Human Hair Follicle Germinative Epidermal Cell Culture

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Isolated human hair follicle germinative epidermal cells were observed *in vitro* for the first time. When cultured alone, this small, round, novel cell type did not grow, divide, take on an outer root sheath–type appearance, or display any obvious signs of epidermal differentiation. We have previously described comparable cells from rat vibrissa follicles. However, in combination with human hair follicle dermal papilla populations, the germinative epidermal cells were stimulated into proliferative and complex interactive behaviors. This included the formation of composite organotypic structures containing not only impressively intact basement membrane, but also the hair-specific form, glassy membrane. Key words: dermal-epidermal interaction/dermal papilla/basement membrane/glassy membrane. *J Invest Dermatol 101:634–638, 1993*

Culture methodology, as applied to hair follicles and their cellular components, has progressed rapidly in recent years. Complete follicles at different stages of maturity can be maintained *in vitro* with continued fiber production [1–6]. An alternative approach has been the isolation and culture of the principal dermal [7,8] and epidermal [9–14] cell populations that make up the follicle. Most follicle epidermal cell cultures have been initiated from plucked outer root sheath (ORS) material [10–14]. Whole tissue pieces, or dispersed cells, prepared from the bases (or matrix regions) of plucked hairs, have been cultured with dermal feeder layers [15], although these populations have not been shown to be morphologically, behaviorally, or biochemically distinguishable from ORS cells.

The hair matrix, or formative region of the fiber, consists of some epidermal cells that are committed to hair-type differentiation, and others that are apparently undifferentiated. We selectively call the cells at the source of the latter group germinative epidermal (GE) cells. In the rat vibrissa follicle this population was seen to correspond to the group of cells left behind after the fiber and the major part of the hair matrix had been plucked. This locational cue was used as the basis for the isolation of biochemically distinct GE cells from rat vibrissa follicles by microdissection [16]. Alone in culture, vibrissa GE cells revealed a highly distinctive morphology and unlike ORS cells, or cells from plucked matrix, they did not grow in epidermal cell culture medium. However, when combined with living dermal papilla (DP) populations, the GE cells multiplied and displayed complex interactive behavior, including the formation of a basement membrane (BM) [16].

For these findings to have broader biologic applicability, and to widen their investigative potential for dermatologists, it was considered important to investigate whether a comparable population of GE cells could be isolated from the smaller human hair follicle, which also displays certain structural and behavioral differences. Here we show that GE cells isolated from the extreme base of the human hair fiber epidermal matrix have distinctive morphology and interact with papilla cells to make complex structures involving dermal and epidermal cell organization: a basal lamina and another junctional extracellular layer that resembles follicular glassy membrane (GM).

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Abbreviations: BM, basement membrane; DP, dermal papilla; GE, germinative epidermal; GM, glassy membrane.

MATERIALS AND METHODS

Isolation of Epidermal Material

Hair Follicle GE: Fat and connective tissues were cleared from the undersurface of excised non-neoplastic human skin (scalp, facial, axillary, pubic, or abdominal) obtained during routine biopsy, to expose the embedded hair follicle end bulbs (Fig 1*a*). The most proximal tip of each (less than one sixth of the entire length) was removed and transferred to a dish containing minimal essential medium (Gibco) with antibiotics (penicillin 50 U/ml, streptomycin 50 U/ml, kanamycin 150 μg/ml, and fungizone 2.5 μg/ml; Gibco) at 4°C. Fine needle points were used to invert the outer dermal sheath tissue layer and fully expose the hair matrix (Fig 1*b*). By pressing one needle point down on the center of the dermal sheath tissue the rest of the specimen could be held steady while the hair matrix was very carefully eased from over the DP with the other needle point. During this procedure, great care was taken to include the lower, innermost extremities of the hair matrix, because this was the location of the small amount of unpigmented tissue (regardless of the degree of fiber pigmentation) thought to represent the GE cells. This tissue was then removed from inside the base of the hair matrix by further needle-point manipulation, and material from between 20 and 30 follicles pooled for each culture, and then transferred on needle points into a 35-mm diameter petri dish.

Hair Follicle Matrices and ORS: Hair fibers were plucked from the center of the above-mentioned skin biopsies and the lowermost hair matrix region amputated. This amputated tissue was placed into culture, either immediately, or following enzymatic digestion (0.2% dispase, 30 mins, 4°C), hence, it could be considered similar to that previously isolated by others [15]. ORS tissue was mechanically separated from the remaining, larger portion of each plucked fiber, torn into pieces and tissue from around 10 follicles placed into each petri dish for culture.

Skin Basal Epidermal: Following amputation of any remaining portions of follicle, the skin from the hair follicle GE procedure was cut into approximately 3-mm² pieces, before incubation in a solution of 0.25% dispase (Boehringer) in minimal essential medium at 4°C for 45 min. The epidermis

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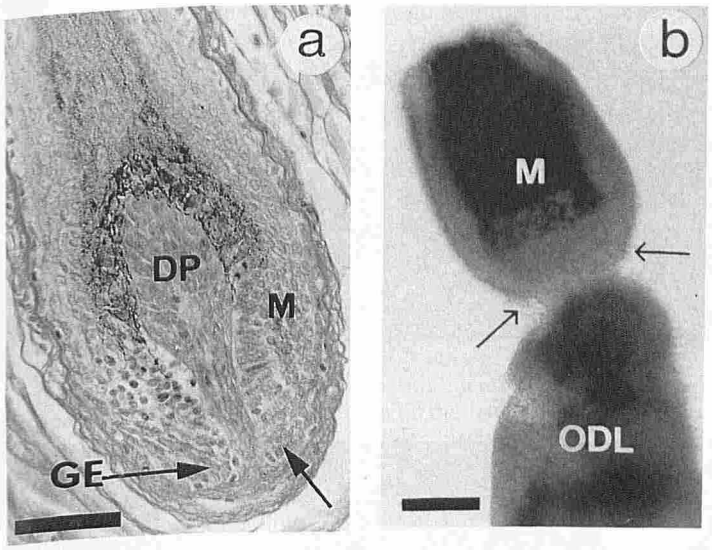

Figure 1. Human scalp hair follicle end bulb region. Histologic section revealing the DP and overlying hair matrix (M), with the GE cells at the lowermost extremities *(arrows) (a)*. During germinative cell isolation the outer dermal layers (ODL) are peeled back to expose the hair matrix (M) and the lowermost germinative cells *(arrows) (b)*. *Bar,* 50 μm.

was then peeled from the dermis, shredded, and placed into culture undersurface down.

Isolation of Dermal Material

DP Cells: Dermal papillae were isolated during the manipulations involved in the dissection of GE material, pooled (20–30 per dish), fragmented, and then forced to adhere on the bottom of petri dishes.

Skin Fibroblasts: Dermal fibroblasts were cultured by explant outgrowth from small fragments of the scalp, pubic, axillary, or abdominal skin from which the follicular tissue had been derived.

Cell Maintenance Both dermal and epidermal cell types were cultivated at 37°C, pH 7.3, with 5% CO_2, antibiotics, and either with or without sterile glass coverslips. The dermal populations were initiated as primary cultures in 20% fetal calf serum (Gibco; reduced to 10% for subsequent maintenance) in minimal essential medium containing 1% L-glutamine at a final concentration of 2 mM. The epidermal populations were initiated in a comparable medium, but with additional epidermal supplements of 140 μg/ml fetal rat pituitary extract, 10 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 0.4 μg/ml hydrocortisone (Sigma), 0.01 μg/ml epidermal growth factor (Sigma); and 10^{-9} M cholera toxin (Sigma). Alternatively, the epidermal cells were grown in the same medium without any fetal calf serum. The recombinations described below were carried out in all three variations of media composition.

Dermal-Epidermal Recombination GE cell tissue isolated from about 30 follicles was positioned over pre-prepared passage two monolayers (approximately 2.5×10^5 cells per dish) of either hair follicle DP cells or skin fibroblasts, derived from the same body region. These recombinations were directly compared with others using equivalent quantities of follicular ORS or basal epidermal cells. Eleven repetitions were conducted for each type of recombination.

Comparative morphologic observations and photographic recordings were made at regular intervals using a Zeiss inverted microscope (ICM 405). Selected material was also processed for transmission electron microscopy as described previously [16].

RESULTS

Comparison of Isolated Epidermal Cells

Hair Follicle GE: Irrespective of the media used, accumulations of unpigmented, loose, sticky material, containing small round cells attached to the culture substrate on only a very low number (one of 11) of occasions (Fig 2*a*), although the use of coverslips kept the material more stably positioned for observation. All of the GE cells remained small, round, and inactive throughout the period of study,

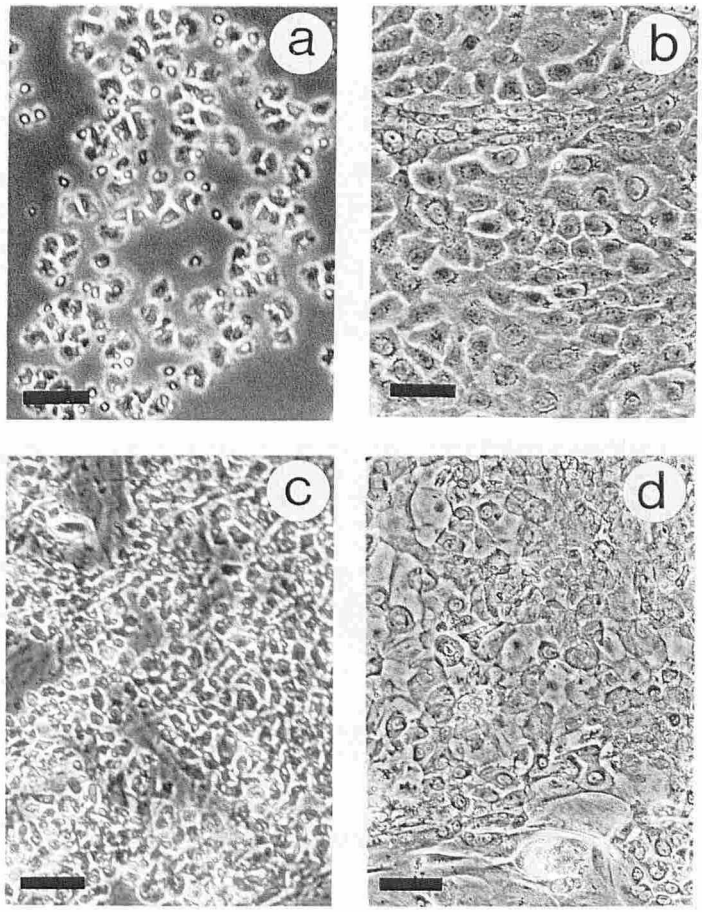

Figure 2. Hair follicle epidermal cells in culture. Isolated germinative cells are small, round, and inactive in isolation *(a)*, whereas outer root sheath populations form flattened pavements of polyhedral cells *(b)*. Following their recombination with dermal papilla monolayers, the proliferating germinative cells retain their distinctive morphology *(c)*, and differ in appearance from recombined outer root sheath cells *(d)*. *Bar,* 20 μm.

displaying no indication of growth, division, or flattening into an epidermal-typical, or ORS-type, morphology. Neither were there any visual signs of these cells deteriorating, differentiating, or enucleating.

Hair Follicle Matrix and ORS: Cells with typical epidermal morphology (pavements of flattened polyhedral cells) grew out from the sides of five of the 11 set-ups involving entire matrix specimens and established colonies in a manner comparable to that previously reported for rat material [16]. Prior treatment with dispase effected more rapid initial cell outgrowth but, after 8 to 10 d, final cell numbers were always similar to those from untreated matrices. A higher proportion (nine of 11) of the freshly isolated ORS tissue produced cell outgrowths whose cells were indistinguishable from those derived from matrices (Fig 2*b*). Under all culture conditions, the epidermal colonies were well established after 7 d and normally grew approximately threefold over the next week before beginning to differentiate, or deteriorate, into dead enucleate cells.

Skin Basal Epidermal: Specimens of skin epidermis attached well, and basal epidermal cells emerged from underneath the edges of all of these explants in the first few days of culture. General cell appearance and rates of outgrowth were similar to those observed for the ORS populations, although the skin basal cells displayed greater stratification prior to their death/differentiation.

Dermal-Epidermal Recombinations

GE/DP: In the presence of DP cells, GE behavior was completely altered. In well over half (seven of 11) of these recombinations the

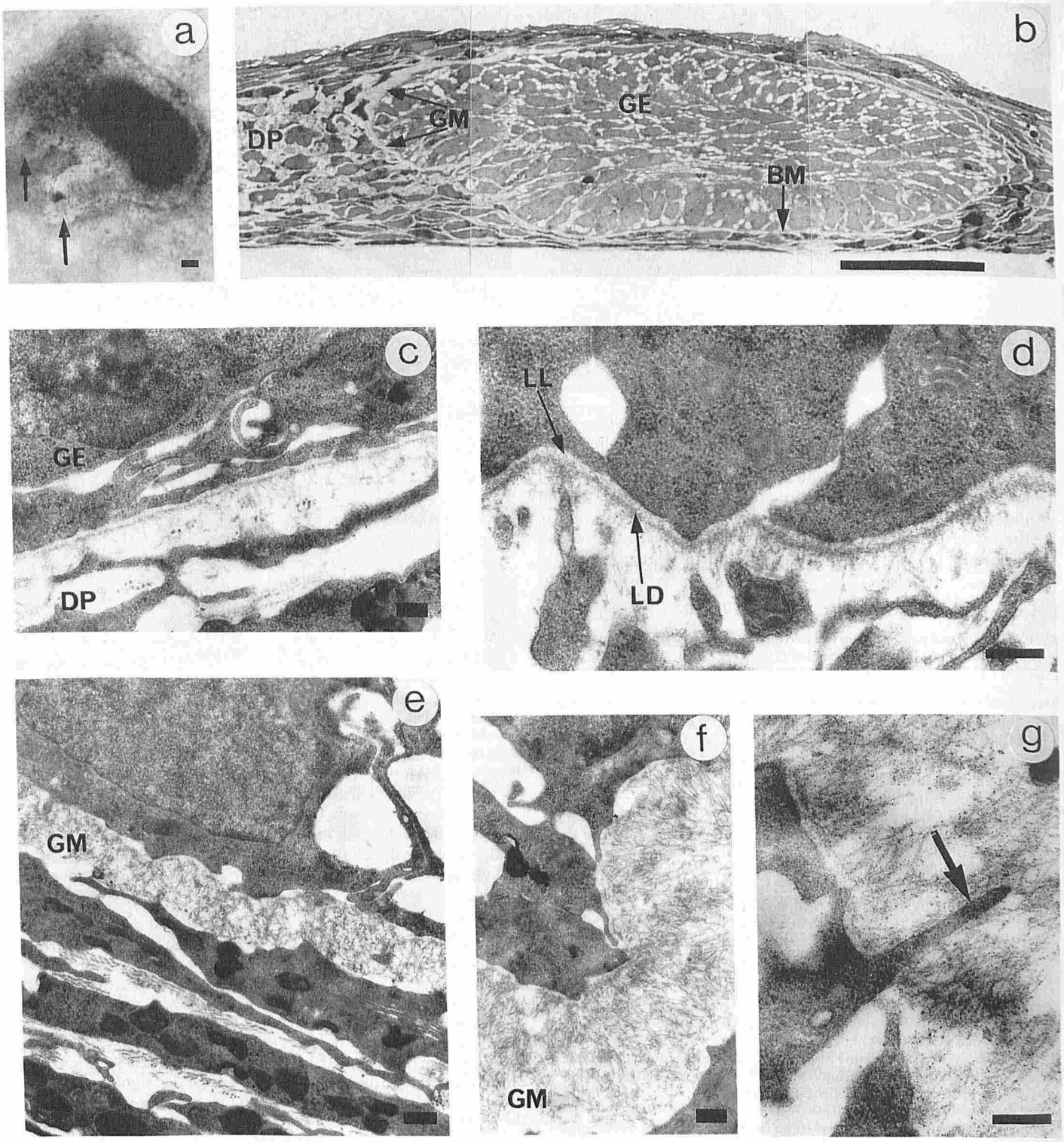

Figure 3. A composite organotypic structure resulting from the recombination of hair follicle germinative epidermal and dermal papilla cells. Viewed by bright field microscopy in culture, the formation appeared to be very dense centrally with a number of peripheral extensions *(arrows) (a)*. A semi-thin toluidine-blue stained section revealing "undifferentiated" GE, some of which appear polarized along the lower basement membrane (BM) *(b)*. The epidermis is completely surrounded by DP cells, which display *in situ*–type morphology and anagen-like loose arrangement within non-fibrous extracellular material. Hair follicle–specific GM is visible, particularly to the sides of the structure *(arrows)*. Transmission electron microscopy reveals basement membrane at the DP and GE interface *(c)*. At higher magnification, dermal cells are seen to associate with extracellular matrix beneath the well defined *lamina lucida* (LL) and *lamina densa* (LD) *(d)*. Broader regions of extracellular material at the dermal-epidermal interface showing remarkable resemblance to hair follicle glassy membrane (GM) *(e,f)*. A GE cell projecting into the glassy membrane *(arrow) (g)*. *Bars: a,b,* 50 μm; *c,d,e,f,g,* 300 nm.

GE cells divided and frequently developed into tightly packed horizontal and sometimes even vertical extensions from their original groupings. As has been found previously with rat GE populations, the human GE cells retained a small round profile and did not flatten (Fig 2*c*). The most pronounced activity observed was the formation of dense mound-like composite structures (Fig 3*a*). Semi-thin sections through these structures revealed that the GE cells had become completely surrounded by the DP cells and that the basal GE cells were organized in a polarized manner (Fig 3*b, arrow*). Examination of the dermal-epidermal interface with transmission electron microscopy revealed the presence of long stretches of extremely well-defined basement membrane (BM), complete with prominent *lamina lucida* and *lamina densa* (Fig 3*c,d*). Moreover, continuous parts of this junction (predominantly at the sides of the formation) were composed of an extracellular structure identical to the thick hair-specific BM that runs up the side of the hair follicle—the glassy membrane (GM; Fig 3*e,f*). Considerable interaction was evident between the most basal dermal and epidermal cells and microfilamentous projections of the extracellular matrix (Figs 3*c–f*, but most obviously in Fig 3*d*), and fingerlike extensions from basal GE cells were clearly observed traversing the BM and GM (Fig 3*g*). Another unusual feature was the morphology and arrangement of the DP cells at the sides of the germinative epidermis. These were widely separated by extracellular matrix and had a highly stellate appearance and multiple cell extensions, remarkably similar to papilla cells *in situ*.

GE/Skin Fibroblast: GE cell growth was never promoted in recombination with skin fibroblasts. Even when GE tissue was forced to adhere in proximity with the surface of the fibroblasts by employing glass coverslips, the GE cells behaved in exactly the same manner as they did in isolation.

ORS/DP: The rate of ORS cell division was promoted by recombination with DP cells. As with rat material, ORS cells grew well both directly over and on the substrate between the papilla cells. Growing on the petri dish surface between the DP cells, the ORS cells appeared to be no different in morphology from that of equivalent cells grown in isolation. However, the ORS cells that grew directly over the DP cells appeared more compact (Fig 2*d*), although never as small and round as the GE cells (Fig 2*c*).

ORS/Skin Fibroblast: ORS cell attachment to skin fibroblasts was poor, so that their overall pattern of growth and maintenance was inferior to that of cells in isolation.

Skin Basal/DP: Growth and division of skin basal epidermal cells was positively influenced by their recombination with hair follicle DP cells, and by a similar order of magnitude as the ORS cells. Their morphology and ability to grow directly over the DP cells as well as between them were also comparable to the ORS cells.

Skin Basal/Skin Fibroblast: While a few small groups of basal epidermal cells attached to the fibroblasts, their subsequent proliferation was limited, to the extent that the culture of the skin basal epidermal cells was actually more favourable in the absence of the fibroblasts.

DISCUSSION

Over the past decade, most of the major dermal and epidermal cell sub-populations of the hair follicle have been isolated and grown in culture, although until recently it remained questionable whether the crucial epidermal cell progenitor population at the base of the hair matrix could be cultured as a distinct cell type. Indeed, work using plucked matrices as starting material [15,16] suggested, to the contrary, that at least some matrix-associated subpopulations might behave like ORS cells in culture. Our strategy of culturing those cells at the extremities of the matrix with the greatest tendency to associate tightly with the papilla (that is, usually left attached to it after plucking) showed that these GE cells isolated from the rat vibrissa follicle were different from other follicular and inter-follicular epidermal cells in terms of their morphology, *in vitro* behavior, and biochemical composition [16]. However, because there is considerable inter- and intraspecific variation in follicle type and activity [17,18], it remained a possibility that anatomical and cyclical differences might have promoted variability in germinative material dissected from rodent vibrissa and human pelage follicles. Furthermore, hair follicle epidermis represents one of the fastest dividing cell populations in the body [19], so that the basal cells isolated from this region might have been expected to display high rates of replication in culture. In fact, human hair follicle GE cells were found to behave in a very similar manner to their rodent follicle-derived counterparts [16]. One of the most distinctive features of both species of GE cells was that under a broad range of culture conditions they remained inactive but then were stimulated into complex interactive behaviors when associated with DP cells. Thus, although the very small quantities of tissue that were involved in the current study made biochemical evaluation and comparison unfeasible, the size, morphology, and behavior (especially the DP-dependent activity) of the human GE cells all supported the proposition that they represented a distinct epidermal population.

The organotypic structures that developed from recombined human hair follicle GE and DP cells displayed features of their *in vivo* site of origin consistent with those produced by comparable associations involving rat vibrissa follicle–derived populations [16], but the human structures revealed additional complexity. In both cases, the dermis had completely enclosed the epidermis, or, conversely, the epidermis had invaded the dermis, suggesting a parallel with the typical arrangement of end bulb tissues. Similarly, the dermal and epidermal cells from both composite developments had constructed an organized, microscopically well-defined BM at their interface, but the human cells had also produced regions of GM, which represents the broader hair-specific form of BM. Interestingly, these impressive GM regions of rich extracellular diversity were predominantly situated at the sides of each structure, and *in situ* this tissue is also distributed up each side of the follicle. Perhaps the cells at the side of our induced structure were behaving like dermal sheath and those below like DP, that is, similar to the papilla-sheath dichotomy within the follicle. Furthermore, the germinative cells at the base of some induced arrangements appeared to be in polarized alignment on the basement membrane (Fig 3*b*), as is observed *in situ* at the base of the matrix horn (Fig 1*a*). However, the generally sparse cytoplasm and absence of any signs of pre-keratin filaments in the GE cells indicated that they were still in an "undifferentiated" state. In adult hair follicles, direct contact between DP and GE cells is rarely seen, but the fact that basal GE cell projections were observed to extend through to the dermal side of the basal lamina (Fig 3*g*) fits in with observations made during follicle development [20] when cell contacts may be required for information exchange. Moreover, and in contrast to their appearance in isolation, the dermal papilla cells assumed a "three-dimensional" *in vivo*–like morphology as loosely arranged polyhedral cells within an abundant extracellular matrix. Because we have only observed the DP cells looking like this following their combination with GE cells—and GE cells only grow in the presence of lower follicle dermis—positive reciprocal influences are clearly involved (see [16]).

The fact that glass coverslips enhanced epidermal attachment and promoted more rapid cell outgrowth and division suggests that they may provide an improved microenvironment for epidermal cell growth and interaction. Autocrine factors may be important, and it has been shown that newborn epidermal cells condition their culture medium and render it growth stimulatory for other cells in the same culture dish [21]. Also, epidermal cells may prefer a more restricted structural arrangement, because *in vivo* cells are always packed tightly together.

Recognition, isolation, and evaluation of all of the dermal and epidermal cell subpopulations that comprise the enigmatic hair follicle, and perhaps most importantly the progenitor cells, represent vital steps towards a full understanding of basic hair biology. There are substantial data relating to the composition of human hair fibers, the semi-differentiated matrix from which they form, and the

closely associated sheaths [22], but apart from recent observations on follicle bulge stem cells [23], there is very little information pertaining to precursor populations. The ability to isolate and manipulate hair follicle GE cells must now provide considerable scope for advancement in this area. Moreover, and beyond the context of the hair follicle, the ability of GE/DP recombinations to readily create organized BM has intrinsic dermatologic potential, as does the availability of cultured progenitor-type cells, to study questions relating to the involvement of progenitor epidermal cells in skin biology and pathology.

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