Epithelial Growth by Rat Vibrissae Follicles In Vitro Requires Mesenchymal Contact via Native Extracellular Matrix

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An in vitro assay utilizing the rat vibrissa anagen follicle as a model for studying the epithelial-mesenchymal interactions (EMI) in hair growth is described. Through selective disruption of the epithelial-mesenchymal interface, we investigate whether the specialized extracellular matrix (ECM) of the dermal papilla and basement membrane zone (BMZ) serves a crucial function in hair follicle EMI. Epithelial bulbs incubated intact within their follicular sheaths incorporate thymidine primarily into cells of the hair matrix and outer root sheath, as shown by autoradiography. However, after removal of its mesenchymal associations (dermal papilla and extrabulbar connective tissue), the epithelial bulb showed no incorporation. Neither externally added collagen (type I or IV) nor the basement membrane components in Matrigel could substitute for the growth supporting influence of native surrounding stroma. Mechanical separation of the bulb from the dermal papilla in the basement membrane zone inhibited thymidine incorporation by the epithelium even though mesenchyme was still in close proximity. Enzymatic digestion of the dermal papilla ECM and the basal lamina by Dispase, a fibronectinase and type IV collagenase, also inhibited bulb growth without evidence of cytotoxicity. These experiments suggest that direct epithelial to mesenchymal contact is required for the support of follicular epithelial growth in vitro and that specific ECM components, possibly fibronectin and/or type IV collagen, rather than diffusable factors alone, play a crucial role in the mechanism of hair follicle EMI. The in vitro system described here provides an alternative to developmental EMI models and may serve as a valuable tool for studying EMI in the adult mammalian organism. *J Invest Dermatol 95:202–207, 1990*

Epithelial-mesenchymal interactions (EMI) are implicated in a vast array of biologic and pathologic processes, including morphogenesis, wound healing, neoplasia, metastasis, the regulation of epithelial growth, and the pathogenesis of some skin diseases [1–3]. A wide variety of mechanisms have been postulated for these interactions, including communication by soluble factors, direct cell-cell signalling through gap junctions and cell adhesion molecules [4], and signal transduction mediated by changes in extracellular matrix (ECM) composition [5].

Although the underlying mechanisms guiding the hair through its growing (anagen), regressing (catagen), and resting (telogen) phases have not yet been well characterized, a number of observations highlight the importance of epithelial-mesenchymal interactions (EMI) in the regulation of hair growth [6,7]. i) The size of the follicle and hair fiber appear to be directly related to the volume of one follicular mesenchymal* component, the dermal papilla [8] whose partial disruption in situ has resulted in the extension of the growth cycle and the production of a longer hair shaft [9]. ii) In vivo, whisker growth ceases following the complete removal of the dermal papilla [10], and resumes after either freshly isolated dermal papilla or cultured papilla cells are grafted into the transected follicle [11–13]. iii) Fresh papillae can initiate new follicle development when transplanted between the epidermis and dermis of embryonic murine skin grafted onto nude mice [14].

Although it is clear from these studies that the dermal papilla plays an important inductive role in hair growth and development, very little is known about the detailed mechanism of hair follicle EMI. For example, it is currently not known whether the dermal papilla influences epithelial bulb growth solely through the production of diffusable factors (with the ECM acting only as a support structure) or whether interactions between these factors and ECM molecules are critical.

The cells of the dermal papilla are embedded in a dense matrix which contains an abundance of basement membrane components (type IV collagen, fibronectin, laminin, chondroitin sulfate, and

* For the purpose of this discussion, the term "mesenchyme" in the vibrissa follicle will be taken to include the cells of the dermal papilla, the dermal fibroblasts of the fibrous sheath and the ECM produced by these cells.

heparan sulfate proteoglycan) but little interstitial type I collagen [15]. Interestingly, the amount and distribution of a number of these components (notably fibronectin and type IV collagen) has been shown to vary during the hair cycle [16]. However, whether these ECM molecules play an important functional role in sustaining and controlling epithelial bulb growth is unknown.

Significant progress in answering these questions depends on the development of suitable in vitro models. The in vitro reconstitution of "hair follicle equivalents," for example, has not yet been accomplished; in part, because purified hair matrix keratinocytes have not been successfully grown in culture. Other groups have reported the growth of intact murine follicles in culture [17,18]. Unfortunately, neither of these models utilizes follicles collected from adult animals, and both have additional limitations. One depends on treatment of follicles with matrix-degrading enzymes [17] and the other requires the addition of an artificial mitogen, minoxidil, to the medium before significant epithelial growth is observed in culture [18].

In this report, we introduce a simple organoid model, utilizing rat vibrissae follicles isolated from adult animals, as a first step toward dissecting hair follicle EMI. Through selective disruption of the epithelial-mesenchymal interface, we investigate whether the specialized ECM of the dermal papilla and BMZ serves a crucial function in hair follicle EMI. We report that i) direct epithelial to mesenchymal contact is required for the support of follicular epithelial growth in vitro; ii) reconstituted interstitial collagen or basement membrane components, alone, cannot replace the inductive influence of native hair follicle mesenchyme on bulb growth in this assay; and iii) enzymatic digestion of the papilla's ECM and the basal lamina inhibits thymidine incorporation into the epithelial bulb.

MATERIALS AND METHODS

Animals   Three-to-six-month-old female Long Evans Hooded Rats (Charles River Laboratory, Kingston, NY) were housed in common cages with 12-h light periods and fed ad libitum with water and "rat/mouse chow 3000" (Agway, Syracuse, NY).

Isolation of Follicles   Ten to fifteen anagen follicles were harvested from the upper lip of two to three rats as described by Messenger [19] (rats were killed by ether overdose) and placed into DME [Dulbecco's modified Eagle's medium (Yale Media Lab, New Haven, CT), supplemented with Antibiotic/Antimycotic mixture (Gibco, Grand Island, NY) and 0.05 mg/mL Gentamicin Sulfate (Sigma, St. Louis, MO)], on ice. The hair follicle is epithelial except for a loose surrounding sheath of connective tissue and the dermal papilla. In contrast to pelage follicles, vibrissae follicles are completely surrounded by an additional vascular sinus. Only anagen follicles were selected (identified by melanized epithelial bulbs of characteristic morphology) and cut transversely about 3 mm from the base. Samples were cleaned of extrafollicular tissue, leaving the proximal portion of the cavernous sinus sheath intact. To allow comparison between enzyme-treated and untreated samples (see below), all follicles were pre-incubated at 4°C for 6 h in DME + 0.05 mg/mL Gentamicin Sulfate.

Culture Conditions

The Incorporation of Thymidine into the Epithelial Bulb was Compared for Two General Culture Conditions: 1) bulbs cultured in the presence of native hair follicle mesenchyme with an undisturbed epithelial-mesenchymal interface (controls): follicles were placed individually into wells of a Corning 24-well tissue culture plate (Corning, NY) containing two drops of unpolymerized type I collagen gel (Vitrogen, 3.6 mg/mL; Collagen Corp., Palo Alto, CA). The matrix was polymerized around the sample according to the manufacturer's instructions (37°C for 30 min).

Bulbs Cultured in the Presence of Native Hair Follicle Mesenchyme with a Mechanically Disrupted Epithelial-Mesenchymal Interface: In this group of follicles, the epithelial-mesenchymal interface was disrupted mechanically, prior to plating, by gently separating the epithelial bulb from hair follicle mesenchyme with forceps. Al-

though epithelial-mesenchymal contact was broken by this treatment (presumably at the level of the lamina lucida in the basement membrane zone, as first demonstrated by Messenger [19]), proximity of the two tissue compartments was maintained. Type I collagen gel was then polymerized around the follicles, as above.

Bulbs Cultured in the Presence of Native Hair Follicle Mesenchyme with an Enzymatically Disrupted Epithelial-Mesenchymal Interface: For this group, the pre-incubation medium was supplemented with Dispase (grade II, 0.5 units/mg, 10 mg/mL; Boehringer Mannheim, Indianapolis, IN) and the follicles were washed 3X in PBS (4°C) before plating. Type I collagen gel was then polymerized around the follicles, as above.

Bulbs Cultured Free of Native Hair Follicle Mesenchyme: For these samples, epithelial bulbs were removed from the follicular sheath and dermal papilla. These "extracted" bulbs were placed into two drops of (D) type I collagen gel (as above) or (E), a 1:1 mixture of type I collagen gel and Matrigel (EHS tumor matrix, composed of laminin, type IV collagen, heparan sulfate proteoglycan and entactin [20]) or (F) type IV collagen gel (in buffer: 50 mM Tris HCl, 0.5 M NaCl, pH 7.4). Polymerization of matrix around the sample was carried out as above. An additional group of "extracted" bulbs (G) were plated directly on plastic without matrix support.

For all sample groups (1 A–C and 2 D–G), 1.5 mL of culture medium (DME + 0.05 mg/mL Gentamicin Sulfate + 20% Fetal Bovine Serum; Gibco) was added to each well prior to incubation at 37°C and 5% CO_2/air, as outlined below.

Measurement of Thymidine Incorporation   The proliferative capacity of epithelial bulb cells was quantified for all sample groups as a function of incorporation of tritiated thymidine into the epithelial bulb. ^{3}H-thymidine (^{3}H-Tdr; TRK 418, specific activity: 171 mCi/mg, stock: 1 μCi/5 μL in CMF-Tyrodes; Amersham, Arlington Heights, IL) was added to each well after 54 h for an 18-h exposure of 6.67 μCi/mL (total length of incubation, 72 h).

Only incorporation of thymidine into the epithelial bulb was measured, and bulbs that were cultured in the presence of mesenchyme were removed from the fibrous sheath and dermal papilla before measurement. Bulbs were subjected to four serial washes of CMF-Tyrodes solution and placed individually in Eppendorf micro-centrifuge vials. A sample of the fourth wash solution was used as a background control. The bulbs were solubilized in 0.2 mL of 0.5 M NaOH for 1 h at 90°C [21] and then transferred to a clean, dry, scintillation vial. After 6 mL of Opti-fluor scintillation fluid (Packard Instruments, Downers Grove, IL) was added, each vial was vortexed for 20 sec, and the incorporated radioactivity was measured in a Beckman liquid scintillation spectrometer. Mean counts/min/bulb (CPM) and SE were calculated and corrected for background. All experiments were analyzed using analysis of variance, and a significance level of $p < 0.05$ was used throughout these experiments.

Histology and Autoradiography   Localization of incorporated label in control follicles, processed and incubated as above, was determined by autoradiography as previously described [22].

To visualize the action of Dispase on the epithelial-mesenchymal junction, some enzyme-treated and control follicles were prepared for plastic embedding. Samples were fixed for 1 h at 25°C in 3% glutaraldehyde, re-fixed and stained for 1 h at 25°C in 1% osmium tetroxide, dehydrated in ethanol, and embedded in LX resin. Ultrathin sections were stained with a 1:1 mixture of Azur 1 Blue and Azur 2 Blue and examined using light microscopy.

Some enzyme-treated follicles were prepared for electron microscopy by fixation overnight at 4°C in a solution of 2.5% glutaraldehyde plus 2% formaldehyde [23] buffered to pH 7.2 with 0.1 M sodium cacodylate buffer. They were re-fixed and stained for 1 h at 25°C in 1% osmium tetroxide plus 1.5% potassium ferrocyanide [24]. The fixed specimens were dehydrated in ethanol and embedded in Spurr's epoxy mixture [25]. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using transmission electron microscopy.

RESULTS

Bulbs Cultured in the Presence of Native Hair Follicle Mesenchyme with an Undisturbed Epithelial-Mesenchymal Interface (Controls): In these follicles, thymidine was incorporated between 54 and 72 h of incubation (18-h ^{3}H-Tdr exposure) primarily into cells in both the hair matrix and the outer root sheath (ORS; see Fig 1). Although labeling was not found in the dermal papilla, small groups of labeled cells were observed in the connective tissue sheath. This finding merits removal of the bulb before counting and may be of significance when using in vitro models which do not require removal of the epithelial bulb [17,18], because this makes the distinction between incorporation by fibroblasts of the perifollicular connective tissue and by epithelial bulb cells difficult.

Bulbs Cultured in the Presence of Native Hair Follicle Mesenchyme with Mechanically Disrupted Epithelial-Mesenchymal Interface: Mechanical disruption resulted in significantly less thymidine incorporation into these bulbs than into controls (p < 0.02, see Table IB), although these bulbs still incorporated more label than bulbs cultured free from hair follicle mesenchyme (p < 0.01, Table ID–G).

Bulbs Cultured in the Presence of Native Hair Follicle Mesenchyme with an Enzymatically Disrupted Epithelial-Mesenchymal Interface: Follicles exposed to Dispase (Table IC) yield bulbs which incorporate four times less thymidine than controls (Table IA). It is notable that bulbs from Dispase-treated follicles still incorporate more thymidine than extracted epithelial bulbs (Table ID–G), and incorporate about the same amount as bulbs whose contact with mesenchyme was disrupted mechanically (Table IB).

To demonstrate that cleavage of the epithelial-mesenchymal interface had occurred after Dispase exposure, both Dispase-treated and control follicles were examined by light microscopy. The untreated follicle (Fig 2a) shows intimate contact between the epithelial bulb and dermal papilla. In contrast, the Dispase-treated follicle (Fig 2b) shows mesenchymal degeneration and a wide gap between bulb and dermal papilla. While the epithelial bulb appears intact, the dermal papilla is partially disrupted, as documented by its pe-

ripheral loss of definition and its granular morphology. It is also notable that the cells of the dermal papilla clump into a tight mass after the extracellular matrix is digested by Dispase. This corroborates the generally appreciated dependence of fibroblast cell shape and attachment on the composition and arrangement of the surrounding ECM [26] and is reminiscent of the cell aggregates found in dermal papilla cell cultures (compare [13,19]).

To examine the action of Dispase on the hair follicle basement membrane zone, some Dispase-treated follicles were prepared for electron microscopy. These specimens show optimal preservation of the basal layer of keratinocytes in the epithelial bulb (Fig 3a) without morphologic indications of cell damage. Figure 3b demonstrates the absence of the basal lamina and the preservation of anchoring fibrils in the basement membrane zone, observations consistent with the previously reported effect of Dispase-exposure on the dermo-epidermal junction [27].

Bulbs Cultured Free of Native Hair Follicle Mesenchyme: These "extracted" bulbs (Table ID–G) incorporated significantly less thymidine than bulbs cultured in the presence of native mesenchyme (p < 0.01; Table IA–C). In addition, the incorporation of ^{3}H-Tdr by bulbs embedded and incubated in type I collagen gel alone (Table ID) did not significantly differ from that of bulbs incubated without externally added matrix (Table IG) or bulbs embedded and incubated in either a mixture of reconstituted basement membrane (Matrigel: containing type IV collagen, laminin, entactin and heparan sulfate proteoglycans) and type I collagen gel (Table IE), or in type IV collagen gel alone (Table IF). Therefore, the basement membrane components present in both Matrigel and purified type IV collagen were unable to elevate isolated epithelial bulb ^{3}H-Tdr incorporation to control levels and, thus, could not reconstitute the growth supporting influence of native hair follicle mesenchyme.

DISCUSSION

In these experiments, we have developed a simple organoid system for studying hair follicle EMI in the mature follicle. By disrupting the epithelial-mesenchymal interface both mechanically and enzy-
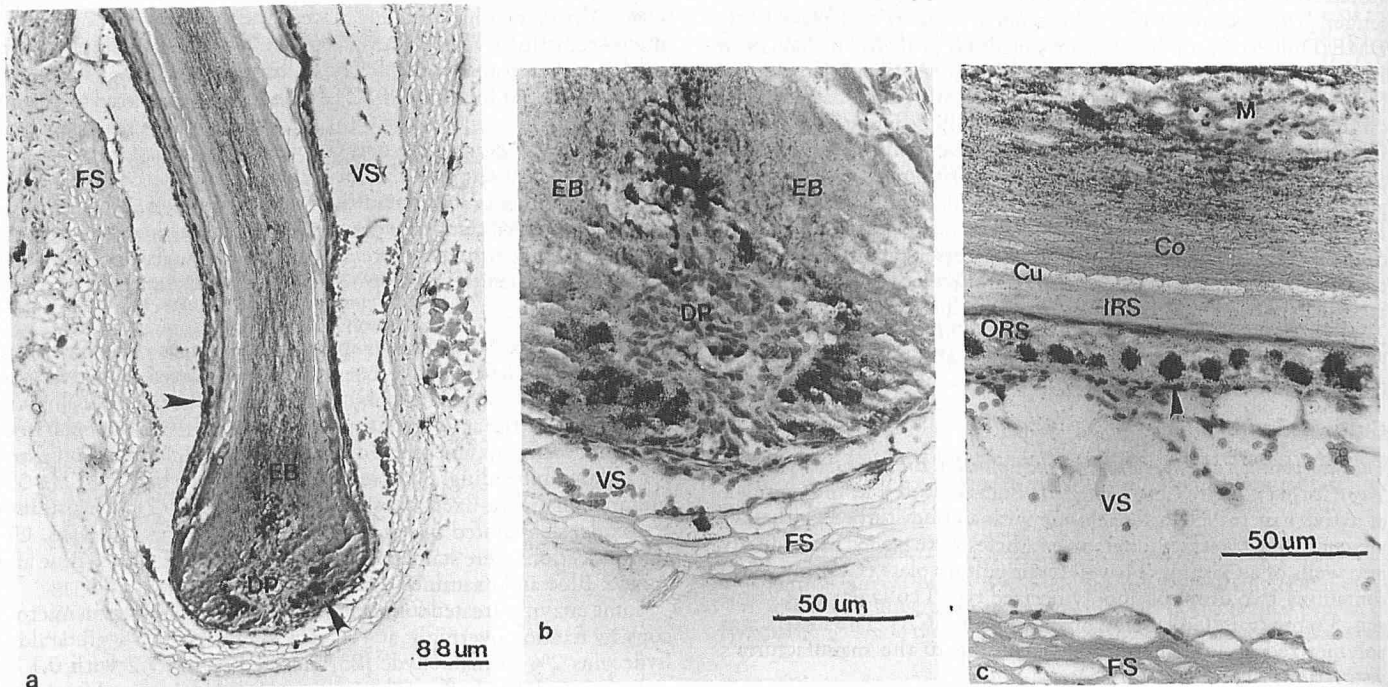

Figure 1. Autoradiographic localization of ^{3}H-thymidine incorporated into the epithelial bulb: This photomicrograph shows an intact rat vibrissa follicle cultured for 72 h and exposed to ^{3}H-Tdr from 54 to 72 h of culture. A longitudinal section (*a*) through the follicle shows labeled nuclei in both the hair matrix and outer root sheath. High magnification photomicrographs of the hair matrix (*b*) and outer root sheath (*c*) are also shown. Sections were stained with hematoxylin and eosin. Notation on these figures is as follows. Co, cortex; Cu, cuticle; DP, dermal papilla; FS, fibrous connective tissue sheath; IRS, inner root sheath; M, medulla; ORS, outer root sheath; VS, vascular sinus; *arrows,* labelled nuclei.

Table I. Comparison of Thymidine Incorporation into the Epithelial Bulb[a]

	n	Mean CPM/ Bulb ± SE	Significant Difference A	B	C	D
(1) Bulbs cultured in the presence of native hair follicle mesenchyme						
A. Undisturbed (Controls)	13	1946 ± 482		Yes (p = 0.012)	Yes (p = 0.006)	Yes (p = 0.002)
B. Mechanically disrupted	12	538 ± 121	Yes (p = 0.012)		No (p = 0.612)	Yes (p = 0.001)
C. Enzymatically disrupted (Dispase-treated)	13	442 ± 142	Yes (p = 0.006)	No (p = 0.612)		Yes (p = 0.021)
(2) Bulbs cultured free of native hair follicle mesenchyme						
D. In type I collagen	10	37 ± 9	Yes (p = 0.002)	Yes (p = 0.001)	Yes (p = 0.021)	
E. In type I collagen + Matrigel	10	42 ± 7	Yes (p = 0.002)	Yes (p = 0.001)	Yes (p = 0.023)	No (p = 0.682)
F. In type IV collagen	10	20 ± 7	Yes (p = 0.002)	Yes (p = 0.001)	Yes (p = 0.017)	No (p = 0.141)
G. No supplement	10	29 ± 5	Yes (p = 0.002)	Yes (p = 0.001)	Yes (p = 0.019)	No (p = 0.415)

[a] All follicles were pre-treated intact for 6 h at 4°C prior to manipulation, either in DME alone (A,B,D,E–G) or in DME supplemented with Dispase (C), embedded in collagen matrix (except for group G), and cultured for 72 h in DME + 20% Fetal Bovine Serum. All the intact follicle samples (A–C) were embedded in type I collagen, while the matrix used for the extracted bulbs (D–F) varied. Samples were exposed to ^{3}H-Tdr between 54 and 72 h of culture (for A–C, bulbs were removed from the follicular sheath prior to solubilization and scintillation spectrometry). n represents the number of individual bulbs measured per group, and all data are corrected for background (10–40 CPM).
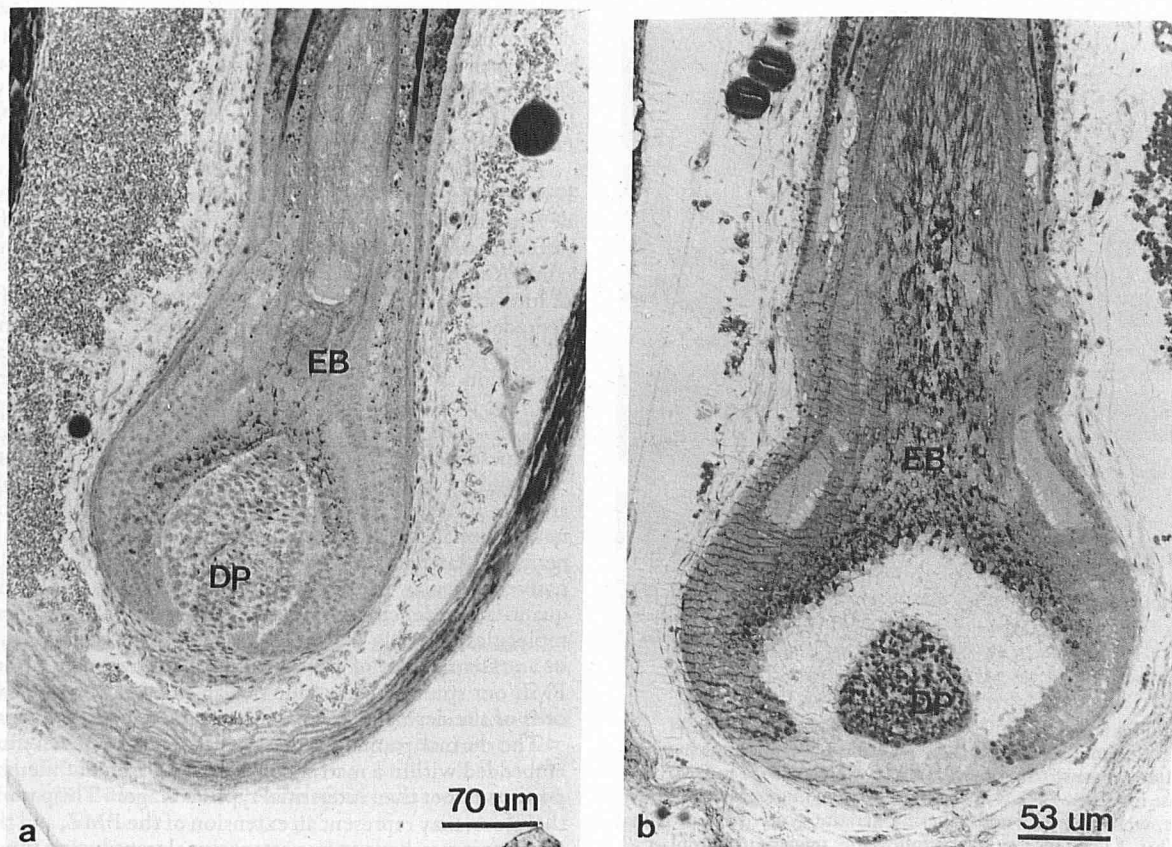

Figure 2. Histologic comparison of Dispase-treated follicles with controls: a: Photomicrograph of the lower portion of an intact vibrissae follicle exposed to DME alone for 6 h at 4°C and then sectioned longitudinally (controls). b: Photomicrograph of the lower portion of an intact vibrissae follicle exposed to DME containing Dispase for 6 h at 4°C and then sectioned longitudinally (Dispase-treated). Note the excellent preservation of epithelial bulb morphology and the wide gap between the bulb and the dermal papilla. Digestion of the papilla's ECM causes the papilla cells to aggregate into a tight mass at the base of the follicle. Sections were stained with a 1:1 mixture of Azur 1 Blue and Azur 2 Blue. Notation in these figures is as follows. DP, dermal papilla; EB, epithelial bulb.
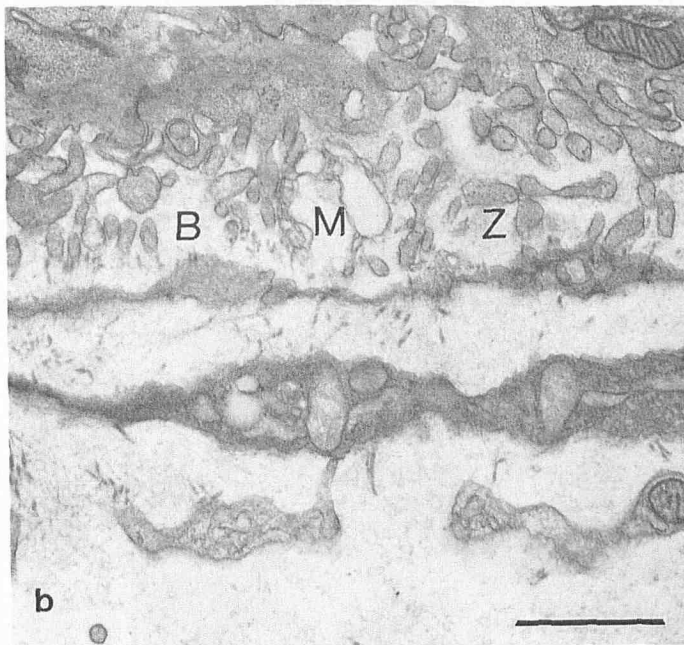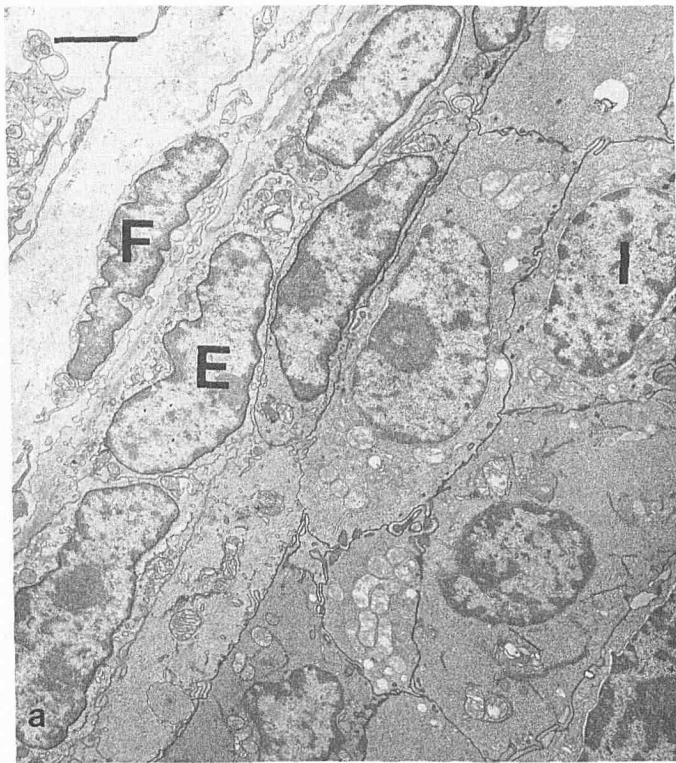
matically, we provide evidence that the EMI regulating epithelial growth in the rat anagen vibrissa follicle depend on epithelial-mesenchymal contact through an intact basal lamina. Because selective enzymatic digestion of the basal lamina and the papilla ECM suppressed thymidine incorporation into the bulb, these data suggest that specific ECM components in their native form play a crucial role in the mechanism of hair follicle EMI.

It is unlikely that the observed reduction of bulb growth was due to epithelial cell damage or disaggregation by Dispase, because 1) this enzyme does not rapidly dissociate epithelial cells [28]; 2) it has virtually no effect on epithelial cell viability, even when used at 37°C, and has been used to successfully passage keratinocytes and harvest intact sheets of these cells for grafting purposes [29]; 3) using suspension culture techniques, a number of cell lines have been grown in media containing Dispase [30]; and 4) neither our light nor electron microscopic studies revealed morphologic evidence of epithelial cell disaggregation or damage in the bulb following Dispase treatment (see Figs 2 and 3). Thus, it is reasonable to conclude that Dispase inhibits follicular epithelial growth by disrupting hair follicle EMI via its digestion of the ECM. While we cannot exclude that, in the mechanical separation experiment (see Table IB), traumatization or loss of the hair matrix cells might have contributed to the reduced ^{3}H-Tdr incorporation as compared to controls, this is unlikely since during separation only the most distal part of the hair shaft was manipulated using forceps, and separation, using this technique, occurs at the level of the lamina lucida [19], not in the hair matrix.

The general role of the ECM in the control of tissue growth is a relatively new focus in developmental biology [4,20,31,32]. The presence of an intact basal lamina is essential for inductive tissue interactions in a wide range of developmental systems, including tooth differentiation [33], limb bud chondrogenesis [34], and the development of the maxillary process [35]. In addition, an intact preformed basal lamina at the dermo-epidermal junction has been shown to be important in the initiation of hair follicle development [36,37]. To date, however, little is known about the ECM's importance in the growth of the mature follicle.

The ECM may act directly as a template to transmit information between epithelial and mesenchymal cells [31]. For example, in the developing submandibular gland, mesenchymal cells influence the differentiation of the overlying epithelium by changing the local composition of the BMZ [5]. The mesenchyme deposits small pads of interstitial collagen and produces a neutral hyaluronidase, which degrades basement membrane hyaluronate. These changes in the BMZ are interpreted by the epithelium and result in branching of the glandular buds [5]. In early anagen hair follicles, increased deposition of two Dispase-sensitive BMZ components, fibronectin and type IV collagen, has been observed [16]. It is possible, therefore, that mesenchymally controlled changes in the amount, distribution, and/or configuration of these molecules in the papilla's ECM and the BMZ are key elements of hair follicle EMI. The fact that neither collagen types I or IV nor the basement membrane components in Matrigel alone, in these experiments, could substitute for native follicular mesenchyme may be due to qualitative and/or quantitative deficiencies in these matrices. While specific ECM molecules (possibly collagen type IV and/or fibronectin) appear to be an essential part of a conduit for signal transmission in follicular EMI, our studies do not rule out an important regulatory role for cells of the dermal papilla and/or connective tissue sheath.

The dermal papilla is unique in that its mesenchymal cells are embedded within a matrix composed of basement membrane components rather than interstitial type I collagen. The papilla's matrix, therefore, may represent an extension of the BMZ, and this papilla/BMZ unit may have an important signal transducing function in the hair follicle. Enzymatic digestion of this basement membrane-like ECM by Dispase, a fibronectinase and type IV collagenase, would thus disrupt the EMI supporting epithelial bulb growth and lead to a decrease in thymidine incorporation into the bulb (see Table I).

Our data do not support the hypothesis that the dermal papilla guides epithelial bulb growth simply through the production of a

Figure 3. Electron micrographs of the basement membrane zone in vibrissae follicles after exposure to Dispase: Follicles were exposed to DME containing Dispase for 6 h at 4°C. A low-power electron micrograph (a) shows representative, well-preserved and adhering keratinocytes of the proximal root sheaths (bar, 3 μm). Notation is as follows: F, nucleus of fibroblast; E, nucleus of keratinocyte of external root sheath (note absence of epithelial basement membrane throughout); I, nucleus of inner root sheath keratinocyte (nascent trichohyalin granules in cytoplasm). At higher magnification (b), details of the BMZ underlying distal matrix cells reveal the absence of a lamina densa and of the lamina lucida as well, because the normally interdigitating keratinocyte processes extend into the BMZ (as notated) and are in direct contact with fibroblasts and collagen fibers (bar, 1 μm).

freely diffusing factor, because the action of such a molecule should not be blocked by the mechanical separation of epithelium from mesenchyme when close proximity is maintained. It is possible, however, that such a factor, originating in the mesenchyme, requires "presentation" or concentration by the ECM for growth-supporting activity, because evidence is accumulating that keratinocyte function in vitro can be mediated by complex interactions between soluble peptide factors (cytokines) and ECM molecules [38].

The organoid assay reported here provides a simple and comparatively physiologic system for further studying EMI which control epithelial cell proliferation in the adult rodent hair follicle.

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