Cyclical Changes in Rat Vibrissa Follicles Maintained *In Vitro*

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In mammals hair growth is cyclical; however, the factors that regulate the hair growth cycle are still poorly understood. The recent development of methods for culturing hair follicles *in vitro* has proved an important tool to investigate many aspects of the regulation of hair follicle growth. At present, however, these models are based on the culture of anagen hair follicles and have only partially been used to address the cyclical nature of hair growth. In this study we have made use of the fact that in rodents the hair growth cycle is synchronized, well characterized, and relatively short. We have isolated vibrissa follicles from 12 d old rats and confirmed by histology that these follicles are in the anagen stage of their first hair growth cycle. We have then maintained these follicles *in vitro*, on Gelfoam supports, for up to 23 d (35 d of age) and compared their histology with *in vivo* follicles from equivalent age littermates. We observed that 12 d old follicles maintained *in vitro* for up to 23 d show changes in morphology that suggest that cultured rat vibrissa follicles retain cyclical activity *in vitro*. Cyclical changes in hair follicle morphology were only seen in follicles maintained on gelfoam supports and moreover, hair follicle size appears to be a key feature in determining the ability of the follicle to cycle *in vitro*. All follicles that showed cyclical changes *in vitro*, however, appeared to remain blocked in pro-anagen. These data suggest that the vibrissa follicle is a *in vitro* good model system with which to investigate hair cycle control. *Key words: cyclical changes/dermal papilla/in vitro/inner root sheath/outer root sheath/rat vibrissa follicles. J Invest Dermatol 115:1152–1155, 2000*

The mammalian hair growth cycle is a complex process involving: (i) an active growth stage anagen, during which a keratinized hair fiber is formed; (ii) a regressive, catagen, stage during which hair growth stops; (iii) and the hair follicle involutes giving rise to a resting telogen hair follicle, which then re-enters anagen. Recent studies on targeted and spontaneous mutations in mice, as well as immunohistochemical, *in situ* hybridization and semiquantitative polymerase chain reaction studies have identified a number of growth regulatory factors that may play important parts in regulating hair growth and the hair growth cycle (Moore *et al*, 1991; Luetteke *et al*, 1993; Hebert *et al*, 1995; Little *et al*, 1996; Rosenquist and Martin, 1996; Rudman *et al*, 1997). Although a large number of putative factors have been identified, however, our understanding of how these factors regulate hair growth are still poorly understood (Paus and Cotsarelis, 1999 for a review), and this reflects in part the lack of *in vitro* models that address regulation of the hair growth cycle.

We have previously shown that human hair follicles isolated from facelift skin can be maintained *in vitro* and continue to produce a keratinized fiber at *in vivo* rates (Philpott *et al*, 1990). Moreover, we have also shown that these cultured hair follicles respond *in vitro* to a number of growth factors, including epidermal growth factor/transforming growth factor-α, insulin-like growth factor-I and -II, transforming growth factor-β, and interleukin-1α (Philpott and Kealey, 1994; Philpott *et al*, 1994, 1996). All of these studies, however, have been carried out using hair follicles from the anagen stage of the hair growth cycle and not catagen or telogen follicles. Catagen hair follicles can be isolated from human skin using the methods previously reported by us (Philpott *et al*, 1990); however, because of the asynchronous nature of human hair growth very few catagen follicles are seen compared with anagen follicles and therefore, it is not usually possible to obtain sufficient numbers of catagen follicles to perform experiments.

The hair growth cycle in rodents, however, is synchronized (Chase, 1954; Ebling and Johnson, 1964). Therefore, it is possible to predict the stage of hair follicle from the age of the animal, and this has been of major use in both *vivo* studies (Ebling and Johnson, 1964; Paus *et al*, 1994) and *in vitro* using whole skin culture (Paus, 1991; Li *et al*, 1992a,b). Anagen hair follicles can be isolated and cultured from the pelage of rats but catagen and telogen follicles are too small to isolate without damage (Philpott *et al*, 1992). Vibrissa follicles from rats and mice are much larger than pelage follicles and can be successfully cultured *in vitro* (Buhl *et al*, 1989; Jindo *et al*, 1994). To date, however, all studies using vibrissa follicles have been carried out on follicles from the anagen stage of their cycle and the ability of these follicles to continue to cycle *in vitro* has not been addressed. In this study we have isolated follicles from 12 d old rats and maintained them for up to 23 d *in vitro*. We show that in culture isolated vibrissae follicles retain cyclical activity and undergo a significant proportion of their hair growth cycle *in vitro*.

MATERIALS AND METHODS

Williams E medium (minus glutamine), L-glutamine, penicillin, and streptomycin were supplied by Gibco (Paisley, U.K.), insulin and hydrocortisone came from Sigma (Poole, U.K.). All tissue culture plastics were supplied by Becton and Dickinson (Plymouth, U.K.).

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Abbreviations: DP, dermal papilla; ORS, outer root sheath.

Isolation and culture of rat vibrissa follicles Rat vibrissa were isolated by microdissection from 12 to 35 d old Wistar rats using methods previously described for the isolation of human pilosebaceous units (Sanders *et al*, 1994). Briefly, the both left and right mystacial pads were removed from the rats and placed in a 1:1 (vol/vol) solution of Earle's balanced salts solution and phosphate-buffered saline supplemented with 100 U penicillin per ml and 100 mg streptomycin per ml. Individual vibrissa follicles were carefully dissected under a dissecting microscope. Considerable care was taken to remove surrounding connective tissue but not to damage the follicle. Both the large posterior and smaller anterior vibrissa were isolated and transferred on to a 5 cm plastic Petri dish containing Earle's balanced salts solution/phosphate-buffered saline (1:1 vol/vol) as described above, using one dish per animal. Using this method we could routinely isolate between 30 and 40 follicles from each animal. From these pooled follicles we then selected five large follicles and five small follicles for histology as described below. A further five large and five small follicles were used for each maintenance condition. By taking some follicles from each animal for histology and using others for maintenance we were able to confirm that the follicles placed *in vitro* came from a population of follicles that were in anagen. Because Ibrahim and Wright (1975) have reported some variation in vibrissa cycling times between anterior and posterior vibrissa we investigated the histology of both. The histology of follicles from 12 d rats, both large posterior and small anterior vibrissae, were always in anagen and showed no signs of catagen, based on the detailed descriptions of Young and Oliver (1976). Isolated vibrissa were either processed for histology as described below, or cultured in individual wells of 24-well multiwell plates. Vibrissa follicles taken for *in vitro* culture were either maintained free floating or on gelfoam supports (Buhl *et al*, 1989; Li *et al*, 1992a; Waldon *et al*, 1993), in 500 μl of Williams E medium supplemented with 2 mM L-glutamine, 10 μg insulin per ml, 10 ng hydrocortisone per ml, 100 U penicillin per ml, and 100 μg streptomycin per ml at 37°C in an atmosphere of 5% CO_2/95% air. Medium was changed every 3 d. Following culture follicles were removed from culture and processed for histology as described below.

Histology Freshly isolated, or maintained follicles were fixed overnight in 3% phosphate-buffered formalin and then mounted in 3% agar to facilitate handling during sectioning. Agar blocks containing follicle were then fixed for a further 24 h in 3% phosphate-buffered formalin and then embedded in wax, processed for sectioning and stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

The aim of this study was to determine whether the *in vivo* cyclic activity of rat vibrissa follicles could be maintained *in vitro* using established methods of hair follicle organ maintenance. Because of the difficulties in locating vibrissa follicles at different stages of their growth cycle (Young and Oliver, 1976) we decided that the most straightforward approach to investigating the ability of follicles to cycle *in vitro* was to isolate follicles in anagen and to then keep them *in vitro* for sufficient time such that, were they *in vivo*, they would have progressed through one cycle and re-entered anagen. We were then able to compare the histology of maintained follicles with the histology of follicles from equivalent aged littermates *in vivo*.

Prior to culturing follicles we first studied the histology of follicles from rats aged 12, 14, and 16 d of age (using five animals for each age and at least 10 follicles from each animal). The reason these preliminary studies were carried out was to ensure that in subsequent studies we used follicles from rats that were in anagen and did not show any indication of onset of catagen before being placed *in vitro*. Of the different aged animals none of the follicles from 12, 14, or 16 d old rats showed any morphologic indication of onset of catagen based on the criteria of Young and Oliver (1976). We chose, however, to use vibrissae from 12 d old rats for further study, because they not only showed no sign of histologic change but also because we felt it less likely that any hair cycle dependent changes in gene expression would have taken place this early in the follicle growth.

Vibrissa follicles isolated from 12 d old rats were found to be in the anagen stage of their hair growth cycle as shown by the histology of the proximal follicle bulb (**Fig 1*a***). In these follicles the elongated dermal papilla (DP) was situated within the follicle bulb and enclosed by the strongly basophilic epithelial matrix cells, which showed normal patterns of lineage restricted differentiation, giving rise to the keratinized hair fiber and inner root sheath.

When anagen vibrissa follicles, isolated from 12 d old rats, were maintained for up to 23 d *in vitro*, marked changes in histology were observed (**Fig 1*b*, *c***). In the maintained vibrissa, the basophilic matrix appeared much reduced in size and the majority of cells within the lower follicle bulb appeared similar to those of the outer root sheath (ORS). In the upper hair follicle the keratinized hair fiber appeared to taper and terminate in the upper epithelial portion of the hair follicle. The histology of these maintained vibrissa appeared very similar to that described by Young and Oliver (1976) for vibrissa in the pro-anagen stage of their hair growth cycle. Pro-anagen is a term used to describe anagen follicles in stages anagen I through anagen V and tends to be restricted to vibrissae follicles. The reason for using this terminology when discussing vibrissae follicles and not using the more detailed classification of Chase (1954) is clearly outlined by Young and Oliver (1976). They showed that, although vibrissae follicles show cyclical activity, they do not undergo the extensive shortening seen during catagen in pelage follicles and more importantly do not have a significant resting (telogen) stage. As a result new fiber formation occurs before differentiation of the club hair is complete. Therefore, Young and Oliver (1976) recommend that because of these differences in vibrissae cycling, that these follicles cannot be accurately accommodated in the existing description of the pelage cycle (Chase, 1954). We have, therefore, restricted our terminology to pro-anagen, anagen and catagen and have not attempted to use pelage follicle terminology. This was confirmed by studying the histology of vibrissae follicles taken from 26 d old littermates (**Fig 1*d***). In these follicles the upper region of the follicle had expanded and contained the tapered base of the keratinized club hair, whereas, in the lower follicle bulb a small basophilic matrix was surrounded by ORS. These observations not only confirm the earlier histologic description of pro-anagen vibrissae reported by Young and Oliver (1976) but also demonstrate that *in vivo*, by 26 d of age, the rat vibrissa follicles have undergone their first hair growth cycle and have re-entered anagen. Moreover, the similarity between the histology of pro-anagen follicles *in vivo* and the histologic changes seen in cultured anagen follicles, suggest that the *in vivo*, cyclical, nature of vibrissa follicle growth is retained *in vitro*.

Young and Oliver (1976) reported that the most striking histochemical changes seen during vibrissa follicle cycling *in vitro* involved loss of basophilic staining in the follicle matrix as they entered catagen and that this reduced basophilic staining of the matrix was also seen in pro-anagen follicles. As shown in **Fig 1**(***b***, ***c***) we observed a similar reduction in basophilic cells in the proximal follicle bulb in our cultured vibrissa. Moreover, in some follicles that showed abnormal growth *in vitro* ((**Fig 2*a***) it is very clear that there is a marked loss of basophilic staining in the cells adjacent to the DP suggesting also, that this follicle was attempting to cycle *in vitro*.

In total 44% of vibrissa maintained free floating (14 of 33) and 62% of vibrissae maintained on gelfoam (13 of 21) showed changes in morphology characteristic of pro-anagen follicles (**Table I**). Other changes in follicle morphology were more abnormal. In 39% (13 of 33) of vibrissae maintained free floating and 19% (4 of 21) maintained on gelfoam, the entire lower follicle bulb was found to have keratinized and the follicles appeared dead. Of the remaining follicles showing abnormal histology (one free floating and four on gelfoam), changes in histology were characterized either by stretching of the DP between its location within the proximal follicle bulb and the ascending club hair (**Fig 2*a***), or by the presence of two DP (**Fig 2*b***). Stretching of the DP is characteristic of the catagen stage of the vibrissa cycle *in vivo*, and is believed to occur because the papilla is attached to both the ascending club hair, as well as the mesenchymal cells and collagen capsule in the lower bulb (Young and Oliver, 1976). Moreover, the presence of two DP within a vibrissa follicle has also previously been reported *in vivo*, and occurs when normal patterns of follicle growth and

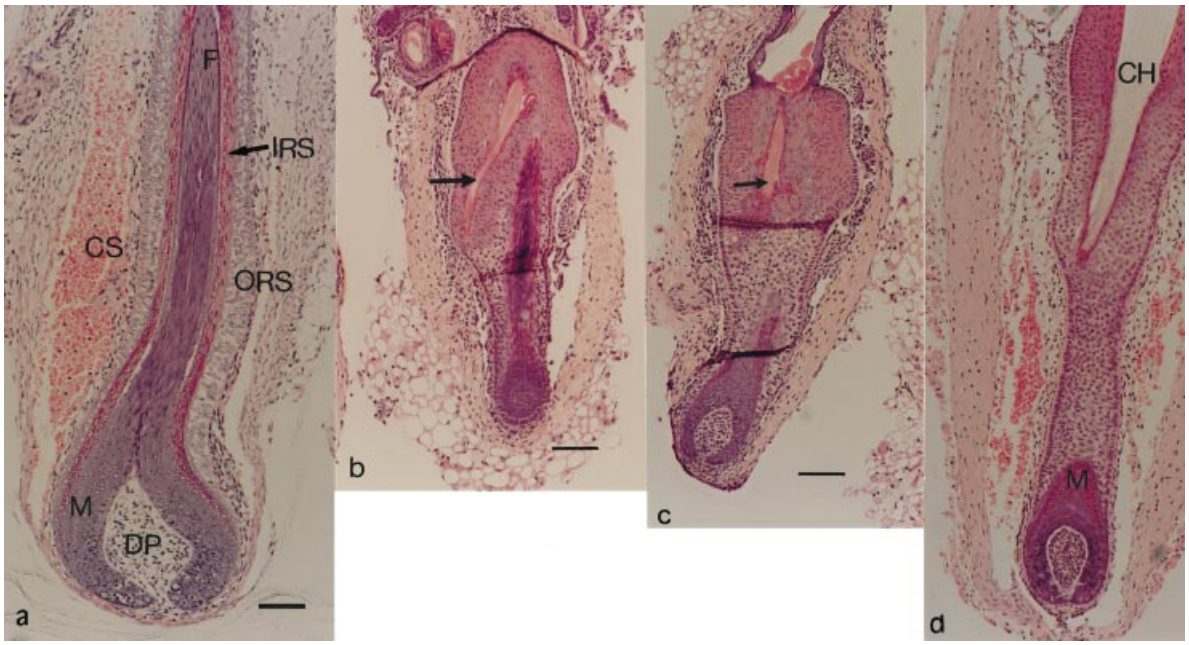

Figure 1. Rat vibrissa follicles maintained *in vitro* show cyclical activity. Characteristics of vibrissa follicles both freshly isolated and following maintenance *in vitro*. (*a*) Freshly isolated vibrissa from 12 d old rat showing hair follicle matrix (M), DP keratinized hair fiber (F), inner root sheath with adjacent ORS and cavernous sinus (CS). (*b*, *c*) Vibrissa follicle isolated from a 12 d old rat and following culture for 23 d on gelfoam showing marked changes in morphology characterized by the formation of a tapered club hair-like structure situated distally within the follicle (*arrow*) also note that in the lower follicle the majority of cells within the follicle bulb are similar to those of the ORS and the basophilic matrix appears much reduced. (*d*) Typical morphology of a pro-anagen vibrissal follicle isolated from a 35 d old littermate with tapered club hair (CH). Note epidermal cells within the lower bulb are identical to the ORS with the exception of the regenerating matrix cells (M) adjacent to the DP. Also note the similarity between the histology of the pro-anagen vibrissa in (*d*) and that of the maintained vibrissa in (*b*, *c*). *Scale bar*: 100 mm.

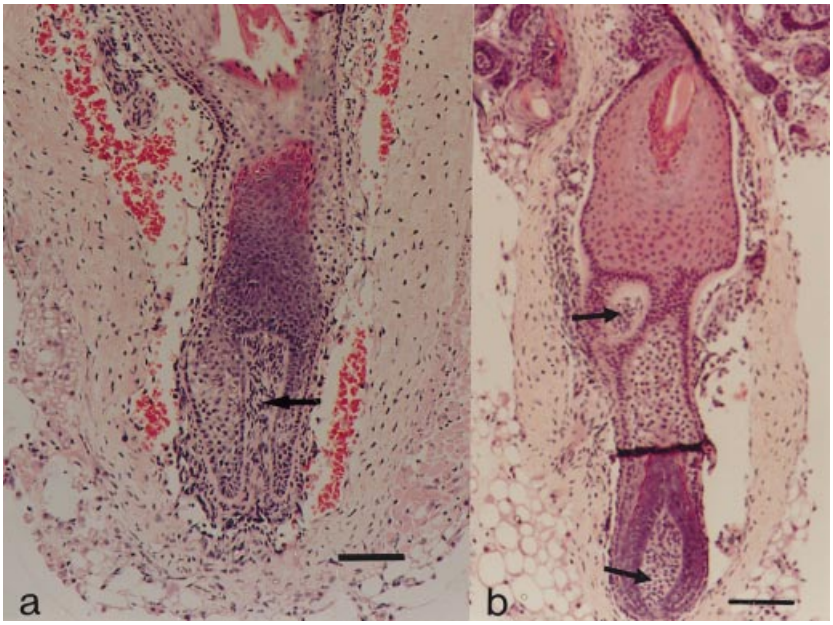

Figure 2. Some cultured vibrissa show abnormal changes in histology. Vibrissa follicles were isolated from 12 d old rats and following maintenance on gelfoam for 23 d showed abnormal changes in histology characterized by either (*a*) a stretched DP (*arrow*) between the ascending matrix cells and the lower follicle bulb or (*b*) two DP (*arrows*). The first is situated within the proximal region of the follicle bulb whereas the second is located distally within the follicle beneath the club hair. *Scale bar*: 100 mm.

differentiation are disrupted, usually following injury to the follicle (Jahoda and Oliver, 1984). That these changes in histology also occur in cultured follicles suggest that, although the cyclical nature of vibrissae follicle growth appears to be retained *in vitro* some follicles may be damaged on isolation. Our observation, however, that cultured follicles *in vitro* exhibit some of the changes in hair follicle morphology characteristic of those seen in damaged follicles *in vivo* is very interesting as it suggests that *in vitro* follicles retain the ability to respond in a similar manner to that seen *in vivo*.

Successful maintenance of vibrissa in culture appeared to be greatly enhanced when follicles were maintained on gelfoam, an expanded collagen matrix. This has been previously reported by Waldon *et al* (1993) and although the reasons for this improved maintenance have not been established; follicles on gelfoam are maintained at the air–liquid interface and this may help support follicle metabolism. We also observed, however, that during the course of these experiments the gelfoam was broken down and appeared to dissolve into the culture medium; this may release extra

Table I. Cultured rat vibrissae follicles showing cyclical changes in morphology

	Free floating	Gelfoam
No. of follicles	33[a]	21[b]
No. of follicles showing cyclical changes *in vitro*[c]	14 (42%)	13 (62%)

[a]Experiment started with 35 follicles from seven rats but two follicles were lost through contamination of culture.
[b]Experiment started with 25 follicles from five rats but three follicles were lost through contamination of culture.
[c]Defined as follicles showing changes in histology representative of pro-anagen. Follicles showing stretched and duplicated DP were not included.

nutrients essential for follicle maintenance. Follicle size would also appear to be important, as in our hands, only the smaller more anterior vibrissa showed cyclical changes and none of the larger follicles isolated from the posterior mystacial pad showed any cyclical changes in histology and instead their bulbs keratinized. This is not altogether surprising as organ maintenance relies on the diffusion of gases and nutrients into maintained tissues, and this is very much dependent on tissue size. *In vivo*, however, the smaller more anterior follicles of the mystacial pad grow at a much slower rate than the larger posterior vibrissa and also have a shorter cycle time (Ibrahim and Wright, 1975); this may also influence cyclical changes *in vitro*.

In this study we did not measure changes in hair follicle length due to difficulties in accurately measuring elongation of unpigmented follicles against the white background of the gelfoam. Furthermore, when follicles were maintained on gelfoam they usually changed orientation as the gelfoam dissolved into the culture medium. To have made accurate length measurements would have necessitated moving the follicles and in our experience this can cause trauma and damage to the hair follicle and is not recommended (Philpott *et al*, 1992) When vibrissa were first isolated, however, the hair fibres were trimmed and we were able therefore, to observe that when follicles were removed from culture and processed for histology that hair fiber elongation had occurred. We do not know whether in our hands this reflects true hair fiber growth; however, these fibres were easily pulled from the vibrissa and had characteristic club hair morphology, which suggests that cyclical changes had occurred *in vitro*. This is further supported by the observations of Robinson *et al* (1997) who have recently shown that *in vitro* growth of mouse vibrissa follicles closely reflected both their *in vivo* origin but also the specific stage of the cycle at which the vibrissa were isolated. Thus *in vitro* vibrissa follicles appeared to follow their *in vivo* patterns of growth and cycle timing.

Histology of maintained follicles indicate that the tapered end of the club hair remained connected to the matrix cells in the follicle bulb. In *vivo* the catagen and telogen stages of the vibrissae follicle cycle are very short and new hair fiber growth is initiated before club hair differentiation is completed (Young and Oliver, 1976). Furthermore, the cycle time of the smaller anterior vibrissa follicles is even shorter than the larger posterior follicles. Therefore, it is possible that in the smaller vibrissa contact may be maintained between the proximal end of the club hair and the distal end of the matrix.

Finally, vibrissa follicles that showed cyclical activity *in vitro* did not appear to progress beyond pro-anagen. This suggests that the transition from pro-anagen to anagen may be a key regulatory step in the growth and development of the hair follicle. This is supported by earlier experiments of Hardy (1949) in which embryonic mouse skin was maintained *in vitro*. In these experiments follicle development frequently arrested in the pro-anagen stage of development. In conclusion we report that the anagen rat vibrissa follicles maintained *in vitro* appear to retain cyclical activity. This model may be of considerable significance in furthering our understanding of the factors that control hair growth and the hair growth cycle.

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