Studies on the biochemistry and morphology of freshly isolated and maintained rat hair follicles

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Summary

We have already shown that shearing can be used to yield large numbers of viable intact hair follicles. We now show that these follicles can be viably maintained on permeable supports for 7 days in vitro as determined by their adenine nucleotide contents, rates of [methyl-³H]thymidine and [U-¹⁴C]leucine uptake, [methyl-³H]thymidine autoradiography, patterns of keratin synthesis and light and electron microscopy. These studies, however, show that after 7 days maintenance the morphology of maintained follicles shows a closer resemblance to the telogen rather than the anagen follicle. We therefore conclude that the failure of previous attempts at maintaining hair growth in culture is due to hair follicles prematurely entering the resting stage of their hair growth cycle, possibly as a response to isolation.

Key words: rat hair follicles, organ maintenance, DNA synthesis.

Introduction

Mammalian hair growth is cyclical (Butcher, 1951). Three stages can be distinguished: an active period or anagen during which hair growth occurs, a transitional or catagen stage and a resting or telogen period, which is followed by shedding of the last hair shaft when the follicle re-enters anagen. Hair growth in the newborn rat is synchronous and for the first few postnatal cycles it follows a predictable course, with each cycle lasting approximately 34 days (Butcher, 1951). The factors that regulate these changes, however, remain unknown, which may in part reflect a lack of suitable in vitro models for hair growth.

A number of authors have reported on the in vitro growth of embryonic hair follicles in isolated skin plugs (Hardy, 1949; Hardy & Lyne, 1956; Kollar, 1966; Bartosova et al. 1971; Frater & Whitmore, 1973; Frater, 1980). However, the ability of the skin plugs to sustain prolonged hair growth decreased with both time in culture and increasing age of the embryo from which skin was removed, so that by birth no in vitro hair growth could be observed. This renders these models unsuitable for studies on the control of the hair growth cycle; furthermore, these in vitro studies did not permit detailed biochemical studies on individual follicles.

Previous studies on isolated maintained hair follicles have been limited to the following: (1) Uzuka (1977) microdissected mouse hair follicles and measured the change in follicle bulb length by time-lapse cinematography, and he observed elongation of the hair root over a 10-h period and also measured incorporation of [³H]thymidine and [¹⁴C]leucine into hair follicle cells. (2) Frater (1980) microdissected rat hair follicles and studied changes in their morphology over 2 days, observing an increase in cellular keratinization in follicles maintained in medium containing rat serum as opposed to foetal calf serum, but did not expand these studies; and (3) Rogers et al. (1987) isolated mouse hair follicles by collagenase digestion and maintained them in collagen matrices for 7 days. The follicles lost their morphology, forming unrecognizable cell aggregates, which may have been a consequence of the disaggregation caused by the collagenase.

We now report on the maintenance of newborn rat hair follicles isolated by shearing (Green et al. 1986). This is a technique derived from the collagenase digestion of skin (Kealey, 1983), which isolates all of the skin glands (Lee et al. 1984; Kealey et al. 1986). For the hair follicle, shearing effects the separation of the follicle from the surrounding dermis through the connective tissue sheath, which surrounds the follicle, and yields large numbers of viable intact follicles (Green et al. 1986).

We have now maintained isolated hair follicles for 7 days on permeable supports. They retain their viability as determined by: (1) ATP, ADP and AMP contents; (2) their rates of DNA and protein synthesis; (3) [methyl-³H]thymidine autoradiography; and (4) their pattern of keratin synthesis. However, during organ maintenance the follicles develop morphological features that resemble a telogen follicle rather than an anagen follicle.
changes that resemble those seen during the in vivo transition from anagen to telogen, which indicates that organ maintenance may prove useful in the study of this aspect of the hair cycle.

Materials and methods

Materials

Williams E medium, L-glutamine, Fungizone, penicillin and streptomycin were supplied by Gibco, all other tissue culture supplements came from Sigma. Polycarbonate filters were supplied by the Nucleopore corporation. All radiochemicals were from Amersham, GF/C filters came from Whatman. The ATP monitoring kits were supplied by LKB Instruments Ltd, pyruvate kinase and adenylate kinase came from Boehrmger and ATP + AMP 20 units ml⁻¹ pyruvate kinase and 150 units ml⁻¹ of adenylate kinase. Samples were incubated for 30 min at 37°C after which 90 μl of 0.1 M-Tris-acetate, pH 7.75, was added and the samples were assayed for ATP content, using a LKB 1250 luminometer.

Adenine nucleotides

These were measured using an adaptation of the method of Spielman et al. (1981), based on the luciferin-luciferase assay of ATP (Stanley & Williams, 1969). Hair follicles were placed in 500 μl of Williams E medium to which was added 100 μl of 20 % PCA, and 20 μl of sample was removed and neutralized with KOH. For the determination of ATP, 10 μl of sample was incubated with 10 μl of reaction buffer (0-3 mM-phosphoenol pyruvate, 9 mM-MgCl₂, 5 mM-KCl, 0-1 M-Tris-acetate, pH 7-75). For the determination of ATP + ADP the reaction buffer contained 20 units ml⁻¹ pyruvate kinase and 150 units ml⁻¹ of adenylate kinase. Samples were incubated for 30 min at 37°C after which 90 μl of 0.1 M-Tris-acetate, pH 7-75, was added and the samples were assayed for ATP content, using a LKB 1250 luminometer.

Microscopy

For light microscopy freshly isolated and maintained hair follicles were fixed for 1 h in 3 % glutaraldehyde and then mounted in 5 % agar to assist handling during subsequent processing. The agar blocks were fixed overnight in 3 % glutaraldehyde and washed three times in PBS. The follicles were then dehydrated in an ascending series of ethanol grades and processed into Historesin (LKB) using three changes over 48 h. The resin was polymerized at 60°C for 48 h. Sections (1-5 μm) were cut and mounted on subbed slides and stained with 0-1 % Toluidine Blue in 0-1 % NaHCO₃. For transmission electron microscopy (TEM) and scanning electron microscopy (SEM) the samples were processed as described by Green et al. (1986).

Authoradiography

Hair follicles were incubated for 6 h with 5 μCi of [methyl-³H]thymidine as described above. After three washes in PBS the follicles were fixed, mounted in 3 % agar and processed as described for light microscopy. Autoradiography was carried out using K2 dipping emulsion (Ilford) as described by Green et al. (1983).

Results

Microscopy

Freshly isolated hair follicles from the skin of 8-day-old rats observed in cross-section (Fig. 1) are shown to have
Fig. 1. Cross-section through an 8-day-old freshly isolated hair follicle showing dermal papilla (dp), hair cortex (c), inner root sheath (irs) and outer root sheath (ors). X250.

been isolated intact and that the following structures can be seen: dermal papilla (dp), hair cortex (c), inner root sheath (irs), and outer root sheath (ors). The ultrastructure of freshly isolated follicles when studied by TEM (Fig. 2) shows that they have been isolated with no apparent structural damage. Fig. 2 shows the ors region of the follicle bounded by an intact basal lamina (bl), adjacent to which can be seen longitudinally and horizontally arranged orthogonal collagen fibres (oc).

SEM shows that after 7 days of organ maintenance on Nucleopore filters (Fig. 3) the overall morphology of the follicle has been maintained, although some cells can be seen explanted from the follicle in the region of the follicle bulb and ors (arrows). Sections studied by light microscopy in Figs 4 and 5 show that considerable changes in follicle morphology have occurred when compared with fresh follicles. In cross-section it can be seen that the majority of the matrix cells have keratinized forming a club-like structure (kb), surrounded by a capsule (ec) of non-keratinized epithelial cells that have probably originated from the ors (Fig. 5). The dermal papilla, which in the freshly isolated follicles forms an elongated structure surrounded by matrix cells, has been transformed to a small spherical cluster of cells situated below the capsule of epithelial cells.

When observed by TEM (Figs 6 and 7) considerable changes in the ors region of the follicles can be seen when compared with that of freshly isolated follicles. The bl appears plicated as do the cell margins of the ors cells, and the cytoplasm of these cells contains numerous filaments. Adjacent to the bl considerable cell debris can be seen, the characteristic orientation of the oc fibres seen in fresh follicles has been lost and instead the fibres are of mixed orientation. These light and electron microscopic obser-
Fig. 3. SEM of maintained follicle on permeable support showing overall maintenance of follicle morphology with some cells explanting from the outer root sheath and bulb (arrows). ×120.

Fig. 4. Cross-section of 7-day maintained follicle showing keratinized bulb (kb), capsule of non-keratinized epithelial cells (ec) and spherical dermal papilla (dp) situated beneath the keratinized bulb. ×250.

Vations made on the morphology of maintained hair follicles are characteristic of those made in vivo on hair follicles during the late catagen and telogen stages of their growth cycle (Sugiyama et al. 1976).

Adenine nucleotides

The adenine nucleotide contents of freshly isolated and maintained hair follicles are shown in Table 1. The energy charge of freshly isolated hair follicles was 0.75 and after 7 days of organ maintenance the value was 0.77, which indicates that the follicles retain their metabolic viability in vitro (Beiss & Newsholme, 1975; Iles et al. 1979).

Rates of DNA and protein synthesis

Rates of DNA and protein synthesis were studied in Table 1.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Freshly isolated pmol/20 hair follicles (mean ± S.E.M.; n = 6)</th>
<th>Maintained for 7 days on Nucleopore filters (mean ± S.E.M.; n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>203 ± 18</td>
<td>281 ± 19</td>
</tr>
<tr>
<td>ADP</td>
<td>139 ± 13</td>
<td>168 ± 19</td>
</tr>
<tr>
<td>AMP</td>
<td>23 ± 2</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>Total</td>
<td>365 ± 16</td>
<td>479 ± 27*</td>
</tr>
<tr>
<td>Energy charge</td>
<td>0.75</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Hair follicles were assayed as described in the text, all experiments were carried out in duplicate. The energy charge was calculated from \([\text{ATP}]+0.5[\text{ADP}]/[\text{ATP}]+[\text{ADP}]+[\text{AMP}]\) as defined by Atkinson (1968).

*Total adenine nucleotide content in maintained follicles differed significantly from fresh follicles (\(P<0.05\)), as determined by Student's \(t\)-test.

Table 1. The adenine nucleotide contents of freshly isolated and maintained rat hair follicles

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fresly isolated and maintained hair follicles by measuring the rates of incorporation of [methyl-3H]thymidine and [U-14C]leucine, respectively, into PCA-precipitable material.

Measurements were made on anagen hair follicles isolated from newborn rats aged 8, 10, 15, and 17 days to determine whether the rates of incorporation remained similar throughout anagen or whether a decrease in rate could be observed in late anagen follicles prior to their entry into catagen and the subsequent formation of the telogen follicle. The results of these studies are shown in Table 2. Follicles isolated from 8-day-old rat skin showed rates of DNA synthesis of 0.24 pmol of thymidine incorporated µg⁻¹ DNA h⁻¹; this value increased significantly to 0.36 pmol µg⁻¹ DNA h⁻¹ for follicles from 15-day-old rats (P < 0.001), which was followed by a significant decrease in the rate of incorporation to 0.12 pmol thymidine µg⁻¹ DNA h⁻¹ at 17 days (P < 0.001). The rates of

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**Fig. 5.** Longitudinal section of maintained follicle showing keratinized bulb (kb), large epithelial cells originating from the ors situated around the keratinized bulb (ec) and stratification of cells of the outer root sheath (ors). ×250.

**Fig. 6.** TEM showing outer root sheath region of maintained follicle with highly convoluted basal lamina (bl), pronounced mutual invaginations of the cell margins (i), cell debris adjacent to the basal lamina (c), orthogonal collagen (oc). ×6500.
protein synthesis showed no significant change between 8 and 15 days, but by 17 days had fallen significantly to 19.0 pmol leucine μg⁻¹ DNA h⁻¹ (P < 0.001).

Measurements were also made on hair follicles isolated from 8- and 10-day-old rats after 7 days organ maintenance (Table 2). This was performed to compare the rates of synthesis in vitro with those observed in the freshly isolated follicles from rats aged 15 and 17 days, to see

Fig. 7. High-power TEM showing plicated basal lamina (bl) and orthogonal collagen (oc). ×50,000.

Table 2. Rate of [methyl-³H]thymidine and [U-¹⁴C]leucine uptake by freshly isolated and 7-day-maintained rat hair follicles

<table>
<thead>
<tr>
<th>Follicles</th>
<th>DNA content (µg DNA/20 hair follicles) (mean ± s.e.m.; n = 6)</th>
<th>[methyl-³H]thymidine (pmol µg⁻¹ DNA h⁻¹) (mean ± s.e.m.; n = 6)</th>
<th>[U-¹⁴C]leucine (pmol µg⁻¹ DNA h⁻¹) (mean ± s.e.m.; n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-day fresh</td>
<td>1.93 ± 0.13</td>
<td>0.24 ± 0.013</td>
<td>79.0 ± 0.9</td>
</tr>
<tr>
<td>10-day fresh</td>
<td>1.98 ± 0.13</td>
<td>0.31 ± 0.054</td>
<td>89.0 ± 0.12</td>
</tr>
<tr>
<td>15-day fresh</td>
<td>2.00 ± 0.12</td>
<td>0.30 ± 0.024***</td>
<td>72.0 ± 0.7</td>
</tr>
<tr>
<td>17-day fresh</td>
<td>1.56 ± 0.17</td>
<td>0.15 ± 0.035</td>
<td>19.0 ± 2.0***</td>
</tr>
<tr>
<td>8-day fresh, maintained for 7 days</td>
<td>1.47 ± 0.13**†</td>
<td>0.12 ± 0.026***††††††</td>
<td>35.0 ± 8.0††††</td>
</tr>
<tr>
<td>10-day fresh, maintained for 7 days</td>
<td>1.35 ± 0.19‡</td>
<td>0.096 ± 0.021‡‡‡‡‡‡‡‡‡‡‡</td>
<td>9.0 ± 2.0‡‡‡‡‡‡‡‡‡‡‡</td>
</tr>
</tbody>
</table>

Experiments were carried out as described in the text, each observation was made over a time course of 1, 3 and 6 h during which time uptake was linear, with 20 follicles in duplicate being used for each time point (720 follicles used for each observation).

* Significant difference from 8-day-old fresh follicles (*P < 0.05; **P < 0.01; ***P < 0.001).
† Significant difference between 8-day fresh follicles maintained for 7 days and 15-day fresh (†P < 0.05; ††P < 0.01; †††P < 0.001).
‡ Significant difference between 10-day fresh follicles and after 7 days maintenance (‡P < 0.05; §§P < 0.01; §§§P < 0.001).
§ Significant difference between 10-day fresh follicles maintained for 7 days and 17-day fresh follicles (§P < 0.05; §§§P < 0.01); as determined by Student's t-test.
Fig. 8. Autoradiograph of 8-day-old fresh follicle showing uptake of tritiated thymidine in epithelial cells adjacent to the dermal papilla (dp); and B, after 7 days of organ maintenance showing the keratinized hair (h), with uptake, which is reduced, restricted to cells explanted from the outer root sheath (ors). ×200.

whether the decrease in the rate of incorporation observed in vivo between 15 and 17 days would also occur in vitro. Table 2 shows that the rate of DNA and protein synthesis in follicles from 8-day-old rats maintained for 7 days, which are the same age as follicles freshly isolated from 15-day-old rats, in fact show greater similarity to 17-day-old-rat hair follicles. This would indicate that in vitro there is a premature decrease in the rate of incorporation of both [methyl-3H]thymidine and [U-14C]leucine.

 Autoradiography
The viability and pattern of cell division in freshly isolated and maintained hair follicles was investigated by [methyl-3H]thymidine autoradiography. The results of this study are shown in Fig. 8A and B. Freshly isolated follicles were shown to incorporate thymidine into the epithelial cells adjacent to the dermal papilla. This observation is compatible with the current understanding of cell division in the anagen follicle whereby active mitotic division occurs in the cells lying in the lower region of the bulb adjacent to the dermal papilla, giving rise to cells that eventually form the matrix, medulla, cortex and irs of the follicle (Butcher, 1951).

In follicles maintained for 7 days, autoradiography shows that very little thymidine uptake occurs, supporting the observations made above on the rates of [methyl-3H]thymidine uptake. Uptake that does occur appears to be in the cells originating from the outer root sheath of the follicle and is probably a result of cells explanting in this region.

Keratin synthesis
The pattern of keratin synthesis in freshly isolated and maintained hair follicles was studied by incubating follicles with [35S]methionine for 24 h at 37°C, after which cell extracts were separated by SDS–acrylamide gel electrophoresis and analysed by autoradiography.

The pattern of keratin synthesis in freshly isolated 8-day-old follicles is shown in Fig. 9 (lanes 4 and 5), major labelled bands were observed at 52, 48, 44, 40 and 37K (K = 10^3 M). Other bands were observed at 120, 79 and 63K, a number of bands were also observed between 16 and 28K. Control experiments using immunoblot analysis of gels with a broad-spectrum keratin antibody identified bands at 60, 52, 48 and 40K as keratins (not shown).

After 7 days of organ maintenance the pattern of labelling is similar to that of fresh follicles, except for the
loss in vitro of the 40K doublet. This loss is observed in both 8- and 10-day-old follicles maintained for 7 days (Fig. 9, lanes 1, 2, 3 and 6, 7, respectively). The loss of this band is also observed in 17-day-old fresh follicles (lane 8). These is, therefore, a similarity in the pattern of labelling seen in hair follicles maintained in vitro to that of freshly isolated late anagen follicles.

Discussion

We have already shown that shearing can be used to yield large numbers of viable intact hair follicles (Green et al. 1986). We now show that these follicles can be viably maintained on permeable supports for 7 days in vitro as determined by their adenine nucleotide contents, rates of [methyl-3H]thymidine and [U-14C]leucine uptake, [methyl-3H]thymidine autoradiography, patterns of keratin synthesis and light and electron microscopy. These studies also show that after 7 days of maintenance the morphology of maintained follicles shows a closer resemblance to the telogen than to the anagen follicle.

We observe by light microscopy that after 7 days of maintenance the majority of cells within the hair bulb keratinize and that this keratinized mass of cells is surrounded by a non-keratinized capsule of epithelial cells originating from the ors (Fig. 6). The dermal papilla, which in freshly isolated hair follicles forms an elongated structure situated within the hair bulb, after 7 days maintenance, forms a spherical structure situated beneath the keratinized bulb adjacent to the epithelial capsule.

In vivo, the transition from anagen to telogen follicle is marked by a cessation of mitotic cell division within the follicle bulb (Parakkal, 1969a). Cells within the bulb that are already committed to differentiate continue to do so, migrating upwards and eventually leading to the formation of a keratinized club hair (Butcher, 1951; Parakkal, 1969a; Straile, 1961). As a result of this migration, the size of the follicle is reduced, a capsule of epithelial cells originating from the ors forms around the keratinized bulb, and the dermal papilla condenses to form a spherical structure attached to the capsule by an epithelial column (Straile, 1961). The cells of this column then retract and degenerate, resulting in an upward
movement of the dermal papilla to a resting position just below the capsule, forming the resting or telogen hair follicle, the morphology of which bears a marked resemblance to that of our maintained follicles.

Electron microscopic examination of the ultrastructure of maintained follicles also shows that considerable structural changes have occurred. The folding of the plasma membrane of outer root sheath cells, and of the basal lamina are typical features seen during the transition from anagen to telogen; however, in vitro these changes are more pronounced (Parakkal, 1969a,b; De Weert et al. 1982), and arise as a direct result of the formation of the club hair, which results in a shortening of the hair follicle to one third of the overall length of the anagen bulb (Parakkal, 1969a). In our study there was no obvious decrease in the length of the follicle during maintenance.

The cell debris found adjacent to the basal lamina in vitro probably originates from degenerating epithelial cells in the hair bulb (Sugiyama et al. 1976; De Weert et al. 1982), or from outer root sheath cells that are modified during catagen to form the epithelial capsule surrounding the club hair in telogen (Parakkal, 1969a). In vivo, this debris along with degenerating collagen fibres is engulfed by macrophages and is therefore absent in electron micrographic studies of telogen follicles (Roth, 1965). The presence of this cell debris in our study may be explained by the absence of macrophages in our in vitro model.

The pattern of keratin synthesis was studied by incubating hair follicles with [35S]methionine; following low- and high-salt extraction the insoluble pellet was analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. Major labelled proteins were identified at 52, 48, 44 and 40K, immunoblot analysis confirmed that bands at 52, 48 and 40K were keratins. The 44K labelled protein was not identified by our antibody but may still be a keratin.

Our studies have shown that the pattern of keratin synthesis is similar in freshly isolated hair follicles from 8-, 10-, 15- and 17-day-old rats except that in 17-day-old hair follicles the 40K band is absent. It was also observed that the pattern of keratin synthesis in freshly isolated and maintained follicles was similar. It was observed that in maintained follicles, as in 17-day-old fresh follicles, the 40K band was absent. The presence of the 40K keratin has been reported in less-differentiated epidermal cells (Eichner, 1986). Its loss, therefore, in 17-day-old and maintained follicles may be a result of the increased differentiation occurring in these tissues.

Our observations on the patterns of keratin synthesis in isolated hair follicles represent a preliminary study and the overall pattern of keratins expressed is likely to be more complex and will need to be investigated using two-dimensional gels, and immunoblot analysis using specific monoclonal antibodies such as the AE1 and AE3 antibodies described by Eichner et al. (1984).

Rates of [methyl-2H]thymidine uptake measured in freshly isolated hair follicles show cyclical changes, with the rate of uptake increasing from 0-009 ± 0-019 pmol μg−1 DNA h−1 (mean ± S.E.M.) in follicles from 8-day-old rats to 0-36 ± 0-024 pmol at 15 days; this was followed by a rapid decrease to 0-15 ± 0-035 pmol in follicles from 17-day-old rats. Similarly, the rates of [U-14C]leucine uptake also showed a cyclical trend, with the rate of uptake increasing from 79-0 ± 9-0 pmol μg−1 DNA h−1 (mean ± S.E.M.) in 8-day-old rats to 89-0 ± 12-0 pmol at 10 days followed by a decrease to 19-0 ± 2-0 pmol at 17 days. Such a marked decrease can be explained when it is considered that freshly isolated anagen follicles contain populations of rapidly dividing cells, and regions of differentiation where keratinized hair and outer root sheath are produced (Straile, 1961). With the onset of catagen, mitotic cell division in the hair matrix stops. Although cells committed to differentiation continue to do so, it would be expected that an overall decrease in the rate of biosynthesis within the follicle would occur, as shown by our observed decrease in the rates of thymidine and leucine uptake. In the rat the onset of catagen usually occurs around day 17 to day 21 in the first hair growth cycle and this would explain the decrease in the rates of [methyl-3H]thymidine and [U-14C]leucine uptake observed in 17-day-old fresh follicles. This study is the first in which such observations have been made on isolated hair follicles. These observations are supported by the in vitro studies of Moffat (1974), who has shown, using autoradiography, a marked decrease in the levels of DNA and RNA synthesis in murine hair follicles by day 17 of their growth cycle, which values falling to zero by day 18.

Our observations on maintained follicles show that after 7 days of maintenance rates of [methyl-3H]thymidine and [U-14C]leucine uptake by 8- and 10-day-old follicles have fallen significantly when compared with their fresh values. This observation and those made on the morphology of maintained hair follicles by light and electron microscopy lead us to conclude that in vitro hair follicles show premature entry into catagen. This gives rise to the formation of a residual structure resembling that of the telogen hair follicle.

The catagen stage of the hair growth cycle results in an orderly transition from the actively growing anagen follicle to a resting state that under the correct stimuli is capable of regenerating a new anagen hair follicle. The reason for the premature entry into catagen is unknown and may reflect a lack of specific growth factors in the culture medium. Another possible explanation is that the follicles are responding to the stress of isolation by entering catagen. It is known that when hair follicles in vitro are exposed to stress they respond by premature entry into catagen and then telogen (Parakkal, 1969a; Mehregan, 1979); this phenomenon is believed to be a form of defence mechanism whereby the follicle enters an embryonic state capable of regeneration.

We therefore conclude that hair follicles maintained in vitro show premature entry into a degradative catagen-like state, and that this is the reason for the failure of previous attempts at growing hair in vitro.

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References


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