

## Cutaneous Biology

# Human follicular stem cells: their presence in plucked hair and follicular cell culture

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### Summary

**Background and objectives** A considerable portion of the hair follicle remains attached to plucked hair and can be used for follicle cell culture. In this study we have phenotyped these cells in an attempt to identify the stem cell fraction. Reports in the literature have indicated that this cell population may be positive for cytokeratin (CK) 19. Because stem cells in general need to be protected from apoptosis, the presence of the apoptosis-suppressing Bcl-2 protein, together with the absence of the apoptosis-promoting Bax and the CK profile may be used as an indicator of the stem cell population in the hair follicle, and in cultures of hair follicle cells.

**Methods** Hair follicles from skin biopsies and plucked hair were derived from the scalps of healthy volunteers. Follicular cells were cultured from the plucked hairs. These hair follicles, plucked hairs and cultured cells were examined for their CK profiles, which are indicative of the type of cell (basal/stem cells) and for their status with respect to the proliferation marker Ki-67, Bax and Bcl-2.

**Results** We found coexpression for CK19 and Bcl-2, but not Bax in two distinct areas, localized in the upper and lower third of the follicle from both skin biopsies and plucked hairs, while proliferation markers were negative in these areas. CK19 and Bcl-2 were also coexpressed in combination in a fraction of the follicular cell culture. The skin basal cell marker CK14 could be found throughout the outer root sheath of the hair follicle from both skin biopsies and plucked hairs, as well as in the follicular cell culture.

**Conclusions** Thus, CK19/Bcl-2-positive and Bax-negative cells can be obtained from cells derived from plucked hair and are retained in cultures made from these cells. If this phenotype represents follicular stem cells, our finding endorses the assumption that stem cells are located in the bulge area of the hair follicle, as we did not find them in or near the dermal papilla.

**Key words:** cell culture, hair, plucked, stem cells

Although a considerable portion of the hair follicle is attached to plucked hair, transplantation of a plucked hair does not result in normal hair growth,<sup>1</sup> in contrast to hair from micropunch grafts.<sup>2</sup> This can

be explained by the fact that the structures of the hair follicle which are retained in the skin after the hair is plucked are necessary for normal hair growth. However, when outer root sheath (ORS) cells derived from plucked hairs are cultured, they can develop into a differentiated epidermis,<sup>3</sup> suitable for use in skin grafts.<sup>4</sup> We therefore speculated that the plucked hairs themselves may contain epidermal stem cells.

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When plucked hair is dissected, the various transverse sections have been shown to exhibit different proliferative and differentiated characteristics.<sup>5</sup> It has been suggested that adult human follicular stem cells from terminal hairs are situated in the bulge area of the follicle. These cells have been suggested to exhibit various specific biochemical properties. For example, they have epidermal growth factor (EGF) receptors, show  $\alpha_2\beta_1$ - and  $\alpha_3\beta_1$ -integrin expression,<sup>6</sup> high levels of  $\alpha_6$ -integrins and low levels of the proliferation marker 10G7,<sup>7</sup> and stain positively for platelet-derived growth factor (PDGF)-A/PDGF-B ligand chains. They do not contain nectadrin, or heat-stable antigen (CD24), a glycoprotein thought to be involved in cell-cell adhesion and signalling, which is also expressed in the outer epithelial sheath of human hair follicles and in glabrous epidermis.<sup>8,9</sup> However, the CK apoptosis resistance profiles seem to have become key indicators of a stem cell phenotype.

During development into a terminal hair, the CK profile of the follicular keratinocyte changes. It has been proposed that CK19 is an indicator of the stem cell population.<sup>10-13</sup> CK19 is present in immature epithelial progenitor cells,<sup>14</sup> but in the hair follicle it is specific for follicular stem cells.<sup>10,11</sup> In adult hair follicles, CK19 can be found in the outermost cells of the ORS at the isthmus and in some cells of the lower ORS.<sup>10</sup> It is proposed that the actual follicular stem cells are CK19-positive and lack connexin (Cx)43, a specific differentiation marker for a gap junction protein.

A balance between cell proliferation, differentiation and apoptosis is essential for hair growth,<sup>15,16</sup> while stem cells must be protected against apoptosis. This protection is achieved by proteins such as Bcl-2, while Bax, a conserved homologue that heterodimerizes with Bcl-2, promotes cell death.<sup>17</sup> The characteristics of the CK profile, in particular the expression of CK19, but also CK5 and CK14 as basal cell markers,<sup>11</sup> together with the expression of Bcl-2 and absence of Bax expression, may therefore be used as indicators of stem cells in the hair follicle.

The aim of this study was to localize the follicular stem cells in the hair follicle from skin biopsies and to characterize them on the basis of their CK phenotype, presence of Bcl-2, and absence of Bax. Furthermore, we wished to determine whether these cells are present in plucked hair and preserved in cell cultures derived therefrom.

## Materials and methods

### *Skin biopsies*

Five healthy volunteers, three males and two females between the ages of 28 and 53 years (mean age 37 years) donated skin biopsies.

### *Biopsies*

Four 3-mm punch biopsies were obtained from the occipital area of the scalp after local anaesthesia with lidocaine 2%. The tissue samples were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use. Samples were then cut into 5- $\mu\text{m}$  thick sections in a vertical direction and carefully placed on Superfrost plus slides (Menzel-Glaser, Braunschweig, Germany).

### *Plucked hair*

Plucked hairs were obtained by removing the hairs with a depilation forceps from the occipital area of the scalp. Hair follicles in the anagen phase were selected under a dissection microscope, embedded in Tissue Tek (Sakura Finetek Europe BV, Zoeterwoude, the Netherlands), and directly cut into 5- $\mu\text{m}$  sections as described above.

### *Cell cultures*

Plucked hairs were placed in a Petri dish with defined serum-free keratinocyte growth medium (dSFk; Life Technologies B.V., Breda, the Netherlands). The non-viable, keratinized part of the hair follicle was removed under a dissection microscope. The hair follicles were subsequently put in a sterile culture dish and incubated in dSFk containing 20 U mL<sup>-1</sup> dispase (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) for 30 min in a CO<sub>2</sub> incubator at 37 °C. After this preincubation step, the hair follicles were transferred to a 24-well culture dish containing dSFk with 500  $\mu\text{g mL}^{-1}$  penicillin (Life Technologies B.V.) and 0.25  $\mu\text{g mL}^{-1}$  streptomycin (Life Technologies B.V.), and placed for 14 days at 31 °C (as suggested previously by Imai *et al.*<sup>18</sup>) in a humidified atmosphere containing 5% CO<sub>2</sub>. The culture medium was carefully removed every 3 days and replaced by fresh culture medium. The cells remained attached to the hair follicles during this culture period. After 14 days

the culture medium was removed and replaced by a 0.5-mg mL<sup>-1</sup> trypsin, 0.2-mg mL<sup>-1</sup> EDTA (ethylenediamine tetraacetic acid) solution (Life Technologies BV), and incubated for 5 min at 37 °C in this medium. After this incubation period clusters of cells were released from the hair follicles. These were harvested by centrifugation at 300 g at 4 °C for 5 min in an Eppendorf 5804R Centrifuge (VWR International, the Netherlands). Subsequently the trypsin/EDTA medium was removed and replaced by culture medium. Cytospins were made from these cells in the Cytospin 3 (SHANDON, Zeist, the Netherlands) by centrifugation at 300 g for 5 min.

These cells were immunostained as described below. Because the number of cells obtained in these cultures was very limited, reliable quantification of the immunopositive cells could not be performed. Therefore, wherever possible, an indication of the percentage of positive cells is provided.

#### *Immunocytochemistry and antibodies*

The sections and cytopins were dried at room temperature for at least 1 h, fixed in acetone (at -20 °C) and processed for immunohistochemical staining.

For the single immunostaining procedure, the slides were incubated overnight at 4 °C with the primary antibodies. All incubations were carried out in phosphate-buffered saline (PBS) pH 7.4 at the appropriate dilution (Table 1). The following day the slides were

washed with PBS and incubated at room temperature for 30 min with the appropriate immunofluorescent-labelled secondary antibody (Table 1). After extensive washing, the slides were mounted with a 4',6-diamidino-2-phenylindole (DAPI)-containing mounting agent (Vector Laboratories Inc., Burlingame, CA, USA) and stored at -20 °C.

For double-immunostaining, the primary antibodies were selected on the basis of their isotypes, or a combination of monoclonal (mouse source) and polyclonal (rabbit source) antibody was applied. The first primary antibody was incubated overnight at 4 °C and the next day, after washing, the second primary antibody was incubated at room temperature for 2 h. After extensive washing in PBS, a mix of appropriate secondary antibodies (obtained from Dako A/S, Glostrup, Denmark or ITK Diagnostics, Uithoorn, the Netherlands) was applied and incubated for 30 min at room temperature. Subsequently the slides were washed in PBS, mounted as described above, and stored at -20 °C.

Amplification of the signal was achieved by incubation with biotinylated goat-antirabbit (BIO-GAR) or biotinylated goat-antimouse (BIO-GAM) and avidin-biotin complex (Vectastain ABC kit, Vector Laboratories Inc.) after incubation with the primary antibody. Detection of peroxidase activity and simultaneous signal amplification was achieved by incubation with tetramethylrhodamine isothiocyanate (TRITC)-labelled tyramide or fluorescein isothiocyanate (FITC)-labelled tyramide.<sup>19</sup>

Antibody/clone	Antibody specificity	Dilution	Isotype monoclonals	Source/reference
<b>Basal cell markers</b>				
LL002	CK14	UD	Mouse IgG3	40
RCK107	CK14	UD	Mouse IgG1	41
RCK108	CK19	1 : 5	Mouse IgG1	42
LP2K	CK19	UD	Mouse IgG2b	43
<b>Proliferation markers</b>				
LL025	CK16	1 : 10	Mouse IgG1	41
MIB-1	Ki-67	1 : 25	Mouse IgG1	Immunotech-Coulter, Marseille, France
Polyclonal	Ki-67	1 : 100	Rabbit IgG	Dako A/S, Glostrup, Denmark
<b>Apoptosis markers</b>				
N-19 polyclonal	Bcl-2	1 : 200	Rabbit IgG	Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.
1 24	Bcl-2	1 : 80	Mouse IgG1	Dako A/S
PC66	Bax	1 : 750	Rabbit IgG	Oncogene Research Products, Cambridge, MA, U.S.A.

**Table 1.** Markers and protocol used for staining of the tissue, plucked hairs and cell culture

UD; undiluted culture supernatant.

## Results

### *Haematoxylin and eosin staining of skin biopsies and plucked hair*

Comparison of the hair follicles derived from skin biopsies and from plucked hairs using light microscopy revealed that most of the epithelial structures from the hair follicle remain attached to the plucked hair (Fig. 1).

#### *Basal cell markers: CK14 and CK19*

Cells positive for the basal cell markers CK14 (RCK107, LL002) were found in the basal cells throughout the whole length of the ORS of the hair follicle and in the basal cell layer of stratified epithelium of the epidermis, both in the skin biopsy samples and in the plucked hairs.

In the hair follicles from the skin biopsy, the basal keratinocyte cell marker CK19 (RCK108, LP2K) was found in the most peripheral cell layer in two distinct areas in the upper (Fig. 2A) and lower third (Fig. 2B) of

the ORS, but not in the epidermis. In the plucked hair, the same regions were found to be positive for CK19 (results not shown). In follicular cell cultures approximately one-fifth of the cells were CK19 positive. These CK19-positive cells were mostly found as cell clusters, as shown in Figure 3(B).

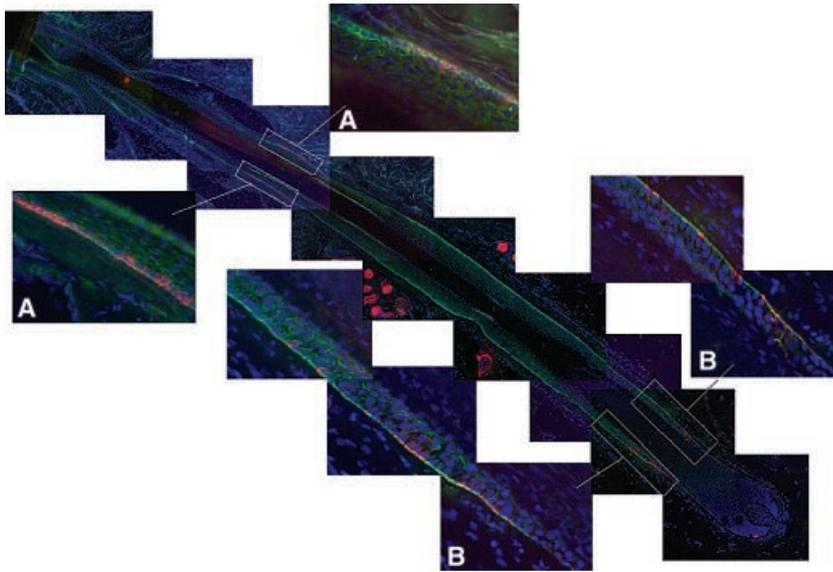
A complete overlap of CK19 (RCK108) and CK14 (LL002) was found in the biopsy samples and in plucked hair, and in approximately 40% of the cultured cells (not shown).

#### *(Anti)-apoptosis markers: Bcl-2 and Bax*

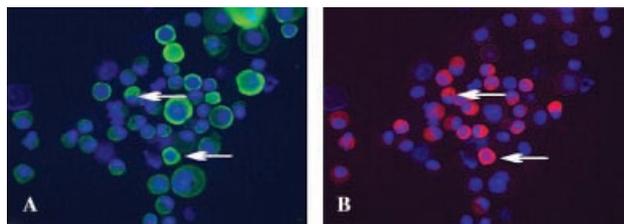
Bcl-2 (N-19)-positive cells were found in the outermost cell layer throughout the whole length of the ORS of the hair follicle from skin biopsies, as well as in the basal layer of the epidermis. We also found Bcl-2-positive cells in the most peripheral cell layer of the whole length of the ORS of the plucked hairs. Bcl-2-positive cells were not found in the dermal papilla. Double-staining for CK19 (LP2K) and Bcl-2 (N-19) revealed that CK19-positive cells were also positive for



**Figure 1.** Haematoxylin and eosin staining of plucked hair. Most epithelial structures from the hair follicle remain attached to the plucked hair. Adjacent to the longitudinal section of the plucked hair (original magnification  $\times 10$ ) the respective areas are shown at a higher magnification ( $\times 40$ ).



**Figure 2.** Longitudinally sectioned hair follicle from skin biopsies (original magnification  $\times 10$ ) double-stained for CK19 in red and Bcl-2 in green. Higher magnifications ( $\times 40$ ) of areas found to be positive for both markers are shown next to the respective areas of the hair follicle. Two areas were found to be positive for both markers, i.e. the upper third (A) and lower third (B) of the hair follicle. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).

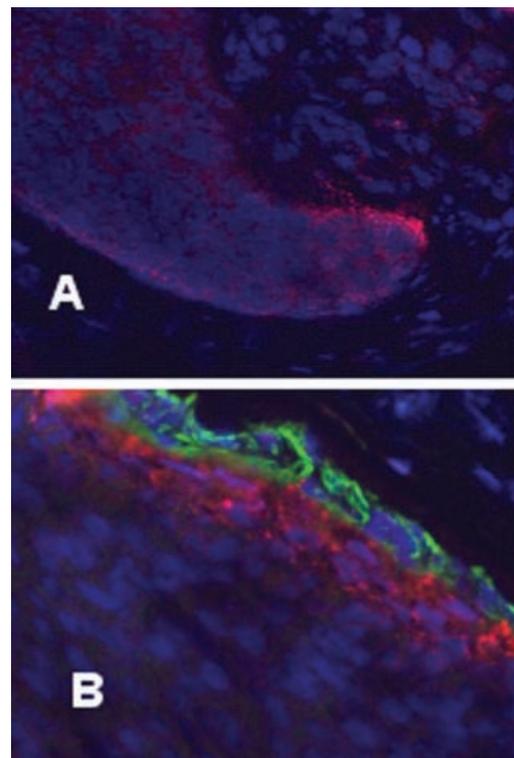


**Figure 3.** (A) Bcl-2 (green) and (B) CK19 (red) staining of cells, derived from plucked hair and cultured for 14 days. Next to cells that are positive for both markers (arrows), some cells are only Bcl-2- or CK19-positive. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).

Bcl-2. However, not all Bcl-2-positive cells were positive for CK19 (Fig. 2A,B).

In the follicular cell cultures subjected to double-staining techniques, some cells stained positive for both Bcl-2 and CK19, while others were positive for either Bcl-2 or CK19 (Fig. 3A,B). Figure 3(B) shows a typical cluster of CK19-positive cells, found in the cytoplasts of the cell cultures.

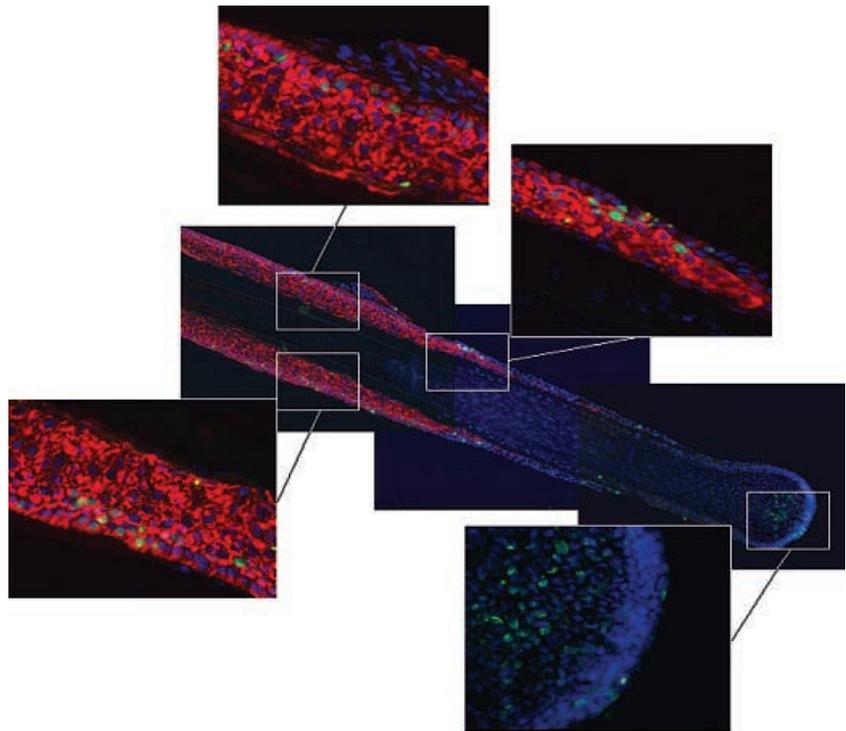
Bax (PC66)-positive cells were present in the internal part of the ORS both in the hair follicles from skin biopsies and in the plucked hair. Bax-positive cells were found in the dermal papilla in the hair follicles from skin biopsies (Fig. 4A) and in the most proximal part of the plucked hair (Fig. 4B). Almost no overlap ( $< 5\%$ ) between Bcl-2 (124) and Bax (PC66)-positive cells was found in either sample. However, in follicular cell cultures there was more overlap (30–40%) between Bcl-2- and Bax-positive cells (results not shown).



**Figure 4.** Details (original magnification  $\times 40$ ) from a longitudinally sectioned dermal papilla (A) and hair follicle (B) from skin biopsies double stained for Bax in red and Bcl-2 in green. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).

*(Hyper)proliferation markers CK16 and Ki-67*

CK16 (LL025)-positive cells were found in the distal internal part of the ORS of the hair follicles, both from

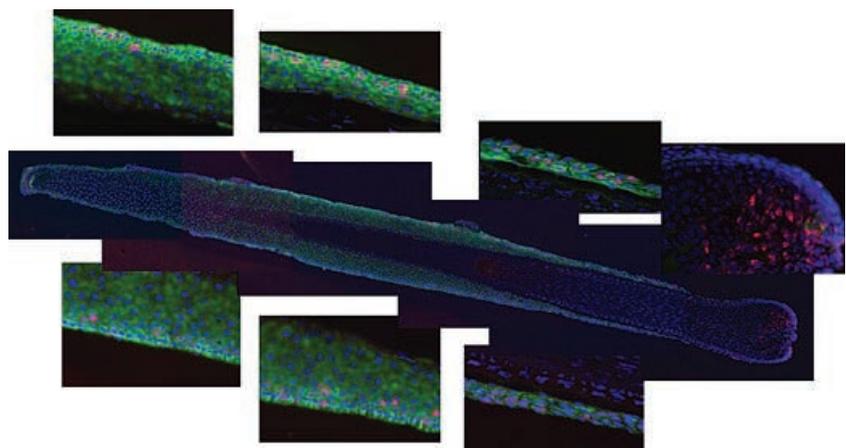


**Figure 5.** Longitudinally sectioned plucked hair (original magnification  $\times 10$ ) double stained for CK16 in red and Ki-67 in green. Higher magnifications ( $\times 40$ ) of areas found to be positive for both markers are shown next to the respective areas of the hair follicle. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).

skin biopsies and plucked hairs (Fig. 5). No CK16 staining was observed in the most proximal part of the hair follicles, either from skin biopsies or plucked hairs. CK16-positive cells were found in the internal part of the ORS, in contrast to CK19-positive cells whose position was more peripheral. There was no overlap between CK16 and CK19 staining in the hair follicles from skin biopsies, plucked hairs or follicular cell culture. There was an overlap in the immunostaining patterns of CK14 (LL002) and CK16 (LL025) in the ORS of the hair follicles from skin biopsies and in the

plucked hair, where CK16-positive cells were located. In follicular cell culture there was virtually a total overlap between CK14- and CK16-positive cells (not shown).

Ki-67-positive cells were sporadically found (Fig. 6). Their location tends to be more toward the internal part of the ORS, although some were found near the external part of the ORS near the CK19-positive cells. Ki-67-positive cells were also found in the dermal papilla (Fig. 5). Less than 1% of Ki-67-positive cells were found in follicular cell culture.



**Figure 6.** Longitudinally sectioned plucked hair (original magnification  $\times 10$ ) double-stained for Bcl-2 in green and Ki-67 in red. Higher magnifications ( $\times 40$ ) of areas found to be positive for both markers are shown next to the respective areas of the hair follicle. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).

## Discussion

Although plucked hair seems to contain all of the epithelial structures that are present in the hair follicle derived from skin biopsies, the transplantation of plucked hair does not result in normal hair growth, in contrast to hair-containing punch grafts.<sup>1</sup> The fact that the structures of the hair follicle remaining in the skin can produce a normal hair, suggests that follicular stem cells are retained in the skin. However, when hair follicle preparations derived from skin biopsies are compared with preparations from plucked hair under a light microscope, it can be seen that the majority of the epithelial structures from the hair follicle remains attached to the plucked hair. The question thus arises whether or not follicular stem cells are extracted with the plucked hair, and whether the stromal tissue surrounding these epithelial structures is necessary for induction of hair growth. This follicular connective tissue has specific biochemical characteristics, such as the presence of PDGF- $\alpha$  and PDGF- $\beta$  receptors and versican,<sup>20,21</sup> that seem to play a role in (embryonic) hair follicle development and cycling.

We based our search for the follicular stem cell population in plucked hair and cultures therefore on stem cell characteristics proposed in the recent literature,<sup>9</sup> i.e. the expression of CK19 and Bcl-2, in combination with the absence (or low levels) of Bax expression,<sup>22</sup> and an extremely low proliferation frequency.<sup>23</sup> In 1996, Michel *et al.*<sup>24</sup> suggested that CK19-positive cells in hair follicles represent stem cells. Furthermore, stem cells in general express Bcl-2.<sup>25,26</sup> In normal skin, Bcl-2 is only expressed by a limited population of cells in the basal compartment, which can be regarded as the stem cell compartment.<sup>27</sup>

An important finding in this study is that phenotypical characteristics of the hair follicle from skin biopsies are preserved in plucked hair, including the expression of CK19<sup>10</sup> and Bcl-2<sup>16</sup> in cells from two areas of the upper and lower third of the follicle. Positivity for CK19 and Bcl-2 corresponds to infrequent cell division in these areas, as concluded from the absence of Ki-67 staining. The fact that these cells are positive for Bcl-2 and CK19, but Ki-67 and Bax negative, is a strong indication that they represent stem cells in the hair follicle. Our results also indicate that transient amplifying cells in the epidermis, which are expected to be Ki-67 positive, are largely Bcl-2 negative. All the markers found in the hair follicle from skin biopsies were also preserved in the follicular cell culture derived from plucked hair, including CK19 and Bcl-2, and the

basal cell marker CK14. We therefore conclude that viable follicular stem cells can be obtained from plucked hairs. The low frequency of Ki-67-positive cells indicates that differentiation may have occurred in these cell cultures, which can also be concluded from the CK10 staining in a proportion of the cells (unpublished observation).

The observation that two distinct areas in the hair follicle from skin biopsies are positive for CK19 was also found by Commo *et al.*<sup>13</sup> The fact that these areas are also Bcl-2-positive, and Bax-negative, is a strong indication for two stem cell sites. The question therefore arises whether or not both areas are necessary for hair growth induction. In humans, Kim *et al.*<sup>28</sup> found that the proximal part of the hair follicle cannot regenerate into a differentiated hair follicle, but the distal part of the follicle can, eventually resulting in a fully developed hair follicle.<sup>29</sup> Reynolds *et al.*<sup>30</sup> found that, although the dermal papillae of humans cannot induce new hair growth, the sheath of the lower part of the hair follicle can. These apparently contradictory results indicate that both proximal and distal areas of the hair follicle can induce hair growth, which agrees with our finding of two stem cell locations.

In addition to these two areas, the dermal papilla has also been ascribed a key role in hair growth. In rats, cultured dermal papilla cells from whisker hairs can generate a fully differentiated hair follicle,<sup>31</sup> suggesting that follicular stem cells are located in or near the dermal papilla. The cells in this region that are held responsible for hair growth are the germ cells. Recently, a hypothesis of hair cycling was proposed that involves participation of these germ cells next to the bulge region stem cells.<sup>32</sup> In our study, the dermal papilla in the hair follicle of the skin biopsy did not contain Bcl-2. In contrast, other studies have found Bcl-2 positivity in the dermal papilla,<sup>16,33</sup> but the tissues used in these studies were either embryonic or derived from nonmelanoma skin cancers. The apparent discrepancy in findings could be due to the fact that, although Bcl-2 is normally found in the basal compartment of normal skin, there is an expansion of this Bcl-2-positive cell population under pathological circumstances such as basal cell carcinoma (BCC).<sup>27,34</sup>

In this study we found that the dermal papilla is positive for Ki-67 and Bax. This immunophenotype, in combination with the Bcl-2 negativity, is not in agreement with the characteristic definition of a follicular stem cell population, but rather supports the findings of others that follicular stem cells are not

present in the dermal papilla.<sup>15,35</sup> However, taking all these findings together suggests that hair growth involves the cooperation of various stem cell regions.

The inability to successfully transplant plucked hairs containing the two stem cell regions may be because of the lack of appropriate conditions in the stromal tissue of the recipient areas. A similar lack of growth has been observed in the case of transplanted epithelial structures derived from BCC, which could not be induced to proliferate without the transplantation of additional stromal cells.<sup>36</sup> This comparison is justifiable because BCC are most likely derived from follicular germinative cells.<sup>37,38</sup> Another indication of the importance of stromal cells for the growth of keratinocytes is the observation that keratinocyte cultures may require a fibroblast feeder layer.<sup>3</sup>

Unsuccessful regeneration after implantation of plucked hairs may also be caused by the inflammatory response of the receptor area to the plucked hairs, which may result in the destruction of the follicular stem cells. The connective tissue surrounding these epithelial structures in micropunch grafts may be crucial for protection against inflammation, and thus a successful transplantation. Therefore, the importance of the connective tissue of the hair follicle cannot be ignored.<sup>1,39</sup> Follow-up studies will focus on the role of components of the connective tissue and the extracellular matrix surrounding the epithelial portion of the hair follicle.

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