

# Hair Regeneration from Transected Follicles in Duplicative Surgery: Rate of Success and Cell Populations Involved

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**BACKGROUND** The use of bisected hair follicles in hair transplantation has been previously reported, but the capacity of each half to regenerate the entire hair has not been clarified.

**OBJECTIVE** To evaluate duplicative surgery rate of success and to analyze the cell populations involved in hair regeneration.

**METHODS** We screened 28 patients undergoing duplicative surgery. Approximately 100 hair follicles from each patient were horizontally bisected and implanted. Upper and lower portions were stained for the known epithelial stem cell markers CD200, p63,  $\beta$ 1-integrin, CD34, and K19.

**RESULTS** Similar percentages of hair regrowth after 12 months were observed when implanting the upper ( $72.7 \pm 0.4\%$ ) and lower ( $69.2 \pm 1.1\%$ ) portions. Expression of CD200, p63, and  $\beta$ 1-integrin was detected in both portions, whereas K19 and CD34 stained different cell populations in the upper and lower fragment, respectively.

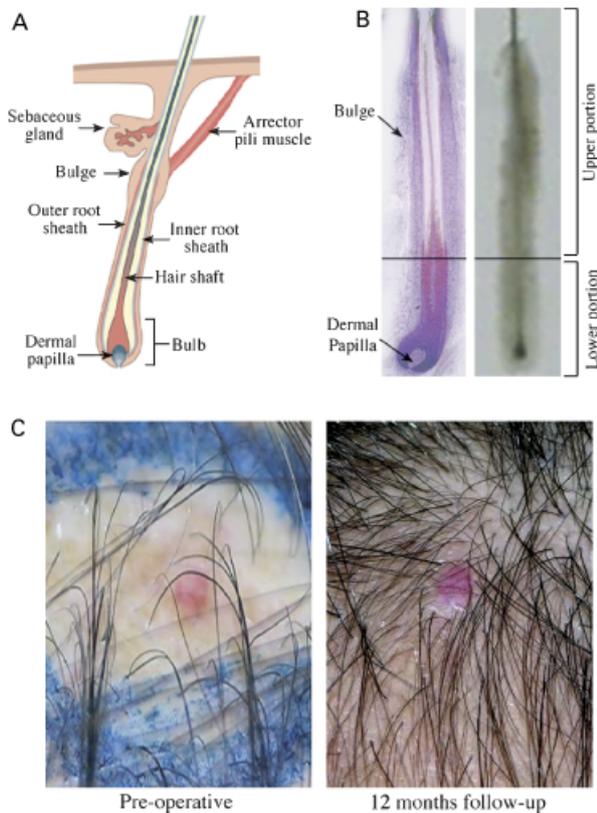
**CONCLUSION** Duplicative surgery might represent a successful alternative for hair transplantation, because both portions are capable of regenerating a healthy hair. Moreover, our results suggest the possible presence of stem cells in both halves of the follicle.

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The most widely used technique for hair transplantation is the implantation of individual hair follicle units (FUs), although the availability of donor hairs limits it. Attempts to create hairlines using bisected hair follicles have been previously reported.<sup>1-5</sup> This method is based on the transection of one FU into two growing follicles to regenerate new hairs. The general consensus shows 50% to 70% of success using this procedure,<sup>1</sup> but the biological basis of the capacity of each half to regenerate the entire hair has not been elucidated. Hair follicles consist of a shaft surrounded by concentric layers of epithelial cells, the inner (IRS) and outer (ORS) root sheaths, a mesenchymal layer surrounding the epithelial core, and a sebaceous gland, which is an outgrowth at the side of the hair germ (Figure 1A). Hair follicles have their own stem cell “niche” in a region of the ORS

known as bulge,<sup>6</sup> in which cells with slow cycling potentiality were identified as keratinocyte stem cells (KSCs).<sup>7</sup> Cross-talk between mesenchymal dermal papilla cells (DPCs) and KSCs initiate hair growth.<sup>8</sup> Such cross-talk is crucial for normal development of the hair follicle, as well as for hair cycling, because the multipotent KSCs are stimulated to proliferate and differentiate through interactions with the underlying mesenchymal DPCs.<sup>9</sup> Bulge stem cells are able to generate new follicles at each hair cycle<sup>10</sup> and exhibit a specific repertoire of cell-surface molecules.<sup>11-13</sup> Recently, many studies have been performed to characterize hair follicle stem cells, searching for the expression of various markers previously shown to be involved in hair follicle cycling, such as CD34, p63, cytokeratin 19 (K19),  $\beta$ 1-integrin, and CD200. The CD34 transmembrane

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**Figure 1.** (A) Schematic diagram of hair follicle structure showing the localization of the bulge region near the arrector pili muscle origin and of the dermal papilla within the hair bulb (modified from Fuchs<sup>26</sup>). (B) Longitudinal section of a hair follicle stained with hematoxylin and eosin. The black line indicates the cutting level, localized to one-third of the follicle length starting from the dermal papilla, which give rise to an upper portion containing the bulge region and a lower portion containing the papilla. (C) The transected follicles were implanted on the right and left side of a selected marker (angiomatous lesion), to facilitate recognition of the new hairs originating from the upper or lower portion of the same follicle. Hair regrowth was evaluated 3, 6 (not shown), and 12 months after transplantation.

protein has been found to be expressed in the ORS of mouse and human hair follicles<sup>14,15</sup> in cells whose proliferation contribute to form the lower part of the follicle.<sup>16,17</sup> Transcription factor p63 belongs to a family of structurally related proteins that includes the tumor suppressor proteins p53 and p73; it has been suggested that it is involved in the signalling pathways of the hair follicle cycle.<sup>18</sup> Some studies also indicate that K19, which has been found to be expressed in cells of the basal layer of epidermis as well as in the bulge area,<sup>19,20</sup> is a suitable marker for

epithelial stem cells of human hair follicles. We based the choice of  $\beta$ 1-integrin marker on evidence that  $\beta$ 1-integrin-positive cells show a high clonogenic potential<sup>21</sup> and that  $\beta$ 1-integrin mutant mice exhibit severe skin blistering and hair defects.<sup>22</sup> Moreover, cultures of isolated bulge cells have been shown to strongly express K19 and  $\beta$ 1-integrin.<sup>20</sup> More recently, the transmembrane protein CD200, a modulator of the immune response,<sup>23</sup> has been detected in the outermost layer of the ORS throughout the length of the mouse hair follicle<sup>24</sup> or localized in human bulge cells, suggesting a role of this molecule in affording immune privilege to KSCs.<sup>25</sup> Therefore, we believe that, in the present study, the use of these markers might represent an appropriate approach to assay the presence of cellular elements capable of regenerating the hair follicle.

In this report, we screened 28 patients undergoing hair duplicative surgery to evaluate the rate of success of this procedure. We analyzed the expression of the above-mentioned markers in both hair fragments. Subsequently, we cultured cells from upper and lower portions of the microdissected hairs to establish their *in vitro* behavior.

## Materials and Methods

The Ethical Committee of the University Sapienza of Rome approved the protocol, and written consent was obtained from each donor. Twenty-eight patients (24 men and 4 women) were enrolled. Approximately 100 hair follicles from each patient were horizontally sectioned under light microscope below the origin of the arrector pili muscle. The procedure was standardized by cutting all follicles at one-third of their length from the papilla. The two portions were implanted in androgenetic alopecia bald sites, choosing standardized areas to the right (upper) or left (lower) of selected markers, such as angiomatous lesions (8 patients), melanocytic nevi (14 patients), or little scars (6 patients). To selectively follow up the transplanted hairs, all the grafted areas were photographed before and after transplantation. The percentage of hair regrowth (%HR)

was evaluated as follows:  $\%HR = (y_1 - y_0/z) \times 100$ , where  $y_1$  = number of total hairs in a selected and oriented circular area of 1 cm diameter at the follow-up time (6 or 12 months),  $y_0$  = number of preexisting hairs in the same area at the time of transplantation,  $z$  = number of total FU (entire or transected hairs) transplanted in the same area. At the 12-month follow-up, 20 hair specimens, cut close to the skin surface, were collected from each group, and their diameter was measured using a microscope equipped with an ocular micrometer. The percentage of hair diameter ( $\%HD$ ) was evaluated as follows:  $\%HD = (x_r/x_d) \times 100$ , where  $x_r$  = diameter in  $\mu\text{m}$  of regrowth hairs and  $x_d$  = diameter in  $\mu\text{m}$  of donor hairs. Hair fragments were assayed for the expression of specific markers using immunohistochemistry (IHC) or immunofluorescence (IFA).

For IHC, frozen sections (3–5  $\mu\text{m}$ ), obtained using a cryomicrotome (Microm HM 505N, Thermo Fisher Scientific Inc., Waltham, MA), were fixed in cold absolute methanol for 4 minutes; endogenous peroxidase activities were blocked using 0.03% hydrogen peroxide for 5 minutes; and sections were incubated for 1 hour at room temperature with anti-human CD200 (BD Biosciences Pharmingen, Bedford, MA), CD34, K19, p63, and  $\beta$ 1-integrin (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:100 in phosphate buffered saline; PBS). Sections were then processed using avidin-biotin-peroxidase complex (Dako, Carpinteria, CA), counterstained with hematoxylin and permanently mounted under a coverslip. For anti-CD34 detection, antigen retrieval was achieved by heating sections in 10 mM citrate buffer, pH 6, in a microwave for 15 minutes before endogenous peroxidase blocking. Control sections were prepared by omitting the primary antibody from the immunohistochemical procedure.

For IFA, frozen sections were incubated with the same primary antibodies, followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:50 in PBS; Cappel Research Products, Durham, NC). Nuclei were visualized using nuclear isolation medium-4,6-diamidino-2-phenylindole

dihydrochloride (blue) or TOTO3 (red), a dimeric cyanine nucleic acid dye that stains nucleic acid (1:10,000 in PBS, Molecular Probes, Invitrogen Corporation, Carlsbad, CA). For double IFA, hair fractions were incubated with anti-CD200 followed by anti-K14 antibody (Santa Cruz Biotechnology).

Upper and lower portions were treated with 0.2% collagenase D (Boehringer, Mannheim, Germany) in Eagle minimum essential medium (MEM; ICN Biomedicals, Aurora, OH) containing 10% fetal bovine serum at 37°C for 30 minutes and separately incubated in human hair follicle stem cell expansion media (Celprogen, San Pedro, CA) supplemented with 24.3  $\mu\text{g}/\text{mL}$  of adenine, 5  $\mu\text{g}/\text{mL}$  of insulin, 5  $\mu\text{g}/\text{mL}$  of human transferrin (Sigma-Aldrich, St. Louis, MO), 0.4  $\mu\text{g}/\text{mL}$  of hydrocortisone (Calbiochem, La Jolla, CA), 10 ng/mL of human recombinant epidermal growth factor (hrEGF; Chiron, Emeryville, CA), 100 iu/mL of penicillin (Sigma), and 25  $\mu\text{g}/\text{mL}$  of gentamicin sulphate (Scheering, Pointe-Claire, QC, Canada). For flow cytometric analysis, single keratinocyte suspensions ( $1 \times 10^6$  cells/mL) from hair portions or neonatal foreskin were stained with 1  $\mu\text{g}/\text{mL}$  of allophycocyanin-conjugated anti-CD34 (BD Biosciences) or 1  $\mu\text{g}/\text{mL}$  of anti-CD200 antibodies for 30 minutes at 4°C, followed by FITC-conjugated goat anti-mouse immunoglobulin G (MP Biomedicals, Irvine, CA) for 30 minutes. The percentage of positively stained cells over 10,000 events was evaluated using a FACS-Calibur flow cytometer and Cell Quest software (BD Biosciences).

## Results and Discussion

The diagram of hair follicle structure in Figure 1A, modified from Fuchs,<sup>26</sup> shows the exact localization of the bulge area in the upper two-thirds of the hair follicle, below the sebaceous gland and in correspondence to the arrector pili muscle origin. The dermal papilla is instead localized in the lower portion of the follicle, corresponding to the hair bulb. Figure 1B shows the two generated FU: the upper portion, which encompasses the bulge region, and the lower portion, containing the dermal papilla.

**TABLE 1. Hair Regeneration in 28 Patients Undergoing Duplicative Surgery**

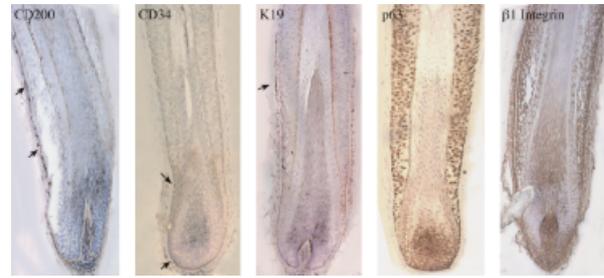
	Mean Percentage $\pm$ Standard Deviation	
	6 Months	12 Months
Upper portion	53.7 $\pm$ 0.3	72.7 $\pm$ 0.4
Lower portion	48.2 $\pm$ 1.1	69.2 $\pm$ 1.1
Recipient's follicle	56.4 $\pm$ 1.3	78.4 $\pm$ 1.5

Bulge and papilla of the same follicle were implanted in marked areas (Figure 1C) and separately followed up. Six months after grafting, transplanted hairs were regenerated with an average efficiency of  $53.7 \pm 0.3\%$  for the upper ( $n = 91$ ) and  $48.2 \pm 1.1\%$  for the lower portion ( $n = 89$ ), similar to the efficiency obtained with the entire follicle ( $56.4 \pm 1.3\%$ ) ( $n = 92$ ) (Table 1). Twelve months after grafting (Figure 1C), the rate of regrowth was  $72.7 \pm 0.4\%$  for the bulge and  $69.2 \pm 1.1\%$  for the papilla, similar to that of the entire follicle ( $78.4 \pm 1.5\%$ ) (Table 1). Some previous works<sup>1,2</sup> have reported that regenerated hair shafts were finer in caliber than the original donor hairs. We found that the caliber of hairs regenerated from entire follicles was  $96.1 \pm 0.2\%$  with respect to original donor hairs (100%). The new hairs obtained from bisected follicles were slightly finer than the donor hairs, although we found no difference in caliber between regenerated hairs derived from the upper ( $75.3 \pm 8.2\%$ ) or lower portion ( $74.4 \pm 4.1\%$ ) (Table 2).

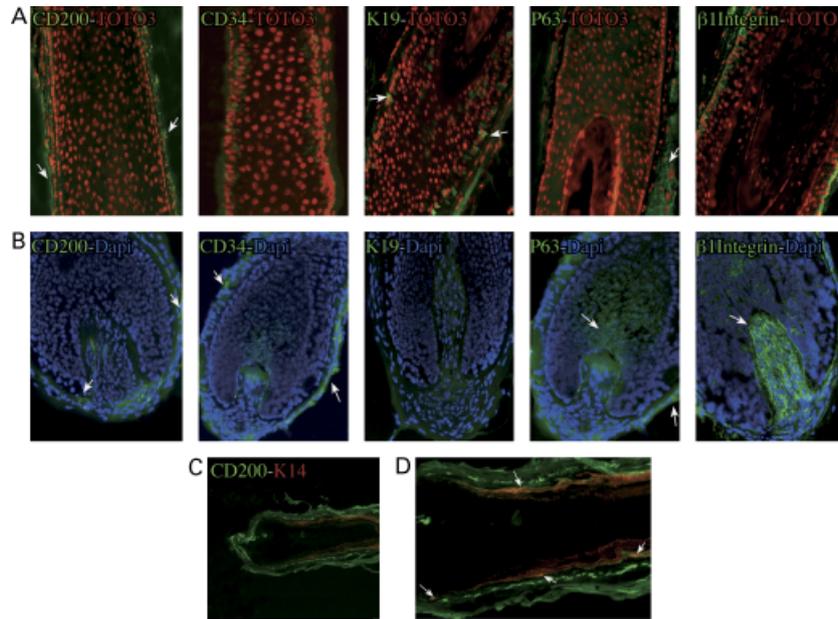
To evaluate the presence of epithelial stem cells in the two portions, we analyzed the expression of known specific markers using IHC (Figure 2). CD200, a recently described marker of bulge stem cells,<sup>27</sup> was detected in the outer root sheath (ORS) of the bulge

**TABLE 2. Caliber of Hairs Regenerated After Duplicative Surgery**

	Donor Hair Caliber, Mean Percentage $\pm$ Standard Deviation
Entire follicle	96.1 $\pm$ 0.2
Upper portion	75.3 $\pm$ 8.2
Lower portion	74.4 $\pm$ 4.1

**Figure 2.** Immunohistochemical analysis of longitudinal serial sections of cryopreserved isolated hair follicles incubated with antibodies to CD200, CD34, K19, p63, and  $\beta 1$ -integrin. Arrows indicate positive immunoreactivity.

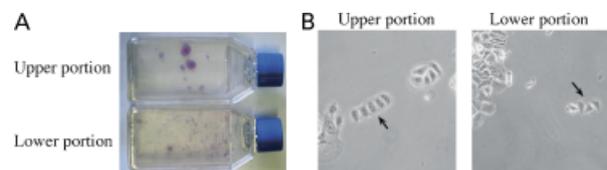
region, as previously shown<sup>25</sup> and, to a lesser extent, in the lower portion (arrows). CD34 was expressed in the ORS of the lower portion (arrows), in keeping with its known downregulation in human bulge cells.<sup>15,25</sup> The cytokeratin K19 was previously found in the bulge and also in a second region of ORS that may constitute a reservoir of stem cells.<sup>19,28,29</sup> In our sections, K19 was consistently expressed in the bulge (arrow) and throughout the upper ORS. A strong signal for the p63 molecule was detected in the ORS of the entire hair shaft and also around the dermal papilla, previously reported to contain only cells with limited growth capacity.<sup>24</sup>  $\beta 1$ -integrin is considered a putative stem cell marker, because cells with higher levels of  $\beta 1$ -integrin showed a higher colony-forming efficiency.<sup>11</sup> Stronger immunoreactivity was evident in the ORS, although the entire follicle was labelled with different staining intensities. We then performed IFA on microdissected hairs to confirm these findings (Figure 3A and B). The upper hair (Figure 3A) clearly expressed CD200 and K19 in the ORS of the bulge region (arrows) and p63 and  $\beta 1$ -integrin throughout the ORS (arrows). The lower hair (Figure 3B) showed the marked expression of p63 and  $\beta 1$ -integrin in the ORS, whereas the K19 signal was virtually negative. Some CD200-positive cells were also detected in the lower portion, although double staining with CD200 and K14 (Figure 3C and D) showed only a partial co-localization of the two molecules (Figure 3D, arrows), suggesting that the positive signal in the lower portion of the follicle might be partially due to cells of the endothelial sheath.



**Figure 3.** Immunofluorescence analysis of upper (A) and lower (B) portions of bisected human hair follicles labelled with antibodies to CD200, CD34, K19, p63, and  $\beta$ 1-integrin (green, arrows). Nuclei were visualized using the dimeric cyanine nucleic acid dye TOTO3 (red in A) or nuclear isolation medium-4,6-diamidino-2-phenylindole dihydrochloride (blue in B). Figures in panels A and B represent merged images of the two signals. Immunofluorescence analysis of the lower portion of bisected human hair follicles double labelled with antibodies to CD200 (green) and K14 (red). Figures in panels C and D represent merged images of the two signals. In panel D, which represents a higher magnification of C, arrows indicate co-localization of the two signals (yellow).

Our screening for putative markers of the epithelial stem cell compartment, performed using IHC and IFA, demonstrated the presence of CD200-,  $\beta$ 1-integrin-, and p63-positive cells in both halves of the follicle, whereas a differential distribution was observed for K19 and CD34 molecules. In particular, K19-positive cells were found localized mainly in the bulge area, although previous reports have provided evidence of the presence of K19-labelled cells in the upper and lower thirds of the hair follicle during the anagen phase. However, a real consensus on the spatial distribution of K19 during the hair cycle has not been achieved, thus providing a possible explanation for our findings. By contrast, CD34-positive cells have been found to be mainly localized in the lower half of the hair, according to previous literature. Our findings about the expression and distribution of epithelial stem cell markers might contribute to identify the role of subsets of cells expressing these molecules on their surface in generating a complete hair.

Primary cultures of keratinocytes obtained from the upper and lower portion of the same FU were different in terms of morphological features and proliferation ratio. Bulge-derived cultures formed large colonies (Figure 4A), and mitotic figures were often observed, suggesting a strong proliferative potential. In cultures derived from the lower portion, cell colonies had a smaller diameter (Figure 4A), and elements in cytokinesis were occasionally found. Hair-derived keratinocytes showed greater motility



**Figure 4.** (A) Colonies derived from the upper or lower portion of the same follicle were fixed in absolute methanol and stained with 1% crystal violet. (B) Cultures derived from bulge-containing (left panel) or papilla-containing (right panel) portions show the presence of rows of cells that display directional proliferation (arrows). Scale bar, 20  $\mu$ m.

in culture and migrated to form rows of cells, an unusual finding for keratinocytes derived from any other source (Figure 4B, arrows).

Cells positive for the indicated markers in the hair follicle might represent interesting target populations for studies aimed at better understanding the biology of stem cells of epithelial origin, as well as for therapeutic use. Therefore, the possibility of expanding such populations in primary cultures, sorting viable positive cells using flow cytometry (fluorescence-activated cell sorting; FACS), might provide a useful tool for further applications. Therefore, using FACS, we assessed the persistence of CD200- and CD34-positive cells in keratinocytes derived from bulge, papilla, or neonatal foreskin, the latest representing a hairless skin control. We found no relevant differences in the percentage of CD34-positive cells between the hair follicle samples (bulge, 1.8%; papilla, 3%) and the control hairless skin (2.5%), indicating that this marker is not specific for hair-derived stem cells. In contrast, the CD200-positive fraction was more highly enriched in bulge (15.6%) and papilla (8.7%) than in control foreskin (0.39%), suggesting that CD200 might represent a specific marker for highly proliferative cells of hair follicle origin (data not shown). The enrichment of CD200-positive cells in bulge-derived cultures is in keeping with previous reports.<sup>25</sup> With regard to papilla-derived cultures, the percentage of positive cells substantially reflects the data obtained using IFA on microdissected hairs, although we cannot exclude the presence of some contamination from endothelial CD200-positive cells in this fraction.

Our results emphasize the idea of a second reservoir of progenitor cells lower down the follicle, apart from the bulge area, that allows for hair growth after follicle bisection, as previously suggested.<sup>2,28</sup> It is known that epithelial–mesenchymal interactions are essential during hair cycle, because KSCs need to be stimulated by DPCs to proliferate. Nevertheless, some studies have reported that the upper half of the follicle could regenerate independently from the bulb.<sup>30–32</sup> Such data could be explained considering

that DPCs in the bulb and dermal sheath cells in the middle of the follicle exhibit striking levels of plasticity, with each able to regenerate both cell types.<sup>4,30,33</sup>

## Conclusions

Our results confirm duplicative surgery as an efficient alternative to single FU transplant, especially in the case of limited availability of donor hairs, because hairs generated from the upper or lower portion of a bisected FU possess a similar rate of regrowth and are almost indistinguishable in terms of thickness and vitality. Concerning the characterization of cellular elements potentially involved in the process, in this report, we hypothesized that each half of the follicle might contain a stem cell reservoir capable of driving the formation of the entire hair. The plasticity of epithelial and dermal stem cells, which might be able to initiate the growth induction even in transected hair follicles, partially supports such considerations. Nevertheless, we cannot exclude the possibility that hair regeneration obtained by transplanting the lower half of the follicle might be at least partially due to the splitting of bulge stem cells during hair bisection, although the size and localization of the bulge area and the accuracy of cutting should minimize the risk of such splitting.

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