Follicular Penetration and Targeting

Jürgen Lademann,* Nina Otberg,* Ute Jacobi,* Robert M. Hoffman,† and Ulrike Blume-Peytavi*
*Department of Dermatology, Venerology and Allergology, Charité, Berlin, Germany; †AntiCancer, Inc., San Diego, California, USA

In the past, intercellular penetration was assumed to be the most important penetration pathway of topically applied substances. First hints that follicular penetration needs to be taken into consideration were confirmed by recent investigations, presented during the workshop ''Follicular Penetration and Targeting'' at the 4th Intercontinental Meeting of Hair Research Societies'', in Berlin 2004. Hair follicles represent an efficient reservoir for the penetration of topically applied substances with subsequent targeting of distinct cell populations, e.g., nestin-expressing follicular bulge cells. The volume of this reservoir can be determined by differential stripping technology. The follicular penetration processes are significantly influenced by the state of the follicular infundibulum; recent experimental investigations could demonstrate that it is essential to distinguish between open and closed hair follicles. Topically applied substances can only penetrate into open hair follicle. Knowledge of follicular penetration is of high clinical relevance for functional targeting of distinct follicular regions. Human hair follicles show a hair-cycle-dependent variation of the dense neuronal and vascular network. Moreover, during hair follicle cycling with initiation of anagen, newly formed vessels occur. Thus, the potential of nestin-expressing hair follicle stem cells to form neurons and blood vessels was investigated.

Key words: cyanoacrylate skin surface stripping/hair follicle/laser scanning microscopy/nestin/penetration/stem cells/tape stripping/targeting
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In the past, intercellular penetration was considered to be the predominant pathway for transdermal delivery of topically applied substances (El Maghraby *et al*, 2001; Neumann *et al*, 2001; Essa *et al*, 2002; Chatelain *et al*, 2003). A high number of experimental studies have been performed to investigate the influence of the structure of the lipid layers on this process (Fisher and Tjarnhage, 2000; Grant and Tiberg, 2002; Tareste *et al*, 2002; Bouwstra *et al*, 2003). Hair follicles and sweat glands accounted for only approximately 0.1% of the skin surface area (Schaefer and Redelmeier, 1996). Therefore, they were not considered to represent significant transdermal penetration routes. Recent *in vitro* and *in vivo* studies on follicular penetration, however, confirm an important significance of this route on follicular penetration processes (Chu *et al*, 1996; Schaefer and Lademann, 2001; Lampen *et al*, 2003; Toll *et al*, 2004).

Recent investigations of Otberg *et al* demonstrated a comparable follicular reservoir in different skin areas, despite their different hair follicle densities. Characteristics of hair follicle sizes and potential follicular reservoir were determined by cyanoacrylate skin surface stripping (Fig 1), taken from seven different skin areas (lateral forehead, back, thorax, upper arm, forearm, thigh, and calf region).

The highest hair follicle reservoir, i.e., the percentage of follicular orifices on the skin surface and infundibular surface, was found on the forehead; the highest average size of the follicular orifices was measured in the calf region. A comparable overall infundibular volume, equal to a comparable follicular reservoir, was calculated for the forehead and calf regions, although the calf region showed the lowest hair follicle density. The calculated follicular reservoir volume of these two skin areas was as high as the estimated reservoir of the stratum corneum. The lowest values for every other parameter were found on the forearm. Thus, at present, we have to correct the statement made in the past that the amount of appendages of the total skin surface is not more than 0.1%, and have to confer the follicular reservoir a high importance, especially during the development of new topical transdermal drug-delivery strategies (Otberg *et al*, 2004).

Abbreviation: GFP, green fluorescent protein

In Vivo Method to Assess Active and Inactive Hair Follicles

Not all human follicles are open for the penetration of topically applied substances. The penetration of a fluorescent dye into hair follicles, analyzing the cross-section of the hair follicles removed by cyanoacrylate skin surface biopsies, was detected by laser scanning microscopy (Lademann *et al*, 2003). The dye was found in nearly 70% of the hair follicles, although some follicles were obviously closed for penetration. These *in vivo* observations confirm the hypothesis of *open* and *closed*, as well as *inactive* and *active hair follicles*, as published earlier (Lademann *et al*, 2001).

The different follicular behavior is possibly due to specific follicular properties. A combination of various tape stripping and staining methods made it possible to measure hair growth and sebum excretion of individual hair follicles in defined skin areas. A correlation between penetration properties, hair growth activity, and sebum secretion was found.

Figure 1
Follicle infundibulum removed by cyanoacrylate skin surface biopsy.

Telogen hair follicles not excreting sebum were identified as being closed for the penetration process. This means that only in the case of *a movement from inside out* of the hair follicle can a topically applied substance penetrate into the follicle. It was proposed that the *inactive follicles* should be closed by a mechanical cover, to prevent the follicular penetration. This effect was investigated by *in vivo* analysis of the hair follicle infundibula cross-sections by optical coherence tomography (OCT) (Lademann *et al*, 2005). The OCT measurements demonstrated that the orifices of inactive hair follicles, i.e., follicles that evidence neither hair growth nor sebum excretion, were filled with a plug. This plug had morphological characteristics similar to those of the stratum corneum and protruded from the surface of the skin. It seems likely that this structure consists of shedded corneocytes glued together with dry sebum and other cell dedritus. This plug on the top of *closed hair follicles* can, however, be opened by soft peeling.

Quantification of Penetrated Substances after Topical Application into Hair Follicle Infundibulum

A new method for quantification of topically applied substances penetrating into the hair follicles has been presented by Teichmann *et al* (2005).

For this purpose, the uppermost part of the SC containing most of the interfollicular topically applied substances was removed primarily by the method of tape stripping. Next, the follicular infundibula were ripped off by cyanoacrylate skin surface stripping. This combination of two methods, tape stripping (TS) and cyanoacrylate skin surface stripping (CSSS), is termed as differential *skin stripping*.

This approach was tested *in vitro* on a porcine skin model after application of an emulsion containing Patent blue V. The dye could be detected selectively in the hair follicles after removal of 30 tape strips. Subsequently, the follicular content was selectively ripped off using CSSS. These qualitative observations were confirmed using histological techniques. The data obtained led to the conclusion that a selective method had been developed to determine the amount of topically applied substances in hair follicles qualitatively.

In further studies, this approach was tested *in vivo* to determine the amount of a topically applied fluorescent dye (Teichmann *et al*, 2005) quantitatively. On the back of human volunteers, approx. 4% of the total amount topically applied was detected in the follicles 30 min after application. These results demonstrate that this new method of *differential skin stripping* is suited to determine the amount of topically applied substances penetrated into hair follicles selectively and quantitatively.

Potential of Nestin-Expressing Hair Follicle Stem Cells to Form Neurons and Blood Vessels

Follicular penetration is mainly intended to influence distinct hair follicle compartments and/or cell populations selectively. The neuronal and vascular network is highly developed in hair follicles at the anagen stages IV–VI, showing hair-cycle-dependent variations with apoptosis of endothelial cells with initiation of catagen. Moreover, at present, angiogenesis is of increasing importance in wound repair and growth control of malignancies (Hoffmann *et al*, 2004). Functional targeting of follicular regions has recently been reported by Hoffman (2004) by investigation of nestin-expressing hair follicle stem cells, which are known to form neurons and blood vessels (Ehrmann *et al*, 2005).

Hair follicle stem cells supply endothelial cells that can form blood vessels in the skin and around the hair follicle (Amoh *et al*, 2004). Thus, targeting of nestin-expressing follicular stem cells with subsequent pharmacological manipulation of this cell population is of key interest in regulating angiogenesis and neuronal growth around the hair follicle.

Hoffmann and co-workers report on recent findings, where they were able to label selectively central nervous system (CNS) stem cells—the progenitor cells of the (CNS)—by placing green fluorescent protein (GFP) under the control of the nestin-regulatory sequences in transgenic mice. During early anagen or growth phase of the hair follicle, nestin-expressing cells, marked by GFP fluorescence in nestin–GFP transgenic mice, appear in the permanent upper hair follicle immediately below the sebaceous glands in the follicular bulge (Mignone *et al*, 2004). This is where stem cells for the hair follicle outer root sheath are thought to be located. The relatively small, oval-shaped, nestin-expressing cells in the bulge area surround the hair shaft and are interconnected by short dendrites. The precise locations of the nestin-expressing cells in the hair follicle vary with hair cycling. During telogen or resting phase and in early anagen, the GFP-positive cells were mainly in the bulge area. In mid- and late anagen, however, the GFP-expressing cells were located in the upper outer root sheath as well as in the bulge area but not in the hair matrix bulb. These

observations showed that the nestin-expressing cells form the outer root sheath. Results of the immunohistochemical staining showed that nestin, GFP, keratin 5/8, and keratin 15 co-localize in the hair follicle bulge cells, outer root sheath cells, and basal cells of the sebaceous glands (Amoh *et al*, 2004). These data indicate that nestin-expressing cells, marked by GFP, in the hair follicle bulge are indeed progenitors of the follicle outer root sheath.

Besides forming hair shafts, the highly organized, metabolically vigorous hair follicle plays several crucial roles in skin architecture. Nestin-driven GFP also labels developing skin blood vessels that appear to originate from hair follicles and form a follicle-linking network. This is seen most clearly by transplanting nestin-driven GFP-labeled vibrissa (whisker) hair follicles to unlabeled nude mice (Amoh *et al*, 2004) New vessels grow from the transplanted follicle and increase when the local recipient skin is wounded. The nestin-driven GFP-expressing structures are blood vessels since they display the characteristic endothelial-cell-specific markers CD31 and von Willebrand factor (vWF). This model displays very early events in skin angiogenesis and can serve for rapid anti-angiogenesis drug screening (Amoh *et al*, 2004). In preliminary experiments, nestin-driven GFP-expressing hair follicle stem cells were isolated and cultured to form neurospheres, which then converted to neurons as demonstrated by immunohistochemical staining with neuron-specific markers. Therefore, the hair follicle stem cells appear to be pluripotent. Future experiments will focus on the multiple therapeutic potential of hair follicle stem cells (Hoffman, 2004), and functional targeting of these nestin-expressing stem cells will be investigated.

Conclusions

Hair follicle has become of special interest in the past years as an efficient penetration pathway for drug delivery (Chu *et al*, 1996; Lampen *et al*, 2003; Toll *et al*, 2004). The reservoir of hair follicles is comparable to the reservoir of the stratum corneum in different body sites. Taking into consideration the properties of the hair follicles, it has to be distinguished between *active* and *inactive follicles* concerning the penetration of topically applied substances and body region-dependent penetration behavior. At present, analytical methods are available to qualify and quantify the amount of topically applied substances penetrating into hair follicle infundibula (Teichmann *et al*, 2005). Together with improved identification and characterization of distinct cell populations, new diagnostic methods are available for the functional targeting of follicular regions, such as nestin-expressing stem cells, highly interesting for innovative strategies of fundamental drug delivery.

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Address correspondence to: Juergen Lademann, Department of Dermatology, Venerology and Allergology, Center of Experimental and Applied Cutaneous Physiology, Charité University Medicine Berlin, Schumannstr. 20/21, 10117 Berlin, Germany. Email: juergen.lademann@charite.de

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