cells are lost continuously through desquamation and are replaced by cells from the lower layers as a consequence of cell division. Such a tissue is likely to contain stem cells, i.e., cells capable of maintaining their numbers throughout the life of an animal despite losses due to differentiation [1]. Even though the epidermis is one of the major sites of self-renewal and the regulation of this process is of central importance in tissue homeostasis, wound repair, and neoplasia, remarkably little is known about epidermal keratinocyte self-renewal. However in all squamous epithelia so far studied the positional distribution of the slow cycling putative stem cells [2–7] and their relationship to other cell types [2,3] does suggest that heterologous cells and tissues may regulate keratinocyte self-renewal.

In the hemopoietic system where clonogenic cell self-renewal is better understood there is compelling evidence that the hemopoietic stem cells are fixed tissue cells [8]. Even in this system, however, it is still uncertain whether stem-cell maintenance is achieved by virtue of the intrinsic "immortality" of the whole stem cell population [9] or whether some stem cells are held in reserve and recruited when required [10–13]. Nevertheless, consistent with both hypotheses is the evidence that the maintenance of the clonogenic progeny of the stem cells (CFU-S) [14] is achieved by their interaction with the heterologous bone marrow stromal cells in their microenvironment [8,15,16].

Despite the evidence for the involvement of cell-cell interactions in hemopoietic self-renewal, far less is known about the role of mesenchymal tissues (such as the dermis of the skin) in the maintenance of clonogenic epithelial cells. The crucial importance of dermo-epidermal interactions in the maintenance of normal tissue homeostasis and in differentiation is widely accepted [17–20] but the possible role of the dermis in the maintenance of keratinocyte self-renewal has not been analyzed intensively, primarily due to the lack of in vivo clonogenic assays for epithelial cells. In an attempt to address the question as to whether epidermal self-renewal is dependent upon its in vivo microenvironment we have developed a mouse model based on previously described grafting techniques [21–23] and culture methods [24].

In the present study we have examined the role of the graft bed by employing, in the mouse, a transplantation technology that uses a silicone transplantation chamber [21] to isolate the graft physically from the wound edge; this effectively prevents host re-epithelialization [21,23]. The graft is placed in the center of the chamber on a graft bed that can be varied experimentally. As there are no in vivo clonogenic assays for epithelial cells, the clonogenic capacity of the grafted epithelium is determined by excising the graft, dissociating the graft into single cells, and measuring the colony-forming efficiency in vitro of the graft keratinocytes using modifications of the techniques of Rheinwald and Green [24]. Although it is uncertain

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Abbreviations:

CO_2: carbon dioxide
DMEM: Dulbecco's modification of Eagle's medium
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
FTSG: full-thickness skin graft
GMEM: Glasgow's modification of Eagle's medium
HIV: human immunodeficiency virus
PBS: phosphate-buffered saline
TGF-β_1: transforming growth factor β_1
TPA: 12-O-tetradecanoylphorbol-13-acetate

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Table I. Clonogenicity of Keratinocytes Recovered from Grafts of Disaggregated Epidermal Cells[a]

Days Post-Grafting	Graft Keratinocytes, Colony Number			Disaggregated Keratinocytes from Skin Adjacent to the Graft, Colony Number		
	Number of Keratinocytes Plated/Dish			Number of Keratinocytes Plated/Dish		
	10^4	10^5	10^6	10^4	10^5	10^6
0	25 ± 5.2	231 ± 51.4	TMC	ND	ND	ND
7	0	12 ± 3.2	ND	113 ± 24.2	TMC	TMC
14	1	ND	8 ± 3.1	30 ± 8.1	361 ± 43.2	TMC
21	0	7 ± 3.8	3 ± 1.8	25 ± 7.2	TMC	TMC

[a] A suspension of 2×10^6 epidermal keratinocytes (prepared from shaved tail or flank skin as described in the text) in 0.2 ml volume of PBS was injected into the transplantation chamber placed over a granulation tissue graft bed. A control group of animals received PBS only in the chamber. Each experimental group consisted of 10 animals, six engrafted with tail keratinocytes and four engrafted with flank keratinocytes. At the designated time after grafting, animals were sacrificed, n = 5 (three tail keratinocyte grafts and two flank grafts), and the chambers containing the grafts excised. Keratinocytes were isolated from the grafts as described in the text; the cells were counted, the cell viability determined, and the suspensions were combined. Cells were cultured as described in the text. After 21 d in culture the feeder cells were removed and the colonies fixed in 10% buffered formal saline and stained with Rhodamine B. Keratinocyte colonies were scored under observation with an inverted microscope at ×40 magnification. The numbers represent the mean values for the numbers from triplicate plates ± the standard deviation from three separate experiments. ND, not done; TMC, too many to count.

whether we are measuring keratinocyte stem cells directly, it is likely that the behavior of the in vitro clonogenic cells is a useful indicator of the requirement of stem cells in vivo [25]. Furthermore the use of in vivo [9,14] together with in vitro [25] clonogenic assays has done much to reveal the possible molecular mechanisms underlying the stem cell–stroma interactions in the hemopoietic system.

MATERIALS AND METHODS

Mouse Grafts and Mouse Keratinocyte Culture

Graft Technique: A granulation bed was prepared on the lateral thoracic wall of 4-week–old BALB/c mice (Department of Pathology, University of Cambridge) as described previously [23]. Briefly, 2 weeks prior to grafting a glass coverslip (Chance), 22 mm in diameter, was inserted under the skin on the lumbo-dermal fascia. This resulted in the formation of a well-vascularized granulation bed [21]. At the time of grafting, a longitudinal incision was made over the disc, which was then removed, and a silicone transplantation chamber (Renner Gmbh) was inserted into the skin pocket that had formed over the granulation bed; this was held in place with 9-mm wing clips [23–26]. Grafts of full-thickness skin or separated intact epidermis were placed onto the granulation bed or directly onto the lumbo-dermal fascia immediately prior to the insertion of the chamber. Disaggregated suspensions of cells were injected directly into the chamber [27]. Animals were grafted in groups of 10 but sampled in groups of three or five. This allowed for losses due to infection or loss of the chamber. Overall a graft take of 80% was achieved.

Preparation of Tissue for Grafting: Skin from adult BALB/c mice was used unless otherwise stated; all tissue was held on ice in sterile phosphate-buffered saline (PBS) prior to grafting for as short a time as possible. Full-thickness skin grafts consisted of 4–5-mm square pieces of skin from tail or shaved flank. Epidermal grafts were obtained by treating full-thickness skin strips with dispase (Boehringer Mannheim) 500 μg/ml for 2 h at 37°C as described previously [22]. Epidermal cell suspensions were prepared from dispase-separated epidermis according to published protocols [23,26]. Disaggregated cell suspensions from full-thickness skin were obtained by stirring minced skin fragments in 0.25% trypsin (SIGMA Type III) for 1 h at 37°C.

Table II. Identity and Viability of Cells Recovered from Epithelial Cell Grafts[a]

Days Post-Grafting	Mean Number of Cells Recovered perGraft	Mean % Viable Cells Recovered per Graft	Mean % Keratinocytes Recovered per graft
7	2.65×10^6	77	94
14	2.67×10^6	82	93
21	2.58×10^6	76	94

[a] Freshly dissociated epidermal keratinocytes were grafted and the grafts isolated as described in Table I. Keratinocytes isolated from the graft were suspended in GMEM/10% serum and counted using a counting chamber. An aliquot (10%) of the cell suspension was removed and divided into two lots. One sample was used to determine the percentage of viable cells by trypan blue exclusion. Cells from the second sample were immunostained with a pan keratin antibody examined under fluorescence optics and the percentage of positively staining cells determined.

Isolation of Graft Keratinocytes: At the designated time after grafting animals were sacrificed (n = 5) and the chamber plus graft excised. The chamber was inverted and a nitrocellulose filter (2.5 cm diameter) was placed on top of the graft bed. Using scissors the graft was removed from the chamber and then floated, epidermal surface down, on dispase [500 μg/ml in GMEM [28]/10% fetal calf serum (FCS)] for 2 h at 37°C. The epidermis detached, the junctional surface was scraped to remove any epidermal cells and a single cell suspension was prepared by stirring vigorously or, if necessary, by treatment with 0.125% trypsin for 15 min at 37°C. The cell suspension was washed twice with Glasgow's modification of Eagle's medium (GMEM)/10%FCS, and counted in a hemocytometer (improved Neubauer), and an aliquot (10%) of the suspension was removed and divided into two samples for immunostaining and viability assessment. For immunostaining cells were fixed in formol saline, cytospun onto slides, washed with PBS, and incubated with a guinea pig anti-bovine hoof pan keratin antibody (ICN) for 60 min at 37°C, washed again with PBS, and then incubated with an anti-guinea pig IgG fluorescein isothiocyanate (FITC) conjugate for 30 min at 37°C. The slides were mounted in Citifluor and viewed under fluorescence, and the number of fluorescing and non-fluorescing cells per high power field (×400 magnification) counted. The number of fluorescent cells was taken as a measure of the number of keratinocytes recovered from the graft. Viability was determined by trypan blue exclusion. Briefly the cells were resuspended in a small volume of PBS to reach a concentration of approximately 10^6 cells. One drop of this cell suspension was mixed with one drop of 1% trypan blue in a counting chamber and the number of stained and unstained cells counted. We inoculated into culture the same number of viable cells from each graft or piece of skin.

Mouse Keratinocyte Culture: Keratinocytes from grafts or matched skin biopsies were plated onto 50-mm plastic petri dishes (Nunc) together with 5×10^5 J2F 3T3 lethally irradiated mouse fibroblasts as described in published protocols [26,29,30]. Cultures were grown at 31°C in a humidified atmosphere containing 5% CO_2 in medium consisting of DMEM (GIBCO) supplemented with 10% FCS (GIBCO), 0.5 μ/ml hydrocortisone (SIGMA), and 10^{-10} M cholera toxin (SIGMA). After 14–21 d in culture the feeder cells were removed as described previously [24], the plates were fixed in 10% buffered saline and stained with Rhodamine B [24].

Human Grafts and Human Keratinocyte Culture: Cultured epithelial grafts from living donors screened for hepatitis B and human immunodeficiency virus (HIV) infection were prepared using published protocols [31]. The epithelial sheets were supported with a woven rayon dressing (N-A, Johnson and Johnson) and applied to the prepared leg ulcers [32]. Biopsies were taken with a 3-mm punch (Stiefel Laboratories) under local anaesthesia (1% Lignocaine). Explant cultures of biopsies were prepared using standard techniques [33] and were maintained for at least 21 d. Clonogenic cultures were established by floating the biopsy, dermal surface down, on 0.125% trypsin (Lorne Diagnostics Ltd.) for 16 h at 4°C. The epidermis separated from the dermis at the dermo-epidermal junction, the junctional surface was scraped and a single cell suspension was prepared by stirring vigorously. After washing in serum containing medium, the cells were counted and 10^4 keratinocytes together with 4×10^5 lethally irradiated J2F 3T3 cells were plated out onto 50-mm plastic dishes (Nunc). Explants and cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and in a culture medium consisting of DMEM (three parts), Hams F12 (one part) supplemented with 10% FCS, 0.5 μg/ml hydrocortisone, 10^{-10}M cholera toxin and insulin, transferrin, and tri-iodothyronine as described [34]. Epidermal growth factor at 10 ng/ml was added 72 h after plating. After 14 d in culture, the colonies were fixed and stained with Rhodamine B as described [24].

Measurement of Colony-Forming Efficiency After Induction of Terminal Differentiation by Suspension Culture: Mouse keratinocytes were prepared from epidermis as described above. Cells were resuspended at a density of 10^5/ml in culture medium. A sample of this suspension was examined under the light microscope to verify that it was a single cell suspension and free from cell aggregates. Four milliliters of this suspension were then added to a 90-mm bacteriologic petri dish (Sterilin) so that the solution covered the base of the dish entirely. Keratinocytes are unable to adhere to the surface of these bacteriologic petri dishes and they remain therefore in suspension. The low input density ensures that the cells also remain dispersed. The plates were checked microscopically to ensure that the dishes were free of cell aggregates and the dishes were incubated at 31°C. At fixed time points, medium was removed from the plates. The plates were rinsed with culture medium and rinses and medium combined and the cells collected by centrifugation at $300 \times g$ for 10 min. Cells were counted using a counting chamber and 10^4 keratinocytes plated onto lethally irradiated 3T3 feeder layers in 50-mm petri dishes as described above. Cultures were grown for 17 d, fixed in 10% formol saline, and stained with 1% rhodamine B and the numbers of colonies scored.

RESULTS

The data in Table I are the results of experiments in which freshly isolated epidermal cells were cultured in vitro either immediately or after epidermal reformation for various times on an experimentally induced granulation tissue graft bed in the chamber. At time zero, isolated and disaggregated epidermal cells had a colony-forming efficiency characteristic of primary murine keratinocyte cultures and exhibited a high frequency of macrocolonies, i.e., macroscopic colonies, many of which had a holoclone morphology (colonies with a smooth perimeter composed of small polygonal cells [35]. However, within 7 d of grafting the macroscopic cloning efficiency of the reconstituted epidermis had declined substantially and the colonies formed were small, abortive, or microscopic (microcolonies). These observations were not due to a loss in viability of the cells recovered from the grafts or to a failure to recover keratinocytes from these grafts, as the data in Table II show, but do suggest that the granulation tissue bed does not support the maintenance of clonogenic keratinocytes.

In a further set of experiments the constitution of the graft was varied and the grafts were placed either on the granulation tissue bed or directly onto the lumbo-dermal fascia. The capacity of these different grafts to form progressively growing colonies in culture was then determined. The results of these experiments are shown in Table III. Epidermis separated enzymatically from dermis at the dermo-epidermal junction, behaved in essentially the same way as the disaggregated epidermal suspension and the clonogenic capacity of the cells was lost. However, when full-thickness skin (which

Table III. Clonogenicity of Keratinocytes Rescued from Grafts of Epidermis, Dissociated Skin Cells, or Full-Thickness Skin on Experimentally Induced Granulation Tissue or Muscle Facsia Graft Beds[a]

Nature of Graft	Graft Bed	Source of Cells Grafted	Days Post-Grafting 0	7	14	21
Disaggregated cells	Granulation tissue	Tail	240 ± 5.1	41 ± 3.0	18 ± 4.2	7 ± 3.5[b]
			240 ± 5.1	3	0	0~
		Flank	236 ± 23.8	9 ± 2.5	8 ± 2.6	ND[b,c]
			236 ± 23.8	1	0	[d]
Epidermis only	Granulation tissue	Tail	236 ± 24.8	7 ± 0.2	10 ± 4.6	7 ± 21[b]
			236 ± 24.8	1	0	0[d]
		Flank	310 ± 42.2	12 ± 3.1	14 ± 2.8	8 ± 2.1[b]
			310 ± 42.2	4	0	0[d]
	Muscle facsia	Tail	125 ± 13.2	ND	ND	4 ± 1.2[b]
			125 ± 13.2			0[d]
Full-thickness skin	Granulation tissue	Tail	240 ± 15.1	41 ± 8.2	17 ± 3.5	10 ± 2.5[b]
			240 ± 15.1	41 ± 8.2	17 ± 3.5	10 ± 2.5[d]
		Flank	107 ± 16.5	13 ± 4.5	7 ± 2.0	11 ± 1.4[b]
			107 ± 16.5	13 ± 4.5	7 ± 2.0	11 ± 1.4[d]
		Newborn	184 ± 33.8	10 ± 3.9	11 ± 1.4	ND[b]
			184 ± 33.8	10 ± 3.9	11 ± 1.4	0[d]
	Muscle fascia	Tail	210 ± 24.1	ND	ND	22 ± 3.7[b]
			210 ± 24.1			22 ± 3.7[d]
Matched flank skin sites disaggregated cells			126 ± 16.7	320 ± 44.1	181 ± 26.2	250 ± 18.4[b]

[a] Tissues and cells for grafting were prepared as described in the text. Grafts were placed inside the transplantation chamber either directly onto the lumbo-dermal fascia or onto a previously induced granulation bed as described in *Materials and Methods.* At the designated time after grafting, the grafts were excised and the graft keratinocytes dissociated and cultured as described in the text. At the same time as the graft was excised, a biopsy of skin was taken from a site immediately adjacent to the graft and epidermal cell suspensions prepared and cultured from these biopsies as previously described [27,29,30]. In these experiments 5×10^4 keratinocytes from individual grafts (n = 3) were plated together with 5×10^5 J2F 3T3 cells onto 50-mm plastic dishes in triplicates and grown for 14 d. The plates were fixed in formol saline and stained with Rhodamine B, and the number of colonies per dish counted under the low power (×40 magnification) of an inverted microscope. The data are the results of two separate experiments.

[b] Mean number of colonies (macrocolonies and microcolonies) per dish ± standard deviation, results from two experiments.

[c] ND, not done.

[d] Mean number of macrocolonies per dish ± standard deviation, results from two experiments.

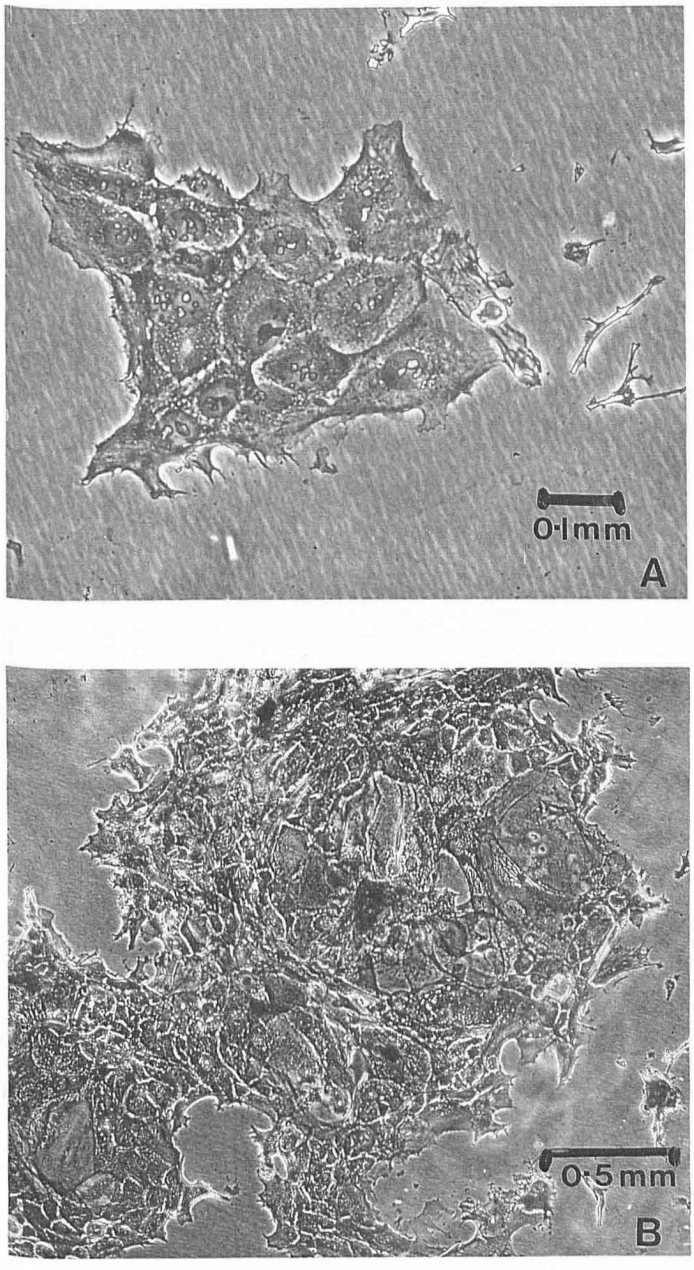


Figure 1. *A*) Morphology of a keratinocyte colony after 16 d in culture and derived from an epidermal graft (graft in situ for 14 d). The colony is small and abortive consisting of squame like cells. *B*) Morphology of a colony derived from a full-thickness skin graft (in situ for 14 d). Many small polygonal cells are present. The colony is stratified and some superficial differentiated squames are present.

includes the dermis) was grafted the macroscopic clonogenic capacity of the keratinocytes was maintained considerably better. The colonies produced from these grafts consisted of many small polygonal cells (Fig 1*B*): these colonies could be serially cultivated indicating that the presence of dermal elements could maintain the self-renewal capacity of at least some of the clonogenic keratinocytes. In grafts other than full-thickness grafts, small aggregates of keratinocytes consisting of no more than 8–12 cells with a squame-like morphology (Fig 1*C*) that stained positively with an anti-keratin antibody were present. These microcolonies, which differed significantly in size from those derived from FTSG (Table IV), could not be serially cultivated. These observations were not related to differences in the number of viable cells or the number of keratinocytes recovered from the epidermal grafts as compared to FTSG. Consistently, after dispase treatment of the graft 10^6 to 2×10^6 cells per graft were recovered irrespective of whether the grafts were epidermal or FTSG or derived from adult or neonatal skin. Trypan blue exclusion showed these suspensions to contain 70–90% viable cells, of which more than 90% were keratin positive by immunostaining.

It could be argued that the data in Table III was due to inadequate separation at the dermo-epidermal junction and that the clonogenic cells were not present in the epidermal grafts. Such a situation would be of particular importance in view of the data [2,3,7], which show that the slow cycling cells in mouse skin are found in both the interfollicular basal layer of the epidermis and in the bulge zone of the hair follicle. However, the effectiveness of the enzymatic separation at the dermo-epidermal junction was examined histologically in all grafts (Fig 2) and all epidermis used in grafts consisted of the complete epidermis with many intact hair follicles. Furthermore when disaggregated cells from full-thickness skin were used (Table III) the clonogenic capacity was not retained and the colonies formed were the same as for epidermal cells alone. The cell suspensions derived by trypsinization of full-thickness skin contained 90% viable cells (by trypan blue exclusion), of which 40% were keratin positive by immunostaining indicating that these suspensions contained both epidermal and dermal cell populations. The loss of clonogenicity observed in grafts of these suspensions suggests that the provision of all the skin cell types was not enough to produce the correct environment for keratinocyte self-renewal. Zero time cell suspensions all had a good colony-forming efficiency with a high frequency of holoclone-like colonies, as observed by others [31,34], suggesting that the enzymatic treatments used in the preparation of the grafts did not alter the in vitro clonogenicity of the keratinocytes. Keratinocyte suspensions derived from skin adjacent to the grafts also had a good cloning efficiency, indicating that the operative procedures used did not have a toxic field effect.

In all cases when microscopic and macroscopic colony scores were combined it could be seen that the numbers of keratinocytes that formed at least a microcolony was similar in all the experimental groups (Table III) indicating that similar numbers of keratinocytes attached to the 3T3 feeder layer and founded colonies in all cases. The major difference observed was that only the colonies obtained from full-thickness skins or their grafts contained keratinocytes capable of significant self-renewal. Keratinocytes lose clonogenicity if maintained in suspension culture [36] and it could be argued that the loss of clonogenicity of the engrafted keratinocytes was due to the possibility that these cells remain in suspension for a longer time period than the zero time controls. However, the data in Fig 3 show that murine keratinocytes held in suspension culture contain a suspension-resistant population and that even after 48 h in suspension the keratinocyte cloning efficiency was still 14% of the zero time controls. Murine keratinocytes engrafted as a suspension onto the granulation bed can reform a stratified epithelium within 36 h (Ramrakha and Stanley, unpublished data) and the results shown in Fig 3 show that the complete loss of progressively growing colonies is unlikely to be explained by the possibility that the experimental keratinocytes seeded into the graft chamber spend longer in suspension than the zero time controls. Furthermore, because the former were seeded onto a preformed fibroblast extracellular matrix (generated by the granulation tissue) it is likely that they attached more rapidly than the zero time controls [37] and probably had a high initial colony-forming efficiency [38].

The loss of clonogenicity observed within the grafts appears to be related to the absence of the dermis and the histologic appearances of long-term epidermal versus full-thickness skin grafts support the notion that pure epidermal populations are, at least in the mouse, incapable of self-renewal. In the mouse model the epidermis reconstituted from cultured keratinocytes will persist for long periods (up to 144 d) [27]. Initially the epidermis is hyperplastic (Fig 4*A*) but after 40–70 d becomes atrophic and grossly thinned (Fig 4*B,C*). This atrophy is not due exclusively to the release of anti-proliferative factors from the granulation bed or to the avascularization of

Table IV. Colony Size at 14 d In Vitro Achieved by Keratinocytes Derived from Full-Thickness Skin, Epidermis, and Disaggregated Skin Cells[a]

Nature of Graft	Graft Bed	Source of Cells Grafted	Days Post-Grafting			
			0	7	14	21
Disaggregated cells	Granulation tissue	Tail	3.1 ± 1.3	0.45 ± 0.1	0.39 ± 0.15	0.48 ± 0.23
		Flank	3.5 ± 1.2	0.6 ± 0.2	0.48 ± 0.24	ND[b]
Epidermis only	Granulation tissue	Tail	3.3 ± 0.87	0.43 ± 0.23	0.39 ± 0.29	0.65 ± 0.29
		Flank	3.8 ± 0.83	0.62 ± 0.26	0.43 ± 0.2	0.48 ± 0.23
	Muscle	Tail	3.5 ± 0.85	ND	ND	0.43 ± 0.24
Full-thickness skin	Granulation tissue	Tail	3.2 ± 0.94	2.7 ± 0.83	2.9 ± 1.2	3.2 ± 1.03
		Flank	3.3 ± 0.9	2.9 ± 0.7	2.8 ± 0.6	2.82 ± 1.3
		Newborn	3.6 ± 1.41	2.6 ± 0.56	2.8 ± 0.6	2.82 ± 1.3
	Muscle	Tail	3.4 ± 1.02	ND	ND	2.8 ± 0.72
Matched flank sites disaggregated cells			2.94 ± 1.42	4.29 ± 1.01	3.45 ± 0.77	2.67 ± 0.48

[a] Numbers are mean colony size in mm ± standard deviation, results from two experiments. Grafts were prepared and the graft keratinocytes cultured as described for Table III. Keratinocyte cultures were grown for 14 d, fixed in formol saline, and stained with Rhodamine B and the individual colony size measured using a micrometer scale in the ×10 eyepiece of an inverted microscope with a ×4 objective.

[b] ND, not done.

the aging wound bed but occurs irrespective of whether the graft is placed on the granulation tissue bed or on the lumbo-dermal fascia. These appearances can be contrasted with that of a FTSG (Fig 5) after 48 d in situ on the granulation bed. In this experiment neonatal hairless skin was grafted; after 48 d hair could be seen growing from the center of the graft and histologically (Fig 5*C*) the skin is characterized by a healthy well-organized epidermis with many mature pilosebaceous units.

In view of these data in the mouse we examined the clonogenicity of keratinocytes in split skin grafts when compared with cultured epidermal grafts in patients (Table V). Clonogenic keratinocytes could be isolated (several weeks post grafting) from the healed sites of ulcers grafted with split skin. However, biopsies taken from the center of healed leg ulcers grafted with cultured allogeneic keratinocytes did not produce keratinocyte colonies in vitro using the feeder culture technique, nor did they result in epithelial outgrowth when explanted as fragments. Control biopsies, taken from a site adjacent to the healed ulcer, had a normal colony-forming efficiency and exhibited epithelial outgrowth in explant culture. The latter observation also suggests that the clonogenic keratinocytes from the graft site were unlikely to have been specifically left behind because of inadequate trypsin digestion of the graft. Several studies have shown that cultured keratinocyte allografts do not persist [39,40] and that the epithelium covering the healed ulcer in these cases is of host origin and must be derived either from the epidermis at the wound edge or from epidermal remnants in the wound bed. In view of this evidence, the epithelium overlying the healed ulcer in those patients grafted with cultured allogeneic epithelium in this study is almost certainly not the donor graft but probably is derived from the clonogenic keratinocytes at the wound edge. This is supported by the clinical observation that the ulcers healed from the edges inward with no evidence of graft take and explains also why the biopsy site healed in every patient. These keratinocytes, which had never been

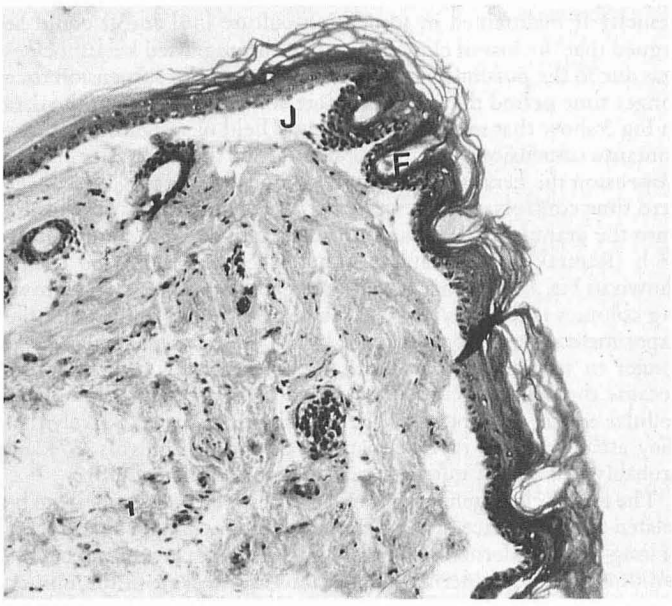


Figure 2. Histological appearance of tail skin after treatment for 2 h at 37°C with the neutral protease, dispase. The dermo-epidermal junction (J) is cleaved cleanly. The separated epidermis that includes hair follicles (F) is grafted as described in the text. Hematoxylin and eosin ×40.

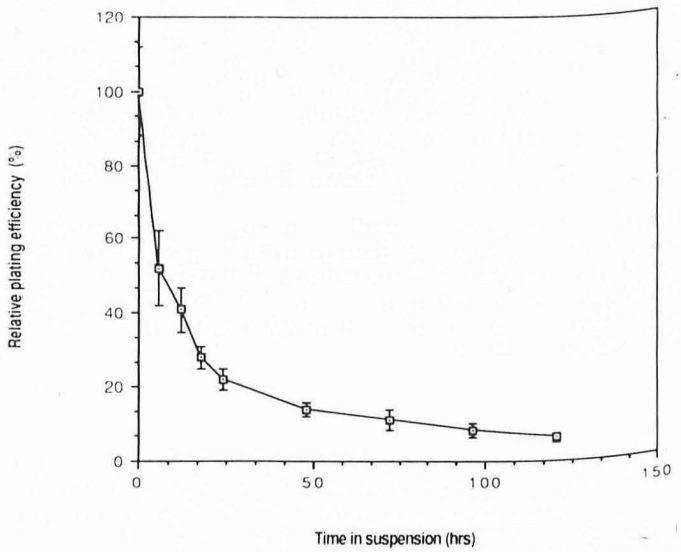
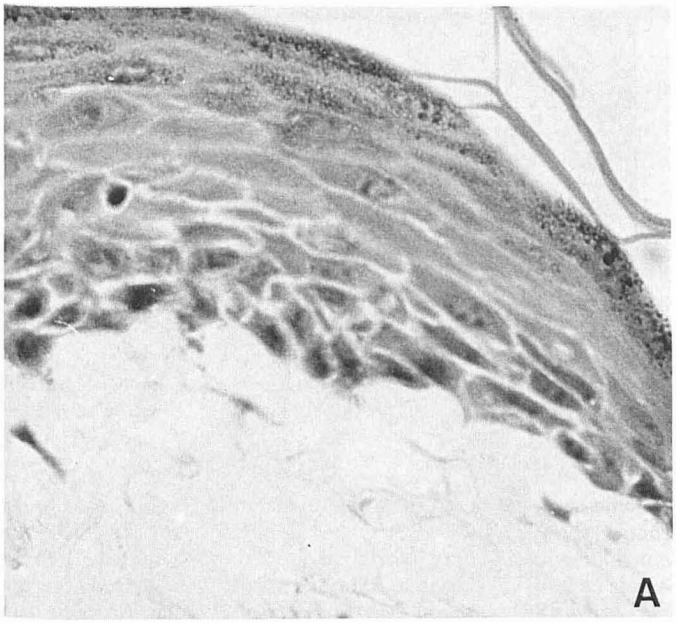
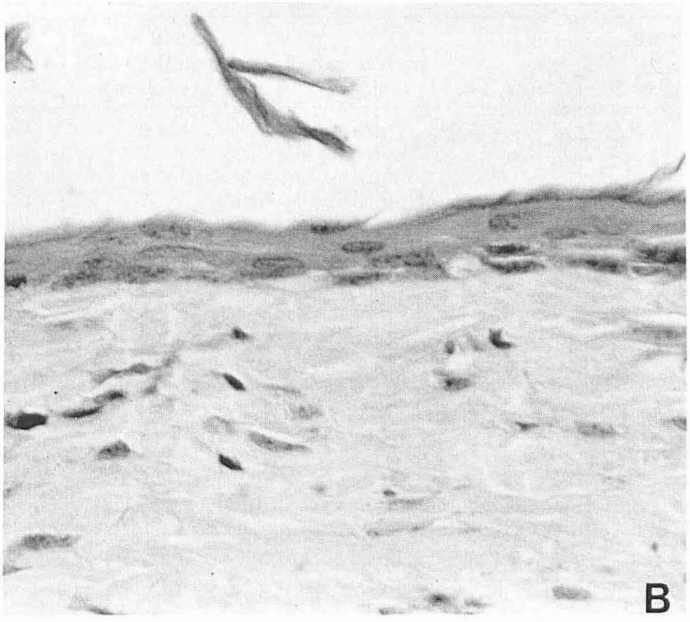


Figure 3. Freshly dissociated mouse (MEK) epidermal cells contain a suspension resistant population. MEK prepared as described in *Materials and Methods* were held in suspension for 6–120 h and then replated on a lethally irradiated 3T3 feeder layer. The relative plating efficiency is expressed as the colony-forming efficiency on replating cells held in suspension as a percentage of the colony-forming efficiency of cells at zero time. *Points,* mean CFE per dish from quadruplicates from two experiments ± the standard error of the mean.





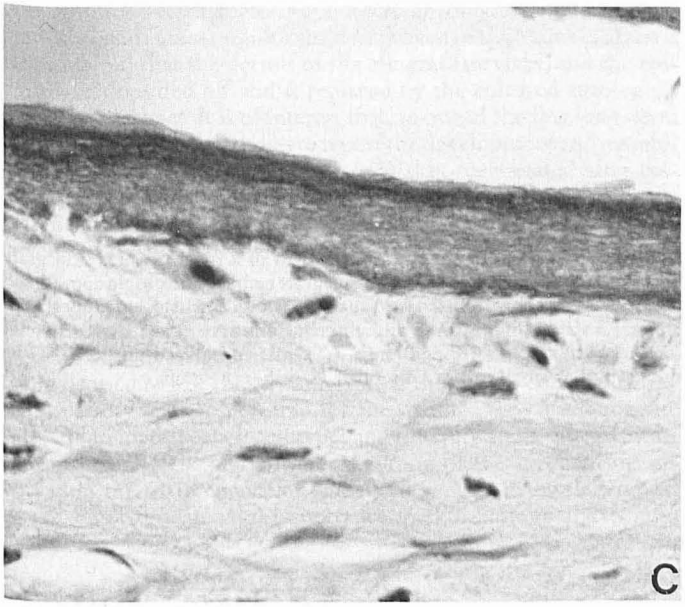


Figure 4. *A*) Histologic appearance of an epidermal graft after 15 d. The reformed epidermis is hyperplastic but well differentiated. Hematoxylin and eosin ×250. *B*) Epidermal graft after 40 d. The epidermis is thin with an atrophic appearance but nucleated cells are still present. Hematoxylin and eosin ×250. *C*) Epidermal graft after 70 d. The epidermis is grossly atrophic and nucleated cells are no longer present. Hematoxylin and eosin ×250.

trypsinised or placed in suspension, had nonetheless lost their ability to self-renew once they had migrated over the granulation bed of the ulcer.

DISCUSSION

The results from both the experimental model and the clinical group strongly suggest that the environment provided by the dermis is required for the maintenance of the clonogenic keratinocytes and, possibly, keratinocyte stem cells. This is consistent with the arguments of Schofield and colleagues [8,41] and Dexter and his associates [42,43], who have provided evidence that clonogenic +hemopoietic cells require functional marrow stroma for self-renewal either in vivo [8] or in vitro [42,43]. In the absence of a functional stroma hemopoietic cells proliferate but become committed to specific lineage differentiation programs [42,43]. This has led Dexter and co-workers to postulate the existence within the marrow stroma of accessory cells that could secrete critical growth factors [42] or extracellular matrix molecules thus constructing a unique environment — a stem cell niche [8,41,42], a hypothesis that has been supported by the demonstration of specific receptors on hemopoietic stem cells and the corresponding ligand on the marrow stromal cell [16]. There are some parallels in our mouse epidermal model.

Initially, the epidermal grafts are hyperplastic and eventually atrophy, but the epidermis of FTSG's retain a normal stratified appearance. In view of the hyperplastic appearance of the epidermal grafts, it could be argued that the stem cells in these grafts had been depleted due to a lack of negative feedback inhibition. This could result in their inappropriate differentiation [43] or their loss due to mass recruitment from the reserve stem cell pool [10] and a resulting fall in their quality of self-renewal with each division [10,13]. However, when keratinocytes are isolated from epidermis made hyperplastic by the phorbol ester TPA the cloning efficiency of these cells is 2–3 times *higher* than keratinocytes isolated from control epidermis (Al-Yaman and Parkinson, unpublished data). Therefore hyperplasia is not sufficient to cause the clonogenic population to deteriorate in the presence of dermis.

The dermis has long been known to be critical for epidermal

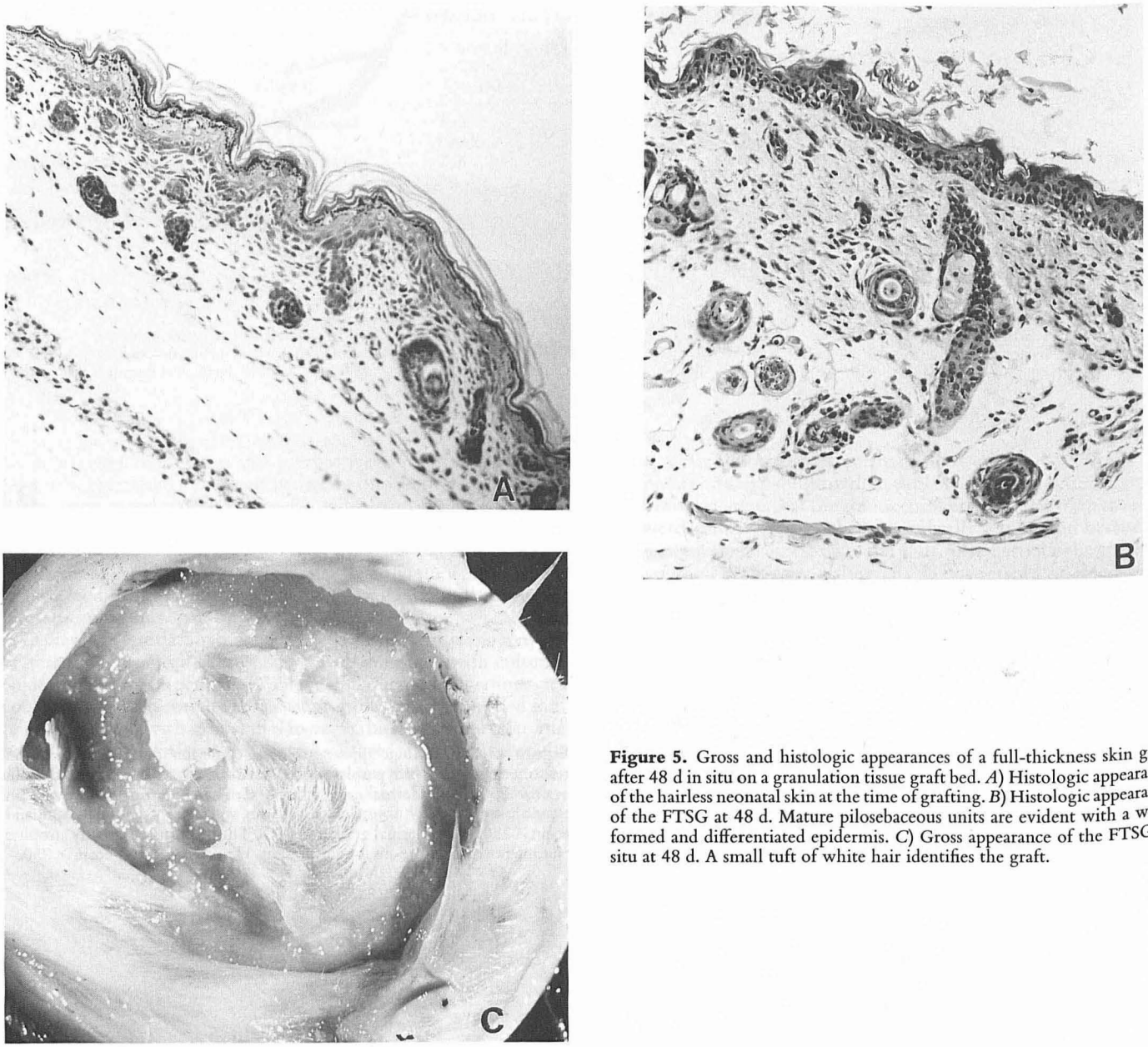


Figure 5. Gross and histologic appearances of a full-thickness skin graft after 48 d in situ on a granulation tissue graft bed. *A*) Histologic appearance of the hairless neonatal skin at the time of grafting. *B*) Histologic appearance of the FTSG at 48 d. Mature pilosebaceous units are evident with a well-formed and differentiated epidermis. *C*) Gross appearance of the FTSG in situ at 48 d. A small tuft of white hair identifies the graft.

growth and differentiation [17–20,44] and it is possible that one essential function is the provision of accessory cells for keratinocyte self maintenance because it is clear that the keratinocytes alone or in conjunction with the other epidermal cell types cannot engender the correct environment. It is likely that the protective environment of the dermis is regulatory, involving the removal of anti-proliferative factors as well as the delivery of growth factors [45]. TGF-β_1, a well-recognized inhibitor of keratinocyte growth [46], is known to be produced by some of the cell types present in granulation tissue [47]. Furthermore, Rollins et al [48] have shown that certain fibroblastic cells have a potent capacity to degrade TGF-β_1, and they suggest that the maintenance in vitro of clonogenic keratinocytes by fibroblasts and of hemopoietic cells by marrow stroma [49] may be related partly to the removal of growth inhibitors. Additionally, cytokines are known to modulate the extracellular matrix [50] and vice versa [51,52]. In particular, heparan sulphate proteoglycans have been implicated in growth-factor binding and presentation [51,52]. Hence specific dermal extracellular matrix molecules by binding growth factors or by altering cell shape, may be crucial to the creation of keratinocyte microenvironments. Finally, because cell shape is known to regulate both cell proliferation [53] and differentiation [54], the morphology of the dermis could also contribute to stem-cell maintenance by physically regulating the three-dimensional architecture of the epidermis in intact skin. The last idea would be consistent with our observation that dispersed cell suspensions of full-thickness skin could not reconstitute a stem-cell niche when injected into the graft chamber (Table III) even though they presumably contained all the skin types.

The results in the present study imply that the dermis has a critical role in epidermal self-renewal. These observations have important implications for the clinical use of cultured keratinocyte grafts, both as wound dressings and as vehicles to deliver exogenous gene products because it is possible that such grafts in the absence of dermis have a limited life span. Our experimental data is, however, consistent with results obtained clinically when cultured keratinocyte grafts have been used as wound dressings in the treatment of burns or varicose ulcers. These grafts have now been used in many centers but the clinical experience with cultured autologous epithelium is variable, the major problem being poor and patchy graft take [55]. The main clinical determinant of graft take in burn patients is the

Table V. In Vitro Growth of Biopsies from Healed Varicose Ulcers Covered with Cultured Keratinocyte Allograft or Split Skin Graft[a]

Patient Code	Age (years)	Type of Graft	Site Grafted	Time of Biopsy Post-Grafting (weeks)	Explant Culture Result	Clonogenic Cell Culture, Colonies/10^4 Cells
A17	78	Cultured allograft	Ulcer	6	No growth	
A39	86	Cultured allograft	Ulcer	2	No growth	
A46	73	Cultured allograft	Ulcer	4	No growth	
A61	49	Cultured allograft	Ulcer	7	No growth	
		Control skin			Outgrowth	
A64	46	Cultured allograft	Left leg ulcer	20		0
		Control	Right leg	20		69
AY	66	Split skin	Left leg	4		33
		Control	Right leg	4		146

[a] Using published protocols [31], cultured epithelial grafts were prepared from living donors screened for Hepatitis B and HIV infection. Biopsies were taken and cultured as described in the text. Clonogenic cultures were maintained for 14 d. The feeder cells were removed and the colonies fixed in 10% buffered formol saline, stained with Rhodamine B, and counted.

wound bed, the best results being obtained where a dermal element is retained and the worse observed on granulating wounds [55,56]. Further problems encountered with these grafts have been blistering and contracture due to the absence of dermis [57] and an abnormal structure at the dermo-epidermal junction [58]. However, when cadaver skin allografts are used prior to grafting with autologous cultured keratinocytes, good results are obtained [59,60] and in particular graft take is significantly improved [61]. In such grafts it is reported [62] that the dermis of the allograft survives, and the epidermis is sloughed off and is replaced by the cultured autologous keratinocyte sheet. It is of interest that, in one of the few long-term and well-documented studies to report the development and maintenance of normal histologic features in skin regenerated after cultured keratinocyte engraftment of full-thickness burns in pediatric patients, all wound beds were excised to fascia and the majority covered at least initially with cadaver allograft or porcine xenograft [63].

It is clearly of importance to identify the function(s) of the dermis in maintaining epidermal self-renewal. The transplantation model used in this investigation or human skin equivalent models [64] could be used to test the hypothesis that the dermis contains essential accessory cells responsible for the maintenance of clonogenic cells and to identify such cell types and their products. The identification of such cells and an understanding of the mechanisms involved in the maintenance of epidermal keratinocyte self-renewal would be of fundamental importance in skin biology and wound healing.

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