In Vivo Induction of Hair Growth by Dermal Cells Isolated from Hair Follicles After Extended Organ Culture

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Successful hair follicle organ culture has been established for some time, but hair growth *in vitro* is limited and generally terminates prematurely in comparison with *in vivo*. The reasons why growth stops in culture are as yet unknown. In this investigation, adult rat vibrissa follicles for which growth in culture is limited to about 10 d, were maintained *in vitro* for a minimum of 20 d after the hair shaft stopped growing. The pattern of fiber growth and long-term follicle pathology reflected the initial hair cycle stage at the time of isolation. Furthermore, there was evidence that a group of follicles put into culture when in late anagen were attempting to cycle *in vitro*. Microscopy showed that, in spite of widespread pathologic changes to the follicle epithelium, dermal cells in the follicle showed remarkable resilience. Their viability was confirmed when primary cell cultures were established from isolated dermal tissue. These cells labeled positively for α-smooth muscle actin, an established marker of hair follicle dermal cell phenotype *in vitro*. Moreover, isolated dermal tissue induced hair growth when implanted into inactivated hair follicles *in vivo*. These data confirm that the cessation in hair growth is not due to a loss of the inductive capacity in the dermal component. Long-term organ culture may provide opportunities to investigate factors that are expressed or lost during hair growth cessation. In addition it may be possible to develop this method further to obtain a reliable and predictable model of hair follicle cycling *in vitro*. *Key words: dermal papilla/dermal sheath/epithelial–mesenchymal interactions. J Invest Dermatol 117:596–604, 2001*

The culture of intact hair follicles has emerged as an important technical advance for studying the mechanisms and molecular control of hair growth. Philpott *et al* (1990) initially grew isolated human scalp follicles for up to 10 d in serum-free medium. This model has been modified and used with different follicle types to investigate multiple aspects of hair follicle activity, including the effects of a wide range of cytokines, growth factors, nutrients, and hormones (Harmon and Nevins, 1993, 1997; Taylor *et al*, 1993; Ibraheem *et al*, 1994; Philpott and Kealey, 1994; Philpott *et al*, 1994, 1996; Harmon *et al*, 1995; Bond *et al*, 1996; Williams *et al*, 1996).

One limitation of most *in vitro* hair growth models is that follicles only grow fiber for a relatively short period of time in comparison with the normal duration of the growing (anagen) phase of the cycle *in vivo*. It thus follows that they do not go through the complete follicle cycle *in vitro*. Most culture systems allow follicles to be grown for a maximum of 10–15 d (Buhl *et al*, 1989; Philpott *et al*, 1990; Waldon *et al*, 1993). Where the morphologic changes associated with the cessation of fiber production in cultured follicles have been investigated microscopically, different phenomena have been described. In some studies, cultured follicles that were stopping growth were observed to have characteristics of catagen follicles (Li *et al*, 1992; Tobin *et al*, 1993; Westgate *et al*, 1993). Catagen is the normal process of hair growth cessation *in vivo*, in which the lower follicle involutes and the nongrowing "club" fiber moves up the follicle. Apoptosis plays a part in normal catagen events *in vivo* and apoptotic events have been reported in cultured follicles (Soma *et al*, 1998). Other work describes the keratinization of the lower matrix resulting in a club-like structure (Philpott *et al*, 1992; Taylor *et al*, 1993), whereas Harmon and Nevins (1994) attributed the cessation in growth of cultured human hair follicles to the complete keratinization of the lower follicle epithelium. Using the mouse vibrissa follicle model we showed previously that the cycle stage a follicle is at when it is first put into culture determines its subsequent fiber growth and behavior *in vitro*. (Robinson *et al*, 1997). Early anagen mouse vibrissa follicles can sustain fiber growth for up to 22 d, which approaches the rate of growth and total fiber production of the smallest of these follicles *in situ*, whereas late anagen follicles grow shorter fibers and terminate growth much more rapidly.

In all of the above follicle studies, morphologic and pathologic changes have only been examined up to the time fiber production ceases. The question of what happens to cultured follicles after hair has stopped growing, and the long-term pathology of follicle components, has not been investigated. The condition of follicle dermal tissues may be crucial, as in skin organ culture the integrity of the skin appears largely to depend on the survival of dermal cells (Varani, 1998).

A diagram outlining the progression of work taken to investigate these questions is shown in **Fig 1**. We maintained rat vibrissa follicles in culture for more 20 d after the hair shaft stopped growing and found macroscopic and morphologic characteristics that still reflected the stage of the cycle that the follicles were in when initially isolated. Indeed, after more than 33 d *in vitro*, histology indicated that many components of the follicle remained

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viable and that some follicles were attempting to cycle. We then addressed the specific question of whether hair growth stops due to a pathologic or irreversible change to the dermal cells of the follicle? Tests were made of the viability and inductive properties of cell cultures derived from follicles that had been left for extended periods after hair growth had stopped. Dermal cells were microdissected from long-term cultured follicles, grown in cell culture and tested for the expression of α-smooth muscle actin (ASMA), a marker of hair follicle dermis *in vitro*. Microdissected dermal tissue was then implanted into inactive follicles *in vivo* using the bioassay described by Oliver (1967) to test their inductive properties. The stimulation of new hair growth, combined with labeling of the dermal cells using the lipophilic dye DiI, confirmed that the dermal component had retained its inductive capacity.

MATERIALS AND METHODS

Rat vibrissa follicle culture—morphologic studies Follicles were isolated from adult male PVG rats aged 10 wk or more (colony University of Durham LSSU) using the method described by Cohen (1961) and Oliver (1967), with some minor modifications (Robinson *et al*, 1997). The individually isolated follicles were immediately placed into Williams medium E with Glutamax, supplemented with 100 U penicillin per ml, 100 g streptomycin per ml, and 2.5 g fungizone per ml (all supplied by Gibco, Life Technologies, U.K.) preheated to 37°C.

The growth cycle stage of each isolated follicle was initially established using external criteria based on the length and arrangement of the follicle fibers. After transection of the upper third (just below the ring sinus, and removing the ringwulst, sebaceous gland, and isthmus) further information as to the precise cycle stage could be gleaned and follicles were reclassified from the internal size and arrangement of fibers, as previously described (Robinson *et al*, 1997). The transected follicles were submerged individually in 1.5 ml of the above media supplemented with sodium selenite at 10 ng per ml (Gibco) and 11.1 mM D-glucose (Sigma, Aldrich, U.K.), in 24-well microplates (Falcon, Becton Dickinson, U.K.) as previously described (Robinson *et al*, 1997). All specimens were incubated at 37°C in an atmosphere of 5% CO_2/95% air. The medium was not changed over the entire culture period. Fiber production was measured at regular intervals and was defined as the increase in fiber length from the top of the cut follicle, measured to the nearest 0.05 mm using a Nikon TMS inverted microscope fitted with an eyepiece measuring graticule.

Histologic analysis using resin embedding Forty-eight follicles were cultured for 33 d and fixed according to the process described by Karnovsky (1965), postfixed in 1% buffered osmium tetroxide, dehydrated and embedded in araldite resin CY212 (Agar Scientific, Stansted, U.K.). Longitudinal sections of 1 μm in thickness were taken using a Reichert Ultracut microtome (Leien, Wien, Austria), stained with 1% toluidine blue, and examined by light microscopy (Zeiss Axiovert 135, Carl Zeiss, Germany).

Isolation of residual dermal tissue After culture, the follicles were classified into those in which the fiber had moved up to the top of the follicle and those in which it had not. By observing each follicle under a dissecting microscope (Zeiss SV-11) with strong illumination, the position of its fiber could be seen through the collagen capsule. End bulbs were transected under the dissecting microscope and then inverted (Cohen, 1961) to expose any residual tissues. Any remaining fiber was removed (plucked) along with its associated epidermal sheaths. The residual tissue, comprising dermal sheath and dermal papilla cells was teased free from the collagen capsule using sharpened watchmakers forceps, and placed in Williams medium E prewarmed to 37°C. Under a dissecting microscope, the dermal specimens were carefully examined and cleared of any adherent epidermal tissue. Discrete dermal tissue was successfully obtained from about half of the dissected cultured follicles.

Culture and immunohistochemical labeling of isolated cells from postgrowing organ cultured follicles The dermal components of 48 PVG rat vibrissa follicles that had been cultured for 29 d were isolated as described above, and used to establish primary explant cultures (Jahoda and Oliver, 1981). After three passages, cells were seeded on top of glass coverslips in 35 mm dishes. The cells were fixed and permeabilized in acetone for 5 min at –20°C before labeling with a monoclonal antibody to ASMA (gift of Prof. G. Gabbiani, University of Geneva) used at 1:10 as previously described (Jahoda *et al*, 1991). Specimens were mounted in Citifluor (Agar Scientific, Stansted, U.K.) medium and examined on a Zeiss axiovert 135 microscope equipped with epiflourescence.

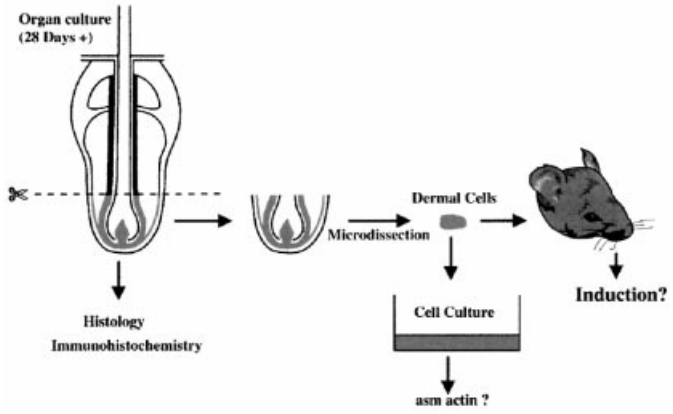

Figure 1. A summary of the series of investigations performed on organ cultured follicles.

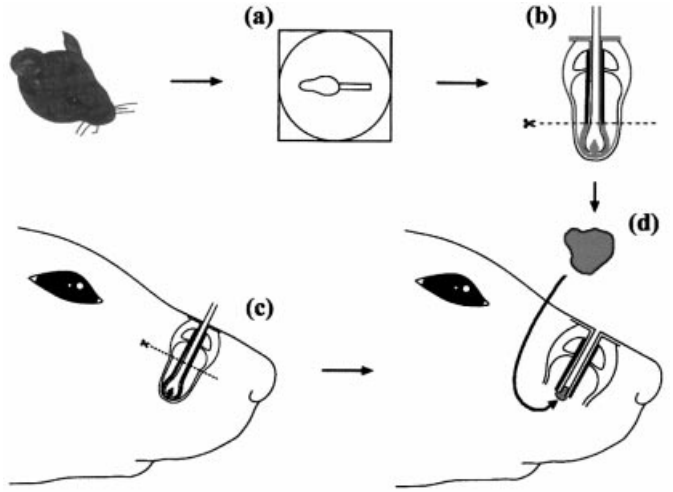

Figure 2. A summary diagram of the essential isolation and transplantation procedures. Rat vibrissa follicles were isolated and cultured for periods up to 35 d (*a*). Their end bulbs were transected and the residual dermal tissue was isolated (*b*). In adult rats major follicles were exposed and transected halfway along their length before plucking (*c*). The isolated dermal tissue from the cultured follicles was then implanted into the open shaft of the transected follicles (*d*).

Implantation of tissue into transected vibrissa follicles An outline of the essential isolation and transplantation procedure is shown in **Fig 2**. From long-term cultured follicles (**Fig 2*a***), tissue was isolated as described above (**Fig 2*b***) and maintained in Williams medium E at 37°C. Prior to implantation, a number of specimens were incubated in Williams medium E containing 5 μg per ml DiI (Molecular Probes, Leiden, The Netherlands) dissolved in dimethyl sulfoxide, for 1.5 h at 37°C. The labeling medium was then removed and the tissue washed three times in fresh Williams medium E at 37°C. Approximately four to eight pieces of isolated tissue, each piece from an individual follicle dissection, were assembled together in small clumps for implantation.

Implantation was essentially as previously described (Oliver, 1966a). Adult male PVG rats were anesthetized and between two and six of the major vibrissa follicles were exposed. Each follicle was transected at least half way along its length, but at a level below the ring sinus (**Fig 2*c***). After plucking of fibers, a clump of material, prepared as described above, was implanted in juxtaposition with the outer root sheath epithelium (**Fig 2*d***). Some of the transected follicles were left as controls. The exposed skin flap was then sutured and the experimental follicle positions were monitored every day. At the end of the experiment animals were killed, the follicles biopsied, and scrutinized for signs of

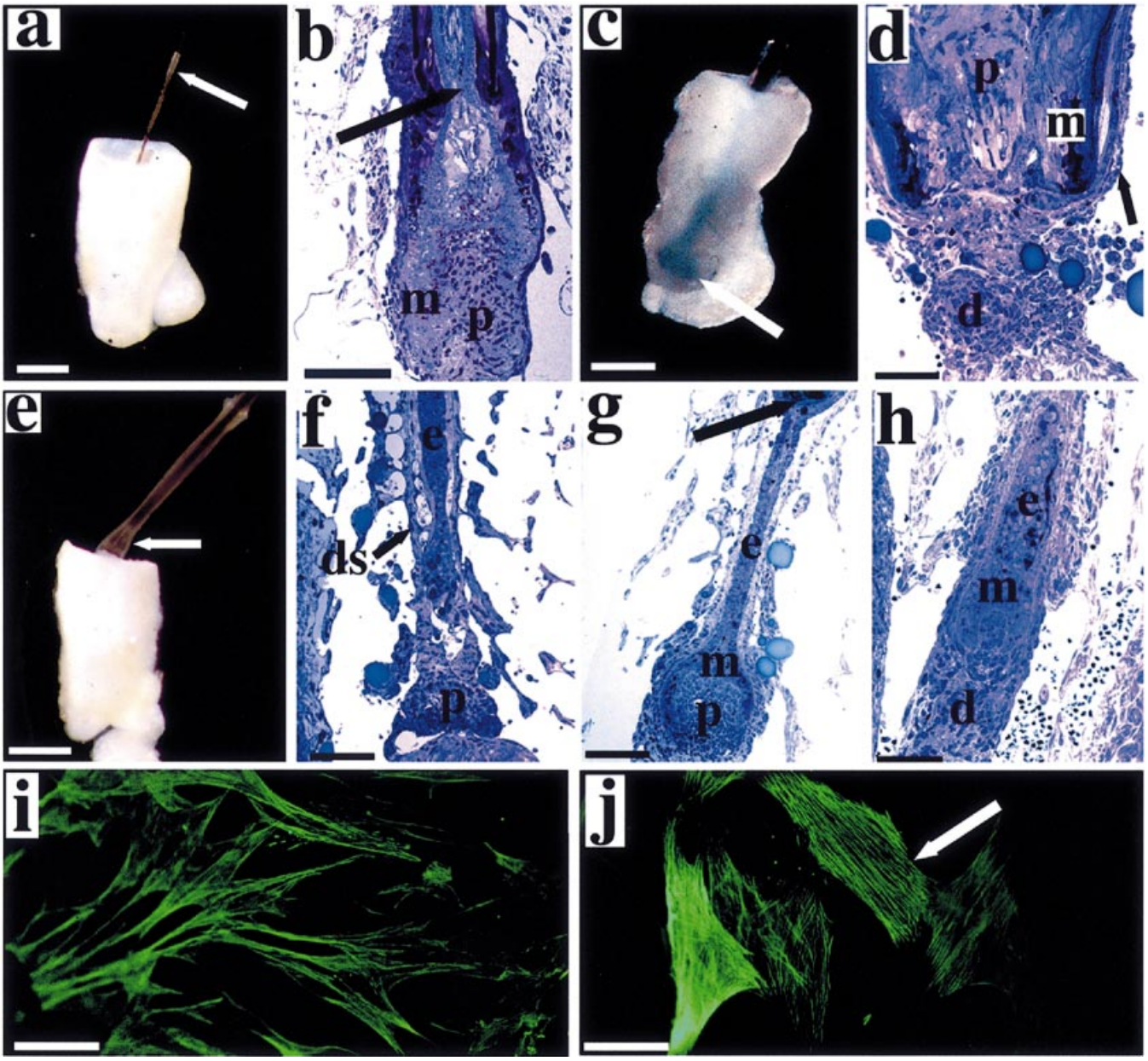

Figure 3. Adult rat vibrissae follicles show hair cycle related growth characteristics and cell viability after long-term culture. Photomicrographs show the external appearance (*a*, *c*, *e*) and toluidine blue stained histology (*b*, *d*, *f*, *g*, *h*) of representative follicles. (*a*, *b*) Early anagen follicles, 33 d. (*a*) Externally there is a fine fiber with very little or no epidermal tissue attached (*arrow*). (*b*) In longitudinal section the essential anagen arrangement has been retained with a dermal papilla (p) enclosed by an epidermal matrix (m), although some abnormal keratinization is occurring (*arrow*). (*c*, *d*) Late anagen follicles, 33d where the fiber has not lifted up. (*c*) The fiber is very thick and the epidermal matrix is clearly visible through the collagen capsule (*arrow*). (*d*) In longitudinal section the epidermal matrix has undergone massive keratinization (m). The dermal papilla (p) contains large amounts of extra cellular matrix, and has a large collection of indeterminate dermal cells (*d*) at its base. The dermal sheath (*arrow*) has remained healthy if slightly thickened. (*e–h*) Late anagen follicles where the fiber has lifted up within the follicle, 33d. (*e*) In this specimen it has been completely expelled (*arrow*). Histologic detail revealed three different morphologies. (*f*) The normal structural arrangement has been lost, but dermal papilla (p) dermal sheath (ds *arrow*), and epidermal cells have been left behind. (*g*) A follicle whose morphology is comparable with a telogen follicle *in vivo*, with an elevated club fiber (*arrow*), separated from the epidermal matrix (m) and papilla (p) by a column of outer root sheath cells (*e*). (*h*) The residual epidermal (e) and dermal cells (d) cells have formed a new pro-anagen-like end bulb structure with a dermal papilla and associated epidermal matrix (m). (*i*) Dermal cells isolated and cultured from rat vibrissa follicles (29d) display strong marking for ASMA. (*j*) Note the broad flattened morphology (*arrow*) of some of these cells. *Scale bars*: (*a*, *c*, *e*) 0.5 mm; (*b*, *f*, *g*, *i*) 100 μm; (*d*, *h*, *j*) 50 μm.

induction and external fiber growth. Specimens were photographed using a Zeiss SMV 11 dissecting microscope fitted with a Contax 167MT camera.

Three separate series of experiments were carried out to investigate the inductive capacity of the dermal component of organ cultured follicles, 20 d or more after they had stopped producing hair.

Series 1 Forty-eight adult PVG rat vibrissa follicles were cultured for 36 d and the residual tissue was microdissected from the bases of follicles at all stages of the cycle. In a single adult PVG rat four amputated vibrissa follicles were implanted with isolated dermal tissue and two amputated follicles were left unimplanted to serve as controls. The follicles were biopsied after 7 wk.

Table I. A summary of follicle organ culture duration, material and procedures used for implantation, and induction success

Animals	No. of cultured follicles and stages	Culture period (d)	Cultured follicles used for tissue isolation	No. of follicles from which dermal tissue obtained	Labelling of tissue	No. of implants/ control follicles	Biopsy time (wk)	% end bulb formation
SERIES 1								
Rat 1	48 EA 8, MA 17, LA 23	36	all follicles	28	–	4 Imp 2 Con	7	100 0
SERIES 2	96 EA 37, MA 28, LA 31			40				
Rat 2		15	all follicles		–	3 Imp	8	100
Rat 3		15	fibres not lifted		–	2 Imp 2 Con	8	100 0
SERIES 3	120 EA 30, MA 39, LA 51			>48				
Rat 4		35	lower 1/3 follicles - fibres not lifted	FM DI-I	3 Imp	3 –	100	–
Rat 5		35	lower 1/3 follicles - fibres not lifted	–	3 Imp	6 2 Con	100	0
Rat 6		35	lower 1/3 follicles - fibres not lifted	FM DI-I	2 Imp	6 -	100	–

Series 2 Ninety-six adult rat vibrissa follicles were cultured for 15 d. The follicles were then separated into those where the base of their fibers had remained attached to the bottom of the follicle, and those that had moved up. In a single PVG rat three amputated follicles were implanted with dermal tissue isolated from both sets of follicles. In a second animal, two amputated follicles were implanted with tissue isolated from selected follicles where the base of the fiber had not moved (keratinized) and two follicles were left unimplanted as controls. Follicles were biopsied from both animals after 8 wk.

Series 3 In this experiment only the lower third of each follicle was cultured. Otherwise culture methods and all subsequent procedures were as described earlier. Follicles were cultured for 35 d and divided into those where the fiber base had lifted up, and those where it had not. Residual dermal tissue was only dissected from the latter group of follicles in which the base of the fiber and epidermal matrix had not moved, as established microscopically. In three rats, eight amputated follicles received dermal tissue implants, five of which had been prelabeled with DiI. Two amputated follicles were left unimplanted as controls. One rat was killed after 3 wk, and two rats were killed after 6 wk.

Analysis of DiI labeled cell implantations All follicles implanted with DiI-labeled tissue were embedded in OCT compound (Agar Scientific, Stansted, U.K.), snap frozen in liquid nitrogen and stored at –80°C. Seven micrometer sections were cut on a Bright OTM cryostat at –22°C and collected on poly-lysine coated microscope slides. The specimens were examined and photographed on a Zeiss axiovert 135 microscope equipped with epiflourescence.

Wax histology The remaining implanted follicles were fixed in 4% paraformaldehyde and processed for routine wax histology. Sections (7 μm) were cut and stained with alcian blue, Wiegert's hematoxylin and Curtis's ponceau S (all supplied by Sigma, Aldrich, U.K.). The sections were analyzed and photographed using a Zeiss axiovert 135 microscope.

RESULTS

Morphology of cultured follicles reflects their cycle stage at isolation

Fiber growth Cultured rat vibrissa follicles grew much less well than their mouse equivalents (Robinson *et al*, 1997) and with high levels of individual variation. When we compared the *in vitro* growth rates, total fiber production, and duration of growth of 240 rat vibrissa follicles, grouped according to stages of the cycle (80 early anagen, 68 mid-anagen and 92 late anagen follicles), no statistically significant differences were found and large error margins were apparent (data not shown). Interval measurements of individual follicles revealed that length increases were greatest within the first 2–3 d and generally halted at about day 6 or 7. (data not shown). Nevertheless, a few individual EA follicles did continue to grow—the longest for 13 d with a 3.4 mm long fiber.

Follicle morphology Combined macroscopic and histologic scrutiny of individual follicles revealed that follicle morphology did largely reflect the follicle cycle stage at their time of isolation. In particular, the pronounced differences between early and late anagen follicles meant that they could be grouped into the following three classes at the end of the culture period (**Fig 3**).

Early anagen follicles in which the fibers had not lifted Early anagen follicles produced fine fibers with little or no associated epidermal sheath (**Fig 3*a***). Only 9% of these follicles had fibers that had lifted—i.e., the base of the fiber had moved up the follicle or had even been lost. Even after 33 d *in vitro*, their basic cellular configuration, resembled that of normal anagen follicles (**Fig 3*b***). In most cases, the epidermal matrix and inner root sheath layers were still visible, although abnormal keratinization was apparent in the epithelial layers of the upper bulb. Dermal papilla and dermal sheath structures appeared to be predominantly intact and healthy.

Late anagen follicles in which fiber bases had not moved up the follicle This group of follicles grew short fibers (**Fig 3*c***) with accompanying epidermal sheaths. Histology of all of these specimens showed extensive keratinization of the epithelial cells of the lower follicle bulb matrix (**Fig 3*d***). In nearly every case, a dermal papilla was still present; however, these often had abnormal-looking extracellular matrix and an irregular distribution of cells. A dermal sheath was universally maintained. Sometimes it was abnormally thick where it joined the papilla basal stalk and additional aggregations of indeterminate dermal cells were frequently visible at the base of the follicle at some distance from the papilla (**Fig 3*d***). Mid-anagen follicles, the base of which had not moved, showed the same histologic characteristics.

Late anagen follicles in which the fiber has lifted up away from the base In 62% of late anagen follicles the fiber had lifted from the base (**Fig 3*e***), and often it had been expelled. Within this class of follicles, there were at least three distinct histologic morphologies. In one, irregular clumps or aggregations of dermal sheath and dermal papilla cells were left with residual epithelial cells in the follicle base (**Fig 3*f***). In other follicles the nongrowing fiber had lifted and was separated by a narrow column of epithelial cells, from a bulb region that resembled that of a "normal" vibrissa telogen specimen (**Fig 3*g***). In the third variation, representing about 50% of the "lifted" specimens (28 follicles in total), papilla cells that had been left behind were associated with an epithelial

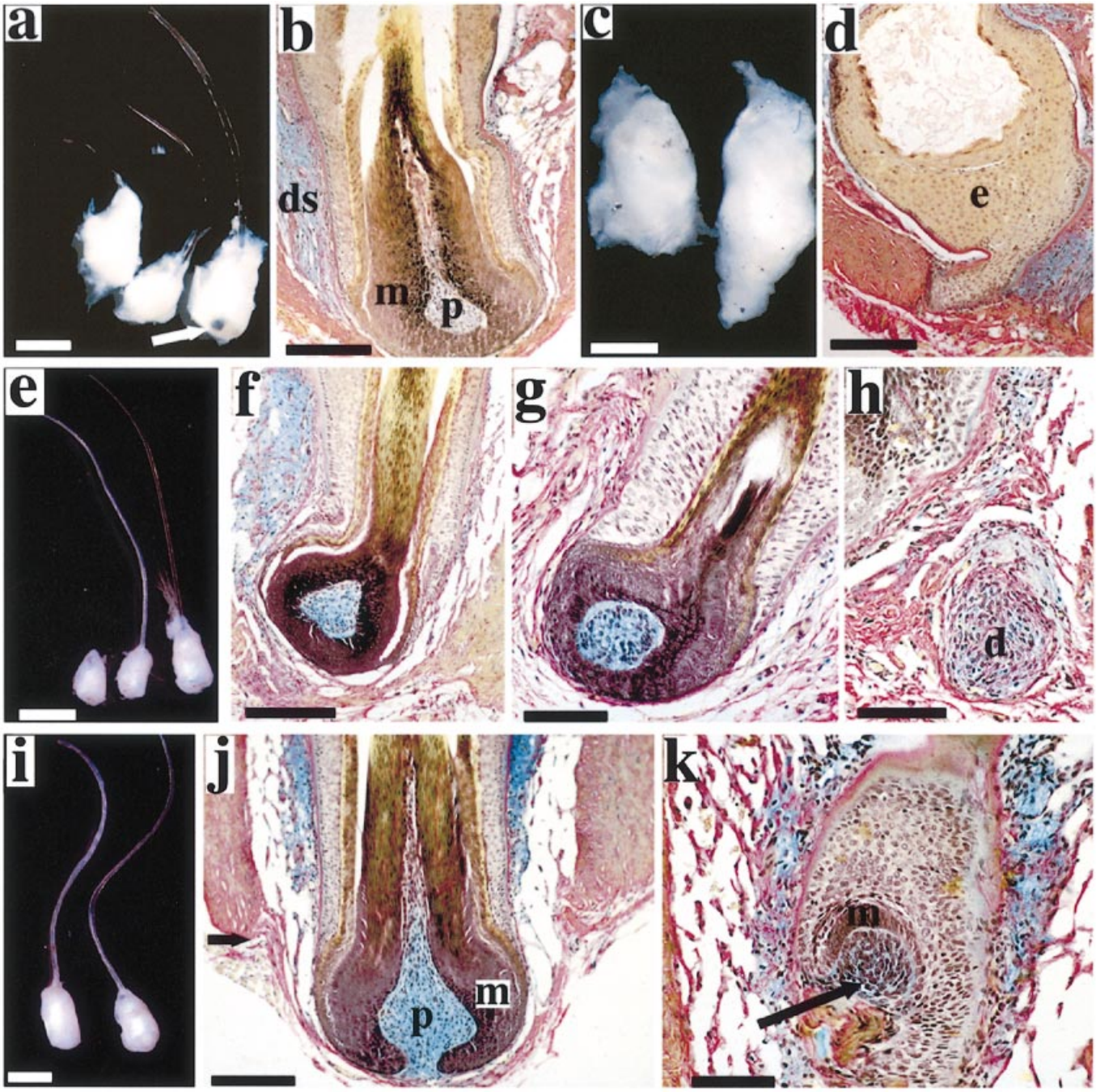

Figure 4. Experimental induction of hair growth by dermal tissue isolated from long-term cultured rat vibrissa follicles. (*a*) Vibrissa-type hairs induced *in vivo* from 36 d culture specimens. Two of the fibers are relatively short, fine, and abnormally curved, whereas the third is much longer and thicker. In this follicle a new epidermal matrix is visible below the level of transection (*arrow*). (*b*) Histologic detail of the same follicle shows a large new anagen-like end bulb, with an elongated papilla (p) surrounded by epidermal matrix (m) and a thickened dermal sheath (ds) stained blue. (*c*) No hair growth was evident in the two control follicles. (*d*) Histologic detail confirmed that in these follicles the space left by fiber removal has been filled by epidermal cells (e) with no hair structures present. (*e*) Vibrissa-type hair fibers induced *in vivo* by dermal cells from 15 d cultured follicles. Two follicles have large external vibrissa-type fibers. (*f*, *g*) Histologic detail of these reveals normal anagen follicle bulb structures. (*h*) Histology of the follicle in (*e*) with no external hair revealed that a new active bulb had formed with a large discrete ball of dermal cells (*d*) located in the connective tissue below it. (*i*) Induction of hair growth by dermal tissue isolated from 15 d cultured follicles whose epidermal matrix had completely keratinized. (*j*) Histologic detail reveals a very large anagen bulb structure extending well below the cut edge of the collagen capsule (*arrow*). m, epidermal matrix; p, papilla. (*k*) High up within the same follicle, dermal cells have interacted with the upper outer root sheath to form a second papilla (p) with associated epidermal matrix (m). (*b*, *d*, *f*, *g*, *h*, *j*, *k*) Alcian blue, Weigerts hematoxylin, and Curtis Ponceau S. *Scale bars*: (*a*, *e*, *i*) 1 mm; (*c*) 0.5 mm; (*b*, *d*, *j*) 200 μm; (*f*, *g*) 100 μm; (*h*, *k*) 50 μm.

matrix, which appeared intact, and whose inner root sheath differentiation in particular was characteristic of early anagen follicles *in vivo* (**Fig 3*h***). In some cases these bulbar structures were observed mid-way up the follicle.

Culture and analysis of dermal cells microdissected from organ cultured follicles Tissue explants isolated from the base of long-term cultured follicles displayed cell outgrowth and proliferation typical of dermal cells from healthy follicles, and the

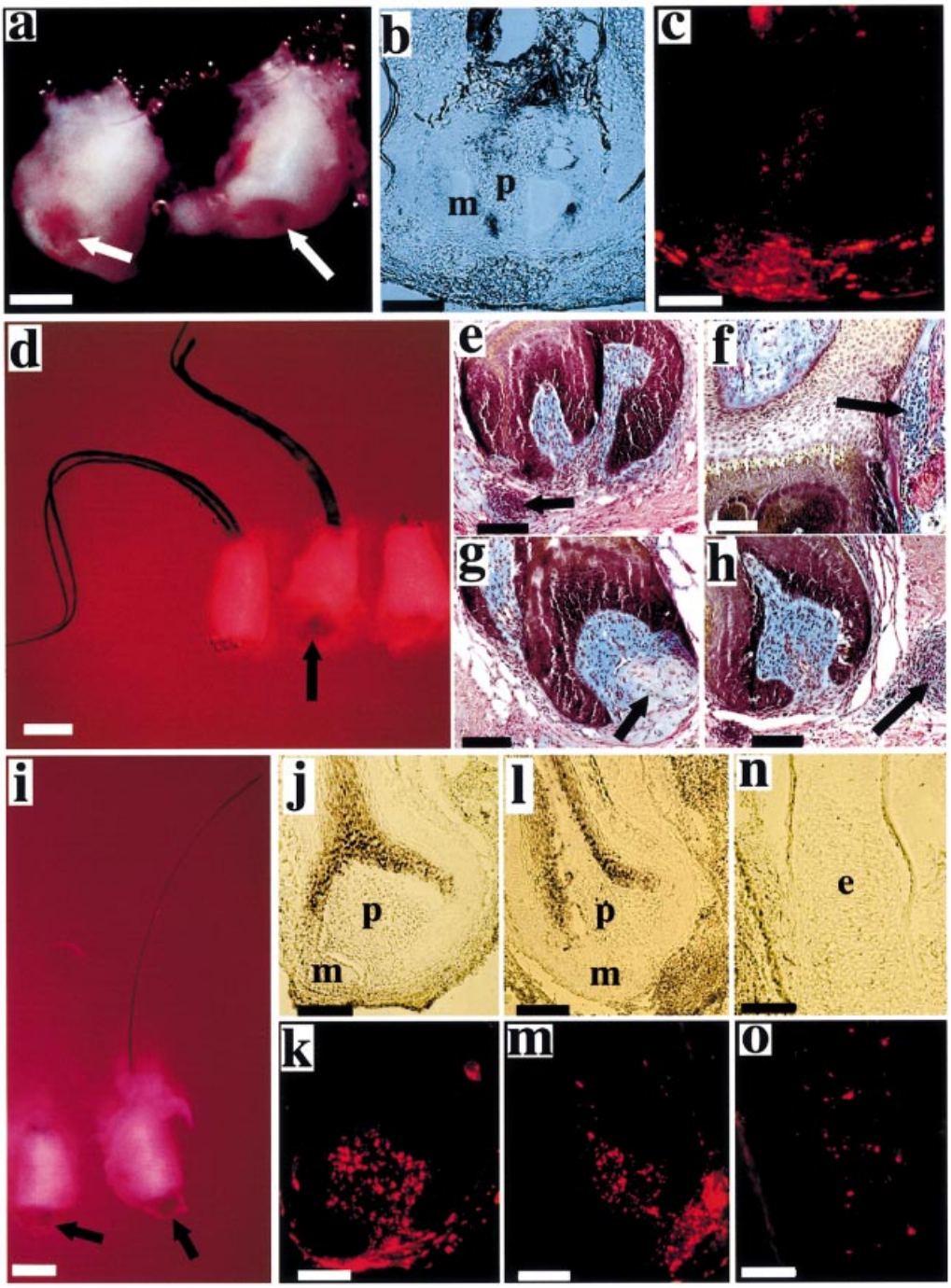

Figure 5. Experimental induction of hair growth by dermal tissue isolated from lower third 35 d rat vibrissa follicle cultures whose fibers had not lifted. Tissue was labeled with DiI prior to implantation (*a–c*, *i–o*). Dermal papilla, p; basal stalk, bs; epidermal matrix, m. (*a*) 3 wk biopsy of two implanted upper follicles. No external fibers are visible, however, a small follicle bulb (*arrows*) has formed in each. (*b*) Frozen longitudinal section of one of these follicles showing a thick basal stalk extending into an abnormal double-lobed papilla, which is associated with an epidermal matrix (phase contrast). (*c*) DiI analysis of the same section shows labeling is restricted to the dermal component. The strong fluorescence (*top*) is autofluorescent fibrous material. (*d*) Vibrissa-type hair induction after 6 wk *in situ*. Two follicles have large double hairs growing, the third a short protruding fiber. In one a very large bulbous end bulb (*arrow*) is visible below the level of the cut collagen capsule. Histology reflects these observations. (*e*) A very large bulb matrix with a multilobed papilla and additional discrete balls of dermal cells below the bulbous matrix (*arrow*). (*f*) Above the matrix, the dermal sheath has become papilla-like (*arrow*). (*g*) End bulb with two separate papilla and matrix structures of different sizes. Note the unusual papilla morphology and abnormal extracellular matrix (*arrow*). (*h*) A normal anagen bulb with additional discrete balls of dermal cells (*arrow*). (*i*) Vibrissa-type hair induction after 6 wk *in vivo*. Only one follicle has a large external fiber; however, a new end bulb structure can be seen in the base of both (*arrows*). (*j*, *l*) Frozen longitudinal sectioning showing a normal anagen morphology in both follicles (phase contrast). (*k*, *m*) DiI analysis of the same sections show the dermal papilla and sheath components packed with labeled cells. (*n*, *o*) Diffuse DiI labeling located in the upper epithelial region (e) of one follicle. (*e*, *f*, *g*, *h*) Alcian blue, Weigerts haematoxylin, and Curtis Ponceau S. *Scale bars*: (*a*, *d*, *i*) 0.5 mm; (*b*, *c*, *e*, *g*, *h*, *j–o*) 100 μm; (*f*) 50 μm.

morphology of these cells when passaged continued to suggest dermal origin. They showed the typically flat and broad multipolar appearance of cells cultured from hair follicle dermal papillae or dermal sheath, and 95% of cells stained positively for ASMA (**Fig 3*i*, *j***) a cytoskeletal marker characteristic of these follicle-derived dermal cells (Jahoda *et al*, 1991). Control sections, incubated in phosphate-buffered saline instead of primary antibody were completely negative (data not shown).

Hair follicle induction by follicle dermal tissue A summary of results is shown in **Table I**.

Series 1 Of the four follicles implanted with cultured follicle tissues, three produced external fibers (**Fig 4*a***). These hairs were of vibrissa-type, but unusual. Two were relatively fine and curved, and the third much thicker. Histology of all four specimens revealed normal anagen end bulbs, each with a large new papilla surrounded by a substantial epidermal matrix, and all level with or below the line of the cut collagen capsule (**Fig 4*b***). The two control follicles did not produce external fibers (**Fig 4*c***) and their histology revealed no evidence of bulb organization (**Fig 4*d***).

Series 2 At biopsy, two of the three follicles implanted with tissue derived from cultured follicles from different cycle stages displayed unusually curved, stout external hairs (**Fig 4*e***). Histology revealed an induced end bulb with a large dermal papilla and organized epidermal matrix in all three specimens (**Fig 4*f*, *g***) and in the third follicle, a large detached ball of dermal cells was present in the vicinity of the active bulb (**Fig 4*h***). Both follicles that had received tissue implants from keratinized cultured follicles, had produced large wavy fibers (**Fig 4*i***), and histology revealed that both had formed large anagen end bulbs below the level of transection (**Fig 4*j***). Away from the main end-bulb papilla additional papilla-like clusters of dermal cells were located in both specimens and in one, a papilla-like structure had associated with, and organized, the local outer root sheath to form a new matrix-like structure midway up the follicle (**Fig 4*k***). No fibers or unusual follicular structures could be seen in either of the control follicles.

Series 3 In the 3 wk postoperative specimens no external fibers were visible from implant sites, but small pigmented end bulbs were identifiable macroscopically at the base of two implanted follicles at biopsy (**Fig 5*a***). Sectioning revealed new end-bulbs in all three cases, with the DiI marking restricted to the new lower dermal sheath and dermal papilla compartments, and no staining of epithelial cells. (**Fig 5*b*, *c***). At 6 wk, hair grew from three implanted follicles—two had double fibers and the other a single very short thick hair (**Fig 5*d***). Histology revealed different morphology in each case. One follicle, with two fibers of equal length and thickness had a central, multilobed papilla surrounded by a large epidermal matrix (**Fig 5*e***). Just above the epidermal matrix the dermal sheath appeared thickened with papilla-like cellular morphology and extracellular consistency (**Fig 5*f***). The second follicle that had produced double hairs, had two separate papillae and epidermal matrices. Unusually, the largest papilla had two morphologically distinct compartments (**Fig 5*g***), the upper typical, the lower containing a higher proportion of collagenous extracellular matrix. The finer and shorter second fiber came from a smaller and separate end bulb above the main one (**Fig 5*g***). In the third follicle, the single stout fiber correlated with a single large papilla and epidermal matrix (**Fig 5*h***). In all three follicles, additional discrete balls of dermal cells were present in the follicle base (**Fig 5*e*, *h***). In the two control follicles, no external fibers, end bulbs or tissue interactions were seen. In the last animal, killed after 6 wk, only one follicle had grown an external fiber, but both had new end bulbs (**Fig 5*i***). DiI labeling was restricted to the dermal components (dermal papilla and lower dermal sheath) in the lower region of both follicles (**Fig 5*j–m***). Labeling was not visible in the germinative epithelium or matrix cells of the bulb, but interestingly, some diffuse cellular marking was observed higher up in the outer root sheath of one follicle (**Fig 5*n*, *o***).

DISCUSSION

Organ culture has shown that different hair follicle types can be grown for variable time periods, although the literature is inconsistent as to the causes of growth arrest (Li *et al*, 1992; Philpott *et al*, 1992; Taylor *et al*, 1993; Tobin *et al*, 1993; Westgate *et al*, 1993; Harmon and Nevins, 1994). Here we have demonstrated that the lower follicle dermal cells, which have well established regenerative and inductive properties (Oliver, 1980; Weinberg *et al*, 1993; Prouty *et al*, 1996), survive in follicle organ culture long after hair growth has terminated. Moreover, they retain their power to induce hair growth when transplanted into inactive amputated follicles. During the course of this study, it also became evident that the behavior of rat vibrissa follicles *in vitro* was hair follicle cycle-related.

Choice of experimental material and hair cycling *in vitro* The limited growth in culture of rat vibrissa follicles made them good candidates for studying postgrowth hair follicle pathology. Insulin has been shown to have important effects on the growth and behavior of cultured follicles *in vitro* (Philpott *et al*, 1994), and the fact that we did not include insulin in our growth media reason is one possible reason for the lack of difference in growth rates between follicles from different stages of the hair cycle. In our previous work growing mouse vibrissa follicles at different stages of the hair cycle there was again no insulin in the culture medium yet significant differences were found. Indeed, early anagen follicle grew for up to 22 d, and never entered a catagen-like phase (Robinson *et al*, 1997). One explanation for why rat follicles grew less well than murine ones is that their large size limited the availability of media. The fact that removal of a substantial section of the upper follicle prior to culturing had no effect on growth, however, argues against this. Interestingly, removal of this sebaceous gland-containing region appeared not to regulate sheath/fiber relationships as previously reported (Williams and Stenn, 1994). Instead we observed that the accompaniment of growing hair shafts with root sheath appeared to correlate with cycle stage—so late follicles consistently had sheath and early ones generally did not. The presence of epithelial sheaths sometimes made fibers look abnormally thick, but occasional measurements (data not shown) indicated normal shaft thickness. Other macroscopic and histologic observations confirmed that rat vibrissa follicle behavior *in vitro* was related to their cycle status when isolated. In particular, the range of behaviors demonstrated by the late anagen follicles was biologically and technically important. In follicles where the fibers remained at the base, the epidermal matrix keratinized. In cases where fibers lifted out, dermal cells were left in the base of the follicle alone, or closely associated with viable epithelial cells and the fact that these late anagen follicles retained viable epithelial cells influenced our operational protocol. Whereas our initial implantations used material recovered from all follicles regardless of their behavior while in culture, subsequent ones used cells dissected exclusively from follicles in which the epidermal matrix had keratinized.

Survival and inductive properties of follicle dermal cells Our histologic observations of viable and healthy dermal papilla and dermal sheath cells after prolonged periods *in vitro* were entirely consistent with other data. For example, although cell death is increasingly seen to be an important feature of hair follicle activities (Seiberg *et al*, 1995; Cotsarelis, 1997; Lindner *et al*, 1997; Matsuo *et al*, 1998; Soma *et al*, 1998; Tobin *et al*, 1998), the dermal papilla is unique in expressing the cell death protective molecule Bcl2 throughout the normal adult cycle (Stenn *et al*, 1994). Moreover, the apparent shift of cells between the dermal papilla and dermal sheath compartments is also in accord with previous observations. These two cell populations derive from the same embryologic precursors (Wessells and Roessner, 1965), and if the dermal papilla, or up to the lower third of the follicle is removed experimentally *in vivo*, the papilla is replaced by adjacent dermal sheath cells (Oliver, 1966b; Jahoda and Oliver, 1992). Similarly, the

finding of dermal cell aggregations at the bases of cultured follicles has also been observed in experimentally wounded whisker follicles (Jahoda and Oliver, 1984a).

As, after extended follicle culture the boundaries between dermal papilla and sheath compartments became poorly defined, they had to be microdissected together. The successful culture of cells from this dermal tissue showed their viability long after the follicles had stopped functioning normally, and their expression of high levels of ASMA, demonstrated that a phenotypic marker of hair follicle dermis had been retained (Jahoda *et al*, 1991). The next question was whether they had maintained the capacity to induce hair growth.

The series of three implantation experiments demonstrated that this was indeed the case. Dermal cells from organ-cultured follicles induced hair growth in all instances. Vibrissa-type fibers were produced, some follicles grew double hairs, and lower follicle morphology and end-bulb positioning was typical of that seen previously after experimental induction (Oliver, 1967; Jahoda *et al*, 1984; Horne and Jahoda, 1992). The fact that both papilla and sheath tissues were implanted was reflected uniquely by some of the newly formed papillae containing extracellular elements consistent with both these elements. The phenomenon whereby excess implanted dermal cells were retained in the vicinity as discrete aggregations was typical, however (Jahoda and Oliver, 1984b), and probably reflects the importance of dermal cell aggregation (Jahoda, 1998). Unequivocal confirmation that the new dermal papilla and sheath structures were derived from the implanted dermal tissue came from specimens prelabeled with DiI. The fact that the labeling of the new bulb region was specific to the dermal compartments was strong evidence that the implanted tissue had interacted with local follicle epithelium rather than epithelial cells carried over with the dermal tissue. In general, the survival and retained inductive capabilities of the dermal papilla/sheath tissue, in a pathologic environment agrees with previous findings. For example in X-irradiated mouse vibrissa follicles the epithelium is destroyed, but the dermal papilla survives and stimulates hair growth when transplanted into other follicles (Ibrahim and Wright, 1977).

Cultured follicle pathology as a model, and follicle cycling The extended survival and retained functional integrity of the papilla and sheath cells is consistent with prolonged skin organ cultures, where survival of the skin as a whole appears to be dependent on maintenance of the dermal cellular components (Varani, 1998). Although cultured adult skin does retain viable and transplantable epidermal cells for up to 6 wk (Summerlin *et al*, 1970), important alterations to normal cellular function are seen within a few days (Siddiqui *et al*, 1974).

In this study, we have confirmed that the cessation of hair growth in organ cultures cannot be attributed to an irreversible loss of the hair growth signaling capacity of these specialized dermal cells. We cannot, however, rule out the idea that some diminution of dermal function is taking place. For example, the papilla may stop producing mitogenic factors while maintaining an inductive capacity. Follicle organ culture has been used to examine the effects of growth stimulatory and modulatory agents, but studying the molecular pathology of follicles after extended culture periods could be another application for this model. Active hair growth requires the maintenance of close range dermal–epidermal signaling, the molecular basis of which is beginning to be unraveled (Hebert *et al*, 1994; Yu *et al*, 1995; Zhou *et al*, 1995; Rosenquist and Martin, 1996; Schallreuter *et al*, 1998; Suzuki *et al*, 1998; Muller-Rover *et al*, 1999; Stenn and Paus, 1999; Kishimoto *et al* 2000). By comparing follicles that are stopping, or have stopped hair growth in both pathologic and nonpathologic circumstances, it should be possible to detect correlations between the loss of expression of specific molecules from the principal interactive components, and growth arrest. Although culture-related expression artefacts must be taken into account, this could provide a rapid means of assaying for molecules that are essential for hair growth maintenance.

The observation that some late anagen follicles that had lost their growing fibers had retained, or produced normal looking hair bulbs, suggests that dermal and epithelial components of these follicles were continuing to interact. In this context Philpott (1999), has found evidence of cycling with vibrissa follicles from young rats on gelfoam cultures. Similarly, sheep follicles can show some elements of bulb regeneration after lower follicle amputation (Hynd *et al*, 1999). This behavior raises the question of whether cultured follicles can be made to renew normal fiber production? The vibrissa follicle is unique in that it undergoes minimal regression and extension between anagen phases (Young and Oliver, 1976). Consequently, it may be possible to find suitable media and substrate conditions such that these follicles can be encouraged to go through one or more complete cycles.

Given the evidence that dermal cells were maintaining inductive properties, perhaps the findings of arrested follicle growth indicate some general tissue-culture-dependent effect on follicle epithelial cells, that constrain their capacity to respond to papillary signals. The results, however, provide no conclusive insight into questions of origin and activity of epithelial stem cells in the hair follicle. On the one hand, the complete keratinization of follicle bulb epithelium lends weight to the argument that bulb germinative cells have limited/finite mitotic potential and that this is a controlling/limiting factor in the follicle cycle. On the other hand, growth arrest in these follicles was clearly pathologic, whereas other follicles that maintained living bulb epithelium revealed that epithelial–mesenchymal interactions could continue for long periods. Under these circumstances the fate of the germinative population is uncertain. Once again, molecular comparisons made between those follicles that ''cycle'' and those that undergo pathologic changes could be rewarding. Studies along these lines are currently underway in our laboratory.

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