

# Cultured Peribulbar Dermal Sheath Cells Can Induce Hair Follicle Development and Contribute to the Dermal Sheath and Dermal Papilla

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**Green fluorescent protein (GFP)-expressing wild-type, and nontransgenic mouse vibrissa follicle cells were cultured and implanted to mouse ears and footpads. Dermal papiller (DP)-derived cells and cells from the peribulbar dermal sheath “cup” (DSC) induced new hair follicles in both implanted ears and footpads, while nonbulbar dermal sheath cells did not. Confocal microscopy revealed that GFP-expressing DP and DSC cells induced hair growth associated with the formation of DP exclusively comprised of fluorescent cells. In mouse ears, but not footpads, fluorescent DP and DSC cells could also be identified in DP along with nonfluorescent cells. DSC cells were characterized *in vivo* and *in vitro* by low alkaline phosphatase activity in contrast**

**to high alkaline phosphatase in DP cells. The results indicate transplanted DP and DSC cells were equally capable of DP formation and hair follicle induction. This suggests the DP and peribulbar DSC may be functionally similar. In addition to observing papillae exclusively composed of GFP-expressing cells, DP and DSC cells may also have combined with resident cells to form papillae composed of implanted GFP-expressing cells and host-derived non-GFP-expressing cells. Alkaline phosphatase expression may be utilized as a simple marker to identify hair follicle mesenchyme derived cells with hair follicle inductive abilities. *Key words: cell transplantation/mouse model. J Invest Dermatol 121:1267–1275, 2003***

**T**he hair follicle unit is composed of dermis and epithelium-derived cell populations in close association. The germinative epithelial cells of the hair bulb proliferate and differentiate to give rise to the mature hair fiber, as well as the root sheaths that surround the hair shaft, within the skin. The dermal mesenchyme-derived component comprises fibroblast-like cells that form the morphologic units known as the dermal papilla (DP), located at the base of the hair follicle unit, and the dermal sheath (DS) that exists around the outer limits of the epithelial hair follicle component (Montagna and Van Scott, 1958).

The DP is regarded as essential to hair follicle development and cycling. Biochemical signaling by DP cells controls the cell dynamics of the epidermal component and the overall physical properties of the hair follicle (Chase, 1955; Van Scott and Ekel, 1958; Van Scott *et al*, 1963; Sengel, 1983; Chuong *et al*, 1996; McElwee and Hoffmann, 2000). The functional importance of these dermal-epidermal interactions has been demonstrated in a series of studies. When the bulb regions including the DP, or the DP alone, of rat vibrissae or human hair follicles were removed by microdissection the amputated follicles were shown to regenerate new DP and renew production of hair fiber (Oliver, 1966b; Jahoda

*et al*, 1992, 1996a; Kim and Choi, 1995). However, when more than one-third of the lower vibrissa follicle was removed, the amputated vibrissae failed to reform a new DP and failed to produce hair fiber (Oliver, 1966a, b). Thus, close association between dermal and epidermal cells of the lower follicle within the hair bulb is fundamental to the production of hair fiber whereas the absence of DP within hair follicles leads to permanent cessation of hair growth.

Generation of hair follicles and related structures from implantation of microdissected components taken from mature follicles has been recognized for some time (Lillie and Wang, 1944; Cohen, 1961, 1969; Oliver, 1967a; Kollar, 1970; Chuong *et al*, 1996). DP cells from adult rat vibrissae have been implanted into vibrissae from which the lower half, including the DP, had been removed and promote formation of new hair follicles (Oliver, 1967b). Moreover, DP can be implanted to adult skin and will induce the formation of new hair follicles from undifferentiated epidermis (Oliver, 1970; Jahoda and Oliver, 1984; Reynolds and Jahoda, 1991). The induced hair follicles retain morphologic and hair cycle characteristics of the donor hair follicle (Reynolds and Jahoda, 1992). DP may also be placed in culture to increase cell numbers, which may then be implanted to induce hair follicle development (Jahoda and Oliver, 1981; Jahoda *et al*, 1984; 1993; Messinger, 1984; Horne *et al*, 1986; Reynolds and Jahoda, 1992; Watson *et al*, 1994; Inamatsu *et al*, 1998; Robinson *et al*, 2001). In theory, this simple but effective method of tissue engineering may be employed to treat hair loss due to a variety of diseases, syndromes, and injuries and may provide significant insights into tissue and organ engineering.

Within this framework we wished to examine the dynamics of cultured hair follicle mesenchyme-derived cells when implanted

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Abbreviations: DP, dermal papilla; DS, dermal sheath; DSC, dermal sheath cup; GFP, green fluorescent protein; Scid, severe combined immunodeficiency.

to epithelium. The LacZ gene and  $\beta$ -galactosidase expression has been used in elegant studies to define potential sources of epidermal progenitor cells in hair follicles (Ghazizadeh and Taichman, 2001; Oshima *et al*, 2001), but inconsistent LacZ expression in mesenchymal tissues prohibits analysis of DP and DS cell dynamics. The use of green fluorescent protein (GFP) expression in cells is an important tool for the study of cell survival and the monitoring of specific gene expression (Okabe *et al*, 1997; Ikawa *et al*, 1998, 1999). Here, cells derived from hair follicles in transgenic, GFP-expressing mice, as well as cells from nontransgenic mice, were employed to elucidate DP, peribulbar dermal sheath "cup": (DSC), and nonbulbar DS cell dynamics associated with the induction of hair follicle development. By using this experimental approach, we describe cultured mesenchyme cells derived from the DP or peribulbar DSC capable of inducing hair follicle development *in vivo* via formation of new DP exclusively comprised of implanted cells and also the formation of chimeric DP composed of implanted fluorescent and recruited, nonfluorescent resident host cells.

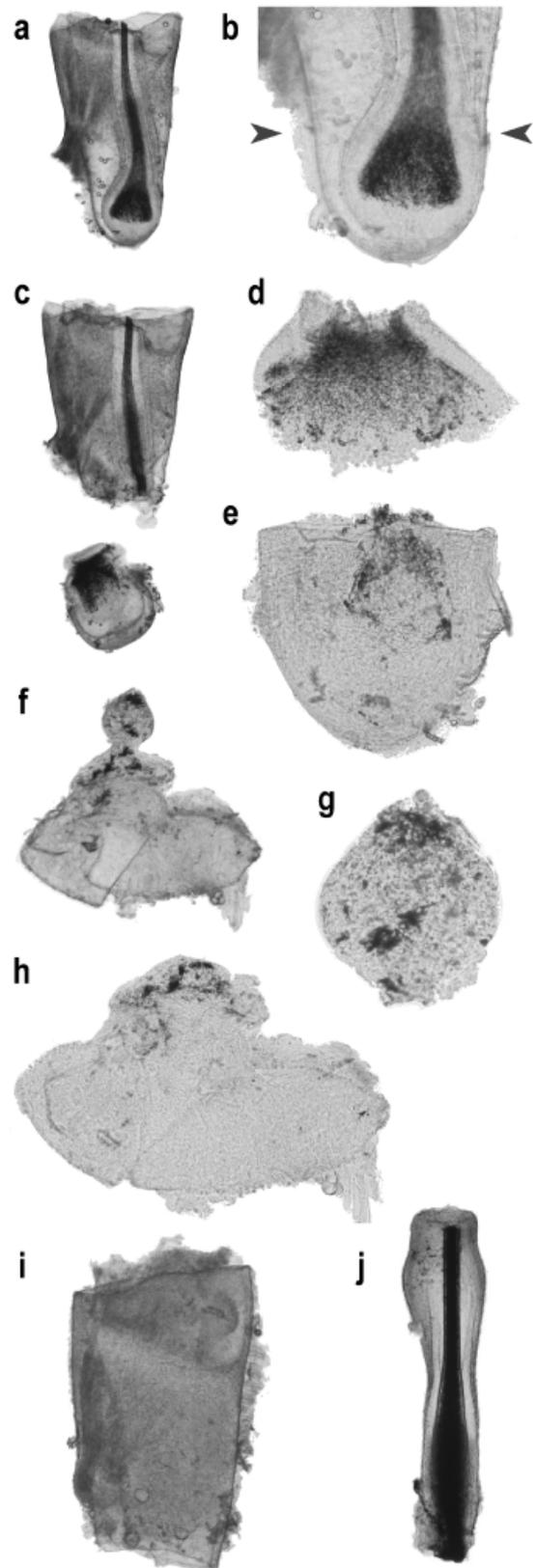
## MATERIALS AND METHODS

**Tissue donors and rodent strains** Commercially available mouse strains obtained from The Jackson Laboratory specific pathogen-free production facility (Bar Harbor, ME) were used. Female CBySmn.CB17-*Prkd<sup>scid</sup>*/J mice and C3H/HeJ mice were employed as both donors and recipients of cells. GFP-expressing cells were derived from female mice of the STOCK TgN(GFPX)4Nagy (hereafter referred to as TgN-GFPX) strain at generation Fx + F4. This transgenic mouse strain carries the enhanced GFP driven by chick  $\beta$ -actin promoter and cytomegalovirus intermediate early enhancer. The construct was electroporated into 129 mouse-strain-derived R1 ES cells and random integration occurred on the X-chromosome (Hadjantonakis *et al*, 1998a, b). All nucleated cells in mice with nonhomologous insertion of the GFP-expressing plasmid are believed to produce the GFP product (Okabe *et al*, 1997; Ikawa *et al*, 1998). Preliminary studies confirmed the *in situ* expression of GFP in DP, DSC, and DS cells as well as epidermal-derived hair follicle keratinocytes with somewhat reduced fluorescence intensity compared to dermis-derived cells (data not shown). Non-GFP-expressing wild-type litter mates were also used as controls for transplanted cells.

All mice received food pellets (altromin 1324, Altromin GmbH, Lage, Germany) and acidified water (pH 2.8–3.0) *ad libitum* for the duration of the study. CBySmn.CB17-*Prkd<sup>scid</sup>*/J (hereafter referred to as Scid) mice were maintained in isolation in a barrier facility. All experiments were conducted in accordance with the German code of practice for the care and use of animals for scientific purposes and with approval from the State of Hessen Animal Research Ethics Committee and in accordance to the Helsinki Principles.

**Amputated hair follicle implantation** Observations made in other studies defined the capacity of rat vibrissae and human hair follicles from which the DP had been removed to reform a new DP and induce hair growth (Oliver, 1966b; Jahoda *et al*, 1992, 1996a; Kim and Choi, 1995). To confirm such observations in mice, the myastacial pads from four wild-type TgN-GFPX mice were isolated as a single unit and the hair follicle bulbs were removed under a dissecting microscope. Each pad was grafted to a Scid mouse using techniques described elsewhere (McElwee *et al*, 1998) and observed for 4 months. At necropsy, tissue across the graft site was fixed in Fekete's acid-alcohol-formalin and processed routinely for histologic examination (Relyea *et al*, 1999).

**Hair follicle subunit derivation and culture** Mice were euthanized under anesthesia by cervical dislocation, and individual vibrissae were dissected from the myastacial pads using a stereoscopic microscope (Eicheler *et al*, 1998). From the reverse side of each myastacial pad, connective tissue was removed and the cleaned hair follicles cut from the epidermis. Whole hair follicles were placed in Williams E medium (Gibco BRL, Gaithersburg, MD) for further dissection.



**Figure 1. Isolation of the dermis derived hair follicle components.**

Whole mouse vibrissa follicles are dissected from donor tissue (a) and cut at a point where the tip of the DP is anticipated (arrows, b) to separate the peribulbar DSC and DP from the nonbulbar DS, root sheaths, and hair matrix (c). Hair matrix and sheath material is removed (d) and the DSC containing the DP (e) is everted (f). Remaining adherent keratinocytes and melanocytes are dislodged, the DP is cut (g) from the DSC (h), and each component is placed in separate cultures. In additional studies, the DSC was also cut from the collagen matrix before culture. The nonbulbar DS, closely adherent to the out root sheath, is separated (i) from the collagen capsule (j) for culture.

**Table I. Implantation of cultured cells to mouse ears**

Cultured cell source	Cell type	Implant recipient	No. of ears or footpads implanted with cells/ No. of ears or footpads with visible hair growth
Scid mice	DP	Scid mouse—ears	4/4
	DSC	Scid mouse—ears	4/4
	DS	Scid mouse—ears	4/0
C3H/HeJ mice	DP	Scid mouse—ears	4/4
	DSC	Scid mouse—ears	4/4
	DS	Scid mouse—ears	4/0
	DP	C3H/HeJ mouse—ears	6/5
	DS	C3H/HeJ mouse—ears	6/0
TgN-GFPX mice	DP	Scid mouse—ears	12/12
	DSC	Scid mouse—ears	12/12
	DS	Scid mouse—ears	12/0
	DP	Scid mouse—footpad	5/5
	DSC	Scid mouse—footpad	5/4
	DS	Scid mouse—footpad	5/0
	DS	Scid mouse—ears	5/0
Wild-type TgN-GFPX mice	DP	Scid mouse—ears	7/7
	DSC	Scid mouse—ears	4/4
	DS	Scid mouse—ears	7/0

For the purpose of this study a subdivision of the hair follicle mesenchyme-derived structures based on morphology and alkaline phosphatase expression in the hair follicle *in vivo* was made such that the DS is defined here as the sheath surrounding the hair follicle that extends from immediately above the bulb region to below the sebaceous gland. The tissue that surrounds the bulb region is defined in this study as the DSC. Mouse DP, DSC, and DS obtained were obtained as shown (Fig 1). With forceps, hair follicles were gripped immediately adjacent to the bulb region and the bulb dissected free with a transverse cut using a 16-gauge needle (Fig 1c). The DSC of the bulb was inverted by the use of forceps and needles and any remaining epithelium-derived tissue removed to expose the DP. The DP (Fig 1g) was separated from the DSC (Fig 1h) with a transverse cut.

In rodent vibrissae the collagen capsule is separated from the nonbulbar DS by the intervention of the blood sinus. The absence of a connective tissue sheath closely associated with DS cells makes the technique for isolation of the nonbulbar DS, as used with human follicles, not possible for rodent vibrissae. Thus, to obtain mouse DS cells, the collagen capsule was removed (Fig 1i) and the remaining fiber, root sheaths, and external DS were placed in culture (Fig 1j). The bulbar DSC closely associates with, and is adherent to, the collagen capsule of both human hair follicles and rodent vibrissae such that culture of the bulbar collagen capsule enables cultivation of DSC cells (Fig 1h). In subsequent studies the DSC was also cut from the collagen capsule and cultured in isolation. However, no significant differences were observed between the two approaches to DSC culture and the results are combined here for simplicity of presentation.

All cells regardless of source were cultured under the same conditions. Hair follicle subunits derived from an individual donor were maintained as separate cultures. Cells from different mouse donors were not combined at any stage of culture or implantation. DP, DS, or DSC units were placed in 1 mL of AmnioMax-C100 basal medium plus AmnioMax-C100 supplement (Gibco) in 24-well culture plates (Falcon, Franklin Lakes, NJ) incubated at 37°C in 5% CO<sub>2</sub>. Culture conditions and medium were such that any contaminating keratinocytes were nonproliferative as confirmed by *in vitro* observation. Proliferating DP-, DS-, or DSC-derived cells were subsequently passaged a maximum of two times into 25 mL culture flasks (Greiner, Frickenhausen, Germany) to produce  $1 \times 10^7 +$  cells per culture.

Samples from cell cultures were also incubated on sterile glass coverslips at each passage, fixed in acetone, and immediately exposed to alkaline phosphatase Fast Red TR substrate in solution (Pierce, Rockford, IL; 10 mg of Fast Red TR as supplied, 10 mL of substrate buffer, 1.5 mL of naphthol AS-MX phosphate concentrate as supplied) at pH 8.1 for 1 h and in the absence of levamisole. Results were contrasted with 6 µm cryosections of mouse vibrissae and pelage hair follicles exposed to the same substrate for 30 min

**Cell implantation and analysis** Cells derived from the DP, DS, and DSC of Scid, C3H/HeJ, wild-type TgN-GFPX, and GFP-expressing TgN-GFPX mouse vibrissa follicles were each implanted into the ears or footpads of Scid mouse recipients (Table I). In addition, cells from C3H/

HeJ mice were implanted into their respective littermates as a control for histocompatibility and possible implant rejection. Rodents were anesthetized with 1.66 mL of xylazinehydrochloride (Rompun, BayerVital, Leverkusen, Germany) in 10 mL of ketamine hydrochloride (Hexal, Holzkirchen, Germany) diluted 1:4 with PBS (0.1 mL/10 g body weight). Each ear or footpad was injected with  $3 \times 10^6$  to  $5 \times 10^6$  cells in 0.05 mL of PBS. In preliminary experiments we had found that hair follicle induction was improved when the cells were injected in association with wounding (data not shown). Thus, a 16-gauge needle was used to cut the skin of the outer ear, and cells were implanted by inserting the injection needle at the wound site and tunneling under the epidermis for approximately 2 mm at either side of the wound. Successful injection was visually apparent with blebbing of the epidermis as the cell suspension was injected. No treatment was applied to the wounds that were dry by completion of the implantation session.

Mice were euthanized at time points 2 or 6 mo after cell implantation, the ears or footpads were removed, and areas where hair fibers were apparent were dissected. Ears implanted with non-GFP-expressing cells were also processed for routine histology. Ears and footpads implanted with GFP-expressing cells, or non-GFP-expressing cells from TgN-GFPX wild-type littermates, were fixed with 4% paraformaldehyde in PBS, embedded in OCT compound (TissueTek, Sakura, Zoeterwoude, the Netherlands), and cryopreserved with a slow freezing technique that provides superior GFP preservation properties, as described elsewhere (Shariatmadari *et al.*, 2001). Sections of 20, 40, and 50 µm thickness were employed in analysis. Images were collected with a LSM 410 inverted laser scanning confocal microscope (Zeiss, Göttingen, Germany). An argon laser provided a 488-nm excitation wavelength and emission was visualized using a 500 to 520 nm window. Confocal microscopy provides a significant advantage in examining GFP-expressing tissues in cryomicrotome sections because fluorescence is generally destroyed at the cut surfaces.

## RESULTS

Previous reports indicated strong alkaline phosphatase presence in DP cells throughout the hair follicle cycle and expression in the lower portions of the DS of anagen-stage hair follicles has been claimed by some (Johnson *et al.*, 1945; Butcher, 1951; Hardy, 1952; Kopf, 1957; Braun-Falco, 1958; Handjiski *et al.*, 1994). This consistent expression of alkaline phosphatase in DP cells has been utilized as a simple and reliable method to distinguish dermis-derived cells of the DP from other hair follicle structures throughout the hair follicle cycle (Paus *et al.*, 1999). In an attempt to demonstrate biochemical distinctions between the DP, DS, and DSC as morphologically defined in this study, cryosections and cultured cells derived from dissected, mature hair follicle components of mouse hair follicles (Fig 2) were subjected to an alkaline

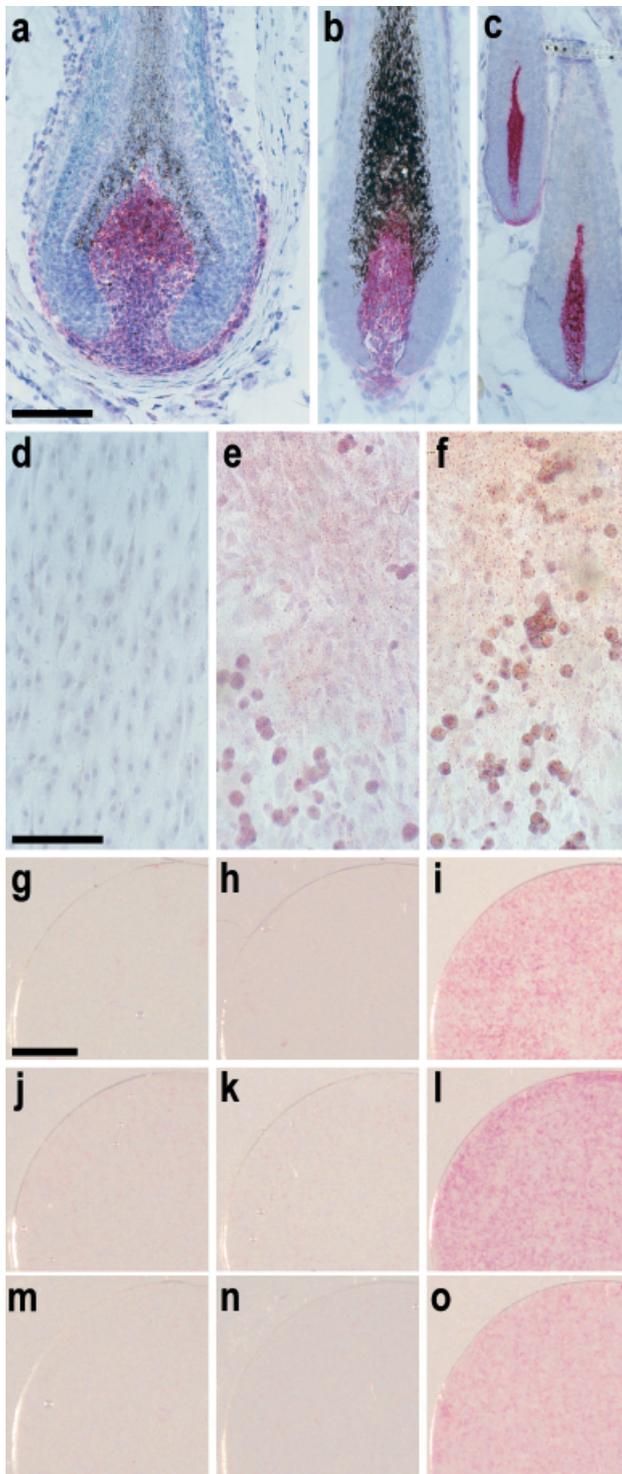
phosphatase substrate commonly used in immunohistochemistry. The DP was strongly alkaline-phosphatase-positive in mouse vibrissae, whereas the DSC, from beneath the DP to the approximate level of the DP apex, exhibited a low, but unequivocal, alkaline phosphatase activity. In contrast, the DS not associated with the bulb region was consistently negative (Fig 2a). Mouse pelage follicles, including guard hairs, showed similarly strong alkaline phosphatase expression in the DP and low expression was observed in cells of the DSC immediately adjacent to the DP, but not in other DSC or DS cells (Fig 2b,c).

To some extent, cells from the DP, DS, and DSC maintained this biochemical distinction *in vitro*. DS-derived cells were devoid of alkaline phosphatase in primary culture (Fig 2d) and subse-

quent passages (Fig 2g,j,m), whereas DSC cells exhibited low alkaline phosphatase expression up to 21 days in primary culture (Fig 2e), but in subsequent passages showed no apparent expression (Fig 2h,k,n). In contrast, DP cells demonstrated strong alkaline phosphatase expression in primary culture (Fig 2f) and in subsequent passages (Fig 2i,l,o) until the time of implantation. As recorded in other studies (Jahoda and Oliver, 1981, 1984), DP cells showed superior *in vitro* aggregative properties compared to DS cells. DSC cells *in vitro* also demonstrated aggregative properties similar to DP cells (Fig 2e,f).

**Amputated mouse vibrissae reconstitute a DP and dermal cup and produce hair fiber.** Although hair regrowth from amputated rat vibrissae and human hair follicles has been shown (Oliver, 1966b; Jahoda *et al*, 1992, 1996a; Kim and Choi, 1995), similar findings for mouse vibrissae have not been demonstrated thus far. Vibrissa from wild-type TgN-GFPX mice from which hair follicle bulbs, including the DP and DSC as defined here, were removed and grafted to Scid mice were capable of reconstituting a new DP and DSC and forming vibrissae-like hair fiber. However, not all amputated vibrissae produced hair fiber visible by gross observation. Histologic examination defined more amputated vibrissae with DP reformation and apparent hair fiber growth. Of 30 amputated vibrissa follicles grafted, 7 demonstrated DP reformation and hair fiber production. The reduced frequency of follicle regeneration compared to studies utilizing rat vibrissae (Oliver, 1966b; Jahoda *et al*, 1992) is likely due to the lack of mouse vibrissa follicle robustness. The reduced "elasticity" of the collagen capsule and small size of mouse vibrissae compared to rat and human hair follicles makes disruption and damage of tissue more likely during microdissection. Two distinct morphologic presentations were demonstrated. Most amputated vibrissae with hair regrowth exhibited reformation of a new DP within the amputated collagen capsule (Fig 3a,b). However, in one amputated follicle DP reformation occurred at a site below the cut end of the collagen capsule suggesting migration of epidermal-derived and dermal-derived hair follicle components in addition to differentiation into DP, DSC, and keratinocyte matrix components (Fig 3c).

**Vibrissae-like hair fiber growth with implantation of cultured DP- and DSC-derived cells.** By gross observation,

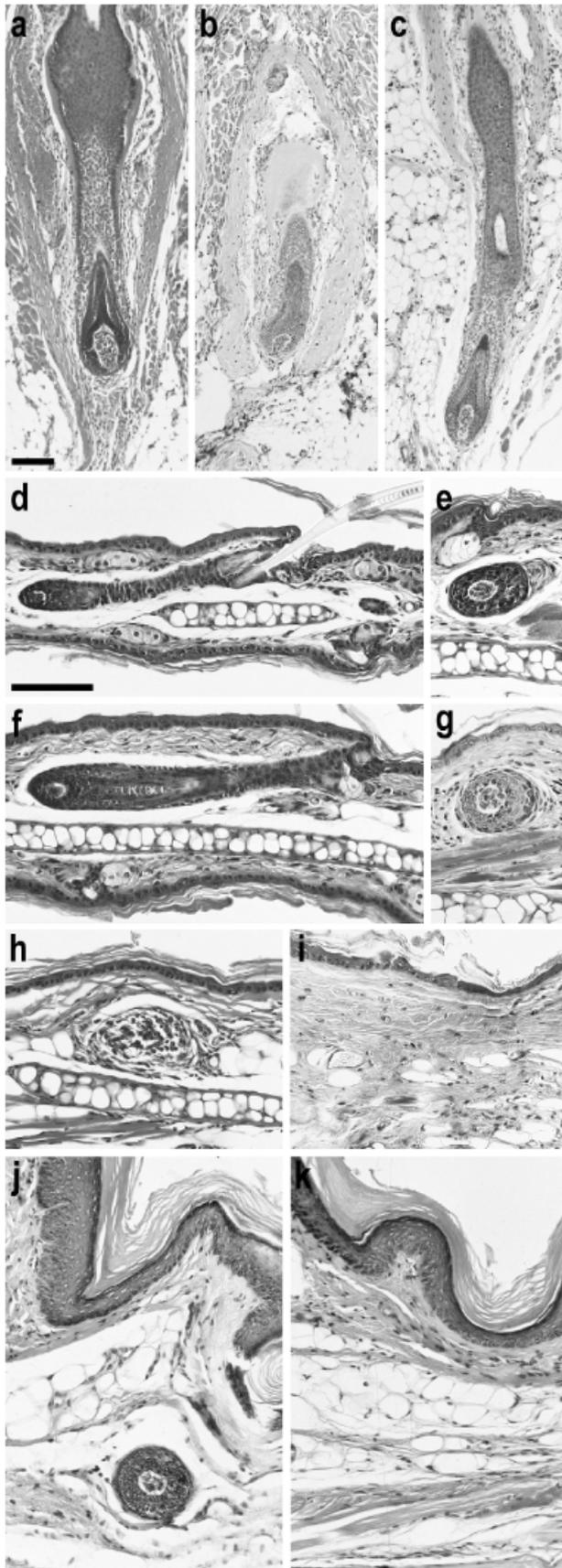


**Figure 2. Alkaline phosphatase expression in the dermis-derived component of hair follicles *in vivo* and *in vitro*.** Section through a C3H/HeJ mouse vibrissa exposed to alkaline phosphatase substrate (a). Expression (red) of alkaline phosphatase is apparent within the DP with greatest intensity in cells towards the apex of the DP above the line of Auber. A reduced alkaline phosphatase expression is apparent in cells of the DSC immediately below the DP as well as in DSC cells associated with the hair follicle bulb to a level approximately equal to the apex of the DP. The non-bulbar DS can be seen contiguous with the DSC but alkaline phosphatase is not expressed. Mouse guard hair (b) and pelage follicles (c) showed a similar presentation with strongest alkaline phosphatase expression toward the DP apex. Cells of the DSC immediately below the DP were moderately positive, whereas other DS cells were negative. Primary mouse cell cultures exposed to alkaline phosphatase substrate at 21 d, when the first cell passage was performed, showed that DS cells were consistently negative for alkaline phosphatase and exhibited a growth pattern of aligned, spindle-shaped cells typical of fibroblast-like cells (d). In contrast, DSC cells were slightly alkaline-phosphatase-positive, the growth pattern was more irregular, and cell morphology was less spindle-shaped (e). DP cells were somewhat more alkaline-phosphatase-positive and showed similar growth patterns to DSC cells (f). Both primary cultures of DSC and DP cells exhibited apparent clustering of cells (e, f) not observed in DS cell cultures. DS- (g, j, m), DSC- (h, k, n), and DP- (i, l, o) derived cell cultures exposed to alkaline phosphatase substrate 14 d after passages 1 (g-i), 2 (j-l), and 3 (m-o), when cells were confluent, illustrate maintenance of alkaline phosphatase expression in DP cells (i, l, o), whereas expression is lacking in passaged DS- and DSC-derived cell cultures. Bars (a-c), 100  $\mu$ m (d-f), 100  $\mu$ m, and (g-o) 2 mm.

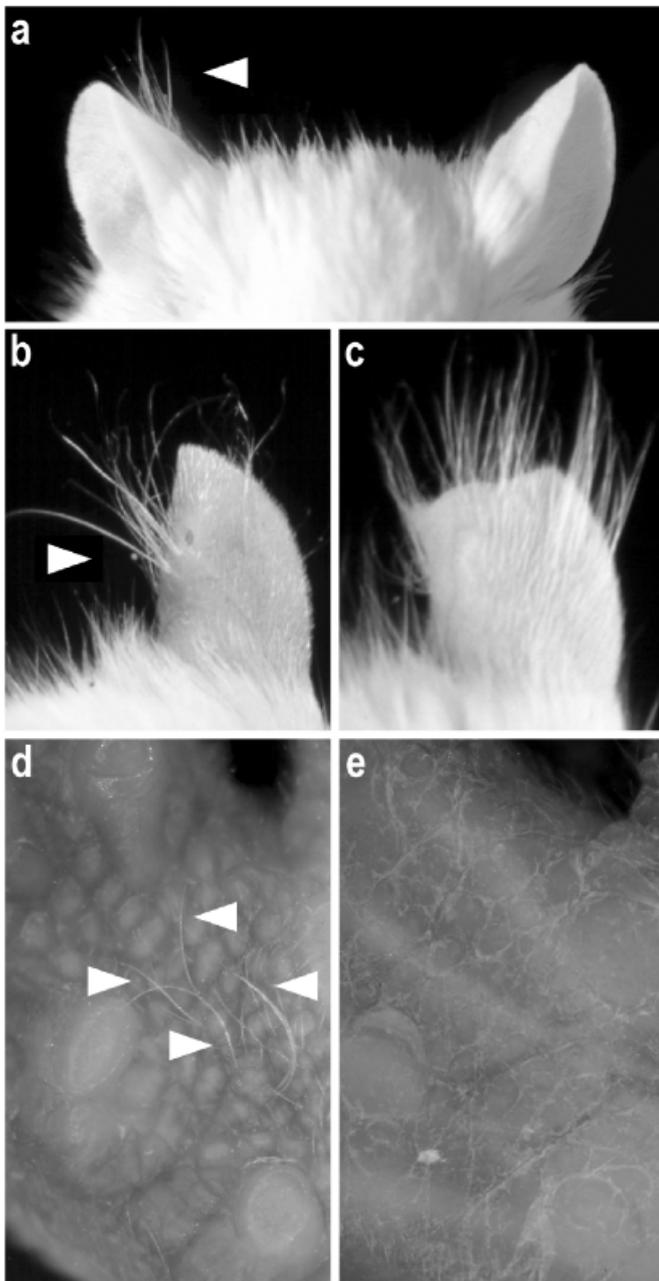
mouse DP- and-DSC derived cells, but not DS-derived cells, induced hair follicles to grow within implanted Scid mouse ears and footpads (Table I). Hair fiber with greater length than that

normally expected from mouse ears was first observed in DP and DSC cell recipients 4 weeks after implantation. By 2 mo after implantation, hair growth was clearly visible compared to DS-cell-implanted ears and persisted for 6 mo (Fig 4). Frequently, emerging hair fibers were clustered within small areas of the ear close to the site of wounding (Fig 4b). Occasionally, hair growth was relatively evenly distributed, with individual hair follicles more consistently oriented, and gave the appearance of a more ordered hair follicle distribution similar to that anticipated with natural, embryogenesis-derived hair follicle distribution (Fig 4c). The cell injections in PBS had caused blebbing of the epidermis over large areas of the ear and this likely permitted the spread of cells under the epidermis once injected. Although similar numbers of cells were implanted to each ear, the number of visible hair fibers varied considerably from a minimum of 3 to an estimated 30. Hair growth was also apparent from the inner ears of some mice, indicating some implantations had involved the penetration of the needle through the collagen layer of the ear. This was subsequently confirmed by histologic examination. DP- and DSC-cell-injected footpads demonstrated first visible hair growth somewhat later than ears at 2 mo after implantation, but also persisted for 6 months until necropsy (Fig 4d), whereas DS cell implantation produced no visible change (Fig 4e).

Routine histology on mouse ears revealed multiple hair follicles with variable orientation after implantation with DP or DSC cells from C3H/HeJ, Scid, and wild-type TgN-GFPX mice. Hair follicles induced or modified by cell implantation were readily differentiated from natural hair follicles by their large size and being in an anagen state, whereas the small, natural hair follicles of the ear were almost exclusively in a telogen state (Figs 3d–g). In general, hair follicles grew in the same general direction away from the body most likely due to the presence of the collagen layer physically limiting greater variability in orientation. However, histology revealed that the hair bulbs of larger hair follicles exhibited no consistent orientation (Fig 3e,g). Hair bulbs were twisted at unusual angles in relation to their respective hair shafts as well as compared to the orientation of natural hair follicles. The presence of extracellular matrix and connective tissue as part of the DS associated with the large, induced hair follicles was highly variable. While some hair follicles had prominent connective tissue, others were virtually devoid of visible connective tissue. By serial sections, clustering of cells was also apparent in DP- and DSC-cell-injected ears, but in the absence of associated



**Figure 3. Histologic presentation of amputated grafted mouse vibrissae and implantation of DP, DSC, and DS cells to Scid mouse ears and footpads.** Of wild-type TgN-GFPX mouse vibrissae grafted to the backs of Scid mice, a proportion reformed DP and generated hair 6 mo after surgery (a–c). Morphologically, DP reformation occurred within the cut collagen capsule with apparent rearrangement of the collagen capsule to encompass the new DP (a) or, in hair follicles with smaller DP, without apparent collagen capsule production or rearrangement such that the DP was “exposed” through the cut end of the capsule (b). In one instance, apparent migration of DP and keratinocyte cells occurred such that the hair follicle bulb lay some distance below the cut end of the collagen capsule (c). Scid mouse ears implanted with Scid mouse derived DP cells (d, e, h) or DSC cells (f, g) exhibited multiple anagen-stage hair follicles and the presence of associated large hair fibers. The presence of larger DP was observed in association with hair follicles of greater disorientation (e, g), whereas hair follicles with smaller DP, but hair fiber of greater length than anticipated from natural mouse ear hair follicles (d, f), were more likely to be appropriately oriented. In addition, ears implanted with DP cells, as well as DSC cells, exhibited clusters of cells in the dermis not associated with epithelial structures (h). Footpads implanted with DP or DSC cells presented with large follicles (j). Nevertheless, mouse ears or footpads implanted with DS cells exhibited occasional patchy diffuse cells within the dermis, but no cell clustering or epithelium derived structures were observed (i, k). Bars, (a–c) 100  $\mu$ m and (d–k) 100  $\mu$ m.



**Figure 4. Hair growth from Scid mouse ears or footpads implanted with mouse-derived DP or DSC cells but not DS cells.** Implantation of Scid mouse ears with TgN-GFPX mouse derived DP cells (left ear, arrowhead) resulted in hair fiber development significantly longer than that normally observed in non manipulated mouse ears or in ears implanted with DS-derived cells (right ear) after 6 mo (a). With DP (b) and DSC cell implantation the most common presentation involved several clusters of hair fibers (b, arrowhead). Nevertheless, a more even distribution and more appropriately oriented production of long hair fiber was observed in two DSC-cell-implanted ears (c). DP or DSC cells implanted to footpads produced comparatively few follicles for the same number of cells implanted, but long hairs emanating from the footpads (arrowheads) were apparent 6 mo after DP or DSC cell implantation (d), whereas DS-implanted footpads exhibited no gross morphologic changes (e).

epithelial structures (Fig 3h), whereas ears implanted with DS cells demonstrated no such phenomena (Fig 3i). DP and DSC cells implanted to footpads were similarly capable of inducing hair follicle formation (Fig 3j), whereas DS cells were not (Fig 3k).

**GFP-expressing DS-derived cells survive *in vivo* but fail to induce new hair follicles.** Confocal microscopy demonstrated that mouse ears and footpads implanted with fluorescent DS cells were devoid of any large vibrissa-like hair follicles as anticipated from routine histologic analysis. Fluorescent cells were observed in a patchy diffuse pattern throughout the dermis at both 2 and 6 mo after implantation. These cells were not associated with any recognizable hair follicle structures whether induced or present as a result of natural development during embryogenesis. Autofluorescence from the sebaceous glands, striated muscle, keratinized hair fiber, and collagen of the ear was present in all mouse ears regardless of the implanted cell type.

**GFP-expressing DP-derived cells form DP *in vivo* and induce new hair follicles.** Confocal microscopy of green fluorescent cell implants from TgN-GFPX to Scid mouse ears provided important information on the dynamics of the dermis-derived cell component in hair follicles. In mouse ears implanted with cultured DP cells, fluorescent DP cells could be identified within DP structures of large, apparently induced, hair follicles. Fluorescent DP cells were observed at both 2 and 6 mo after implantation. The most common presentation was a sharply delimited fluorescent cell-containing DP closely associated with nonfluorescent epidermal cell components of the hair follicle (Fig 5a). In some hair follicles, the DP was exclusively comprised of fluorescent cells. However, some hair follicle DP contained fluorescent cells interspersed with nonfluorescent cells (18 DP of 36 evaluated), suggesting hair follicle development through a combination of implanted and resident DP cells (Fig 5c). The frequency of fluorescent cells in these composite DP was similar at both 2- and 6-mo time points and ranged from 1 to 16 (mean 9.4 at 2 mo, n = 8; mean 9.8 at 6 mo, n = 10). By contrast, hair follicles induced using DP-derived cells from wild-type TgN-GFPX consistently exhibited nonfluorescent DP (Fig 5f). To help understand how implanted cells might combine with resident host dermal cells, subsequent, additional studies involved implanting cells to food pad regions. Confocal microscope analysis revealed that the DP of induced hair follicles in footpads were consistently composed of fluorescent cells (15 DP evaluated). That is, DP with a combination of fluorescent and nonfluorescent cells, as observed in mouse ears, were not identified in implanted footpad skin (Fig 5g).

**GFP-expressing DSC cells form DP *in vivo* and induce new hair follicles.** DSC-cell-implanted ears successfully produced fluorescent-cell-containing DP (Fig 5b,e). Similar to DP cell implantation, 19 of 35 evaluated hair follicle DP exhibited a mixture of fluorescent and nonfluorescent cells (Fig 5d). DSC cells implanted to footpad skin exhibited a composition of only fluorescent cells as observed with DP cells (Fig 5h). In these composite DP, the frequency of fluorescent DSC-derived cells was similar to that observed with DP-derived cells ranging from 4 to 18 (mean 10.2 at 2 mo, n = 9; mean 11.3 at 6 mo, n = 10). Some unusual aberrations within the induced hair follicles could be identified. In one example, two closely associated induced hair follicles apparently shared the same fluorescent cell cluster as a DP (Fig 5f). As identified by routine histology, a lack of appropriate orientation of induced hair follicle bulbs was observed with both DP- and DSC-cell-induced hair follicles.

## DISCUSSION

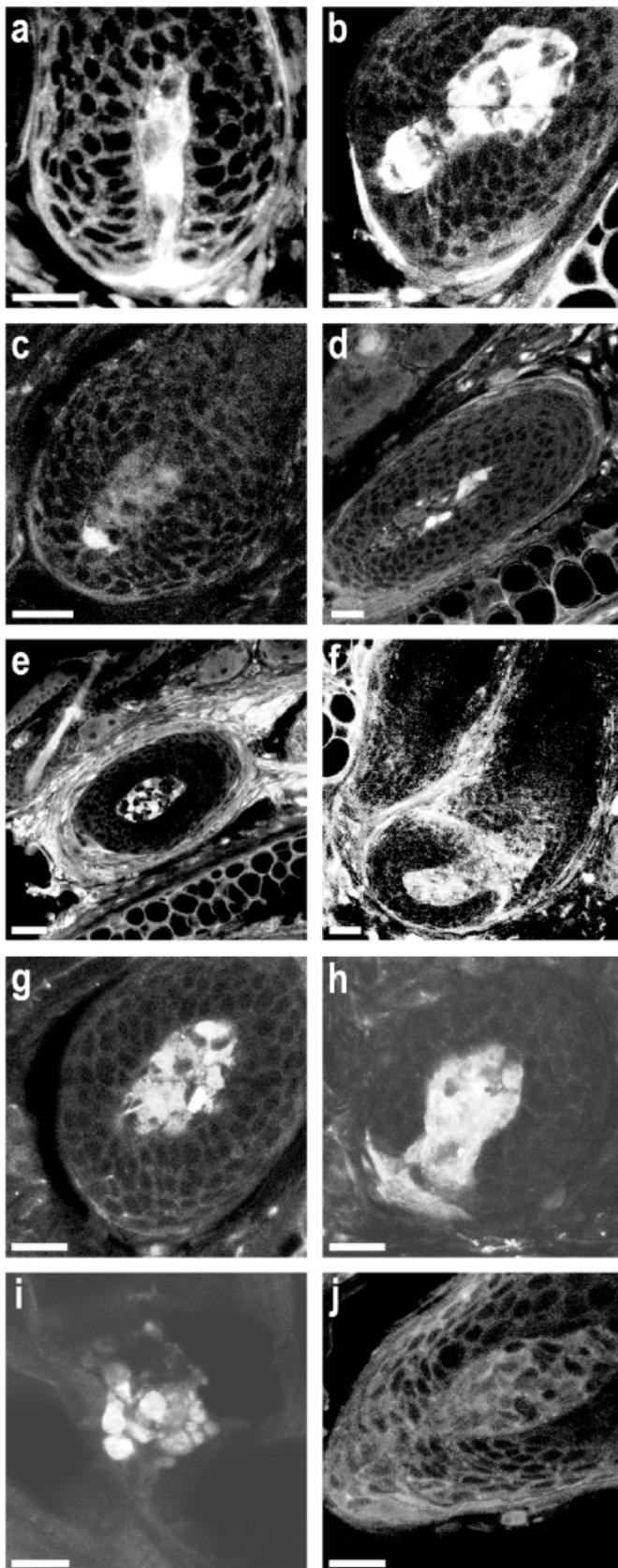
The embryogenic development and cycling of hair follicles and related mammalian structures such as feathers, horns, teeth, and nails require an intimate interaction between epithelium and dermis-derived components (Lillie and Wang, 1944; Chase, 1955; Van Scott and Ekel, 1958; Cohen, 1969; Kollar, 1970; Dhouailly, 1973; Hardy, 1992; Paus *et al*, 1999; Chuong *et al*, 2001). The dermis-derived DP structure of hair follicles can be long-lived with human scalp hair follicles persisting in an anagen growth stage for several

years (Van Scott *et al*, 1957; Saitoh *et al*, 1970; Courtois *et al*, 1995). Morphologically, the DP structure is a permanent feature throughout the hair growth cycle, but whether individual DP cells survive from one complete hair cycle to the next has been a matter

for debate (Segall, 1918; Butcher, 1951; Wolbach, 1951). It has been suggested that epithelial cells may stimulate DP cells to proliferate and perpetuate the DP structure (Cotsarelis *et al*, 1990; Sun *et al*, 1991). However, there is little direct evidence that DP-located cells proliferate other than during early proagen stages (anagen II–IV) of hair follicle development (Wessells and Roessner, 1965; Parry *et al*, 1995; Stenn *et al*, 1999) and such proliferating cells as do occur within the DP have been defined as endothelial or migratory cells (Pierard and de la Brassinne, 1975). DP cells in which nonproliferation is enforced by irradiation are still capable of inducing hair follicle formation after implantation (Ibrahim and Wright, 1977).

Previously it was suggested that the cells that constitute the DP may be derived from the DS (Oliver, 1966b,c 1967a; Horne and Jahoda, 1992; Jahoda *et al*, 1992; Elliott *et al*, 1999). Surgical implantation of microdissected follicular DS to amputated rodent vibrissae promoted the formation of new DP and hair fiber (Horne and Jahoda, 1992). Various combinations of differentiated, epidermal-derived hair follicle cells permit the redevelopment of DP and formation of hair-follicle-like structures (Oliver, 1967a; Kobayashi and Nishimura, 1989; Reynolds and Jahoda, 1991; Matsuzaki *et al*, 1996; Hashimoto *et al*, 2000, 2001). A recent report involving transgener implantation of microdissected human DP and DS indicated DS successfully induced hair follicle formation (Reynolds *et al*, 1999).

Previously, implantation of cultured, DS-derived cells was shown not to induce new hair follicle development and the conclusion was made that disengaging sheath cells from epidermal cell influences through cell culture prohibits a DS cell to DP cell transition after *in vivo* implantation (Horne *et al*, 1986; Reynolds and Jahoda, 1991, 1992, 1996; Horne and Jahoda, 1992; Jahoda and Reynolds, 1996b). Cells derived from the nonbulbar DS, as defined morphologically in this study, may represent a differentiated cell population with a primarily structural or contractile role. As anticipated from these observations, implantation of cultured DS-derived cells in the current study consistently failed to induce hair follicle development. GFP-expressing DS-derived



**Figure 5. Fluorescent cells within hair follicles of Scid mouse ears implanted with TgN-GFPX mouse-derived DP or DSC cells.** The bulbs of large, anagen-stage hair follicles in Scid mouse ears implanted with TgN-GFPX mouse-derived DP cells demonstrated DP-containing fluorescent cells 6 mo after implantation (a). Ears implanted with TgN-GFPX mouse-derived DSC cells also contained anagen-stage hair follicles with fluorescent DP (b). While some DP structures exclusively contained fluorescent cells, hair follicles in ears implanted with DP (c) or DSC (d) cells also sometimes contained a combination of fluorescent and nonfluorescent cells. The number of fluorescent cells observed in an entire DP varied considerably with one hair follicle in DP-cell-implanted ears containing a single fluorescent cell (e), whereas other follicles, in this case from a DSC-cell-implanted ear, showed a mixture of several fluorescent and nonfluorescent cells (d). Fluorescent cells peripheral to the DP region, in DSC and DS areas of apparently induced hair follicles, could also be observed (a, b, e). Occasional anomalies were observed in DP- and DSC-implanted ears. In one DSC-cell-implanted ear, a single fluorescent cell cluster was associated with two separate hair follicles epithelium structures (f) with the bulb regions lying at a different orientation to the rest of the hair follicles. Fluorescence in DSC and DS regions, proximal to the follicle bulb, is due to a combination of fluorescent cells and nonspecific autofluorescence from deposited collagen. DP (g) and DSC (h) cells implanted to footpads also promoted hair follicle formation with associated fluorescent DP. Fluorescent cell clusters in the absence of associated epithelial structures could also be occasionally observed after DP or DSC implantation (i). Hair follicles in ears implanted with cells from wild-type TgN-GFPX did not demonstrate fluorescence within the DP or DSC (j). Mouse ear collagen (b, e, f) and keratinized hair shafts (e) are autofluorescent and non-specific in these images. Bars, (a–j) 20  $\mu$ m.

cells showed no apparent attempt to cluster and form DP and survived as a patchy, diffuse population within the dermis. In contrast, the DS, in association with hair follicle keratinocytes in amputated vibrissae, did reform DP. These observations suggest that to induce hair follicles nonbulbar DS cells must closely associate with hair follicle epidermal cells. However, in contrast to previous work the present studies demonstrate that cultured sheath cells derived from a hair follicle bulb location (DSC) are capable of directly reconstituting a DP and promoting the development of vibrissae-like hair follicles. In this study, DSC-derived cells, but not DS-derived cells, induced formation of new hair follicles. It is conceivable that the DSC is the source for constitution of the DP via cell migration in natural hair follicle cycling.

In addition to the formation of new DP and DSC by implanted cells, the cells apparently also combined with host cells to form composite papillae composed of both fluorescent and nonfluorescent cells. It is possible that the implanted cells recruited, and promoted differentiation of, nonfollicular resident dermal cells or recruited resident, differentiated hair follicle mesenchyme cells to the formation of new DP. Alternatively, the implanted cells may have directly integrated with the mesenchyme component of hair follicles that are already present in the ear skin through natural hair follicle embryogenesis. The presentation of DP exclusively composed of fluorescent cells in the footpads suggests that the implanted cells fail to recruit nonfollicular dermal cells and that cells from resident hair follicles are the more likely source of nonfluorescent cells in chimeric papillae in the ears.

Previously it has been shown that the size of the DP is directly correlated to the size of the hair follicle and the fiber produced (Ibrahim and Wright, 1982; Elliott *et al*, 1999). In the current study, the large DP composed exclusively of fluorescent cells or chimeric DP containing both fluorescent and nonfluorescent cells gave rise to large hair follicles and hair fibers with prolongation of their anagen growth stage. This suggests that the implanted cells largely retained their functional properties from the donor vibrissa follicles. In their capacity to induce or to supplement hair follicle development, the DP and DSC cells contributed to all the relevant dermis derived structures, DP, DSC, and to a lesser extent, the DS.

In other mesenchymal cell systems, alkaline phosphatase expression may be utilized as a marker of cell differentiation. In bone formation, osteoblasts show enhanced alkaline phosphatase expression with the loss of proliferative capacity and progressive differentiation (Stein *et al*, 1990; Aubin *et al*, 1995; Spector *et al*, 2002). The expression of alkaline phosphatase was exploited as a simple method to biochemically differentiate the DP, DS, and DSC cell populations used in this study. The high expression of alkaline phosphatase in the DP, particularly toward the DP apex above the line of Auber (Auber, 1952), may be consistent with the general view that these cells are nonproliferative and highly differentiated. Moreover, alkaline phosphatase expression in hair follicle mesenchyme coincided with hair follicle induction properties. That is, cells from DP and DSC that express alkaline phosphatase could be cultured and implanted to induce hair follicle formation while cells from nonbulbar DS that do not express alkaline phosphatase could not induce new hair follicle development after implantation. The expression of alkaline phosphatase in hair-follicle-inducing DP and DSC cells enables easy *in situ* and *in vitro* distinction from nonbulbar DS cells.

From these and other studies, it would seem that cells capable of generating the dermal component of hair follicles reside in all regions of the hair follicle DS. We suggest that: (1) cultured DP and DSC cells are equally capable of hair follicle induction after transplantation and might be regarded as functionally similar. (2) Both DP and DSC cell populations survive *in vivo* as a distinct entity. As such, their hair follicle inductive properties may persist for a prolonged period of time, 6 mo in the current study, if not indefinitely. (3) Hair follicle mesenchyme cells with hair follicle inductive properties may be identified by application of alkaline

phosphatase substrates both *in situ* and *in vitro*. DSC cells may hold clues to understanding hair follicle dynamics and may yield information on morphogenesis and tissue engineering involving epithelium–mesenchyme interactions.

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