Expert Opinion

The hair follicle and its stem cells as drug delivery targets

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The hair follicle is a skin appendage with a complex structure containing many cell types that produce highly specialised proteins. The hair follicle is in a continuous cycle: anagen is the hair growth phase, catogen the involution phase and telogen is the resting phase. The follicle offers many potential therapeutic targets. Hoffman and colleagues have pioneered hair-follicle-specific targeting using liposomes to deliver small and large molecules, including genes. They have also pioneered *ex vivo* hair-follicle targeting with continued expression of the introduced gene following transplantation. Recently, it has been discovered that hair follicle stem cells are highly pluripotent and can form neurons, glial cells and other cell types, and this has suggested that hair follicle stem cells may serve as gene therapy targets for regenerative medicine.

Keywords: adenovirus, gene therapy, hair follicle, liposome, stem cell

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1. Introduction

There is an increasing interest in the hair-follicle route for the delivery of drugs for both transdermal drug delivery and the delivery of active compounds that affect the hair itself [1]. Feldmann and Maibach [2,3] noted that *in vitro* absorption of various compounds depended on the regional difference in the morphology of the human skin used. Absorption was increased in areas of increased follicular density and size (e.g., forehead and scalp). Illel *et al.* [4] reported that the *in vitro* percutaneous steady-state flux for representative penetrants was two- to four-times higher in normal appendage-containing, hairless rat skin (follicular density 10 – 200/cm^2) relative to their appendage-free (follicle free) skin. Other reports in the literature also suggested a significant amount of penetration via the pilosebaceous apparatus [5-7].

2. Targeting genes to the hair follicle

2.1 Histoculture of hair-growing skin for the study of drug delivery

Hoffman and colleagues have previously developed a relatively long-term histoculture [8] of whole human and mouse skin, using a collagen-sponge gel-supported *in vitro* three-dimensional histoculture system, such that long-term effects of various agents could be studied with regard to toxicology, penetration and hair growth [9]. These authors were able to culture both white [10] and black mouse skin [11] with hair growing for periods of at least 10 – 16 days, respectively, as well as human scalp tissue with hair follicle cells proliferating for at least 40 days [12]. They were also able to determine cytotoxicity and viability using fluorescent dyes to identify dead and living cells (including cells in hair follicles) three-dimensionally in the histocultured skin in the living state, in particular using confocal microscopy [9]. A key aspect of the intact skin that was histocultured in this system is the presence of the major types of cells, including hair follicles, as well as growing hair. The histoculture model for the skin may be an effective replacement of animal systems for many experiments and be superior to the dispersed and reconstituted skin equivalent cell system that has been used previously for the measurement of the effect of manufactured

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products, drugs and pollutants on skin and hair growth [13]. The histoculture of intact skin on sponge-gel matrices offers many opportunities for tissue engineering and for the *in vitro* study of important processes that are intrinsic to the skin, such as hair growth and toxicity to various agents [9-11].

2.2 Using liposomes to target the hair follicle

Liposomes have been widely and successfully used as delivery systems to transport macromolecular substances into the cell that cannot normally cross the plasma membrane [14-16]. It has been reported that calcein dye-entrapped liposomes specifically targeted hair follicles in histocultured intact skin [17]. The results from Hoffman and colleagues were strongly confirmed by studies by Lieb *et al.* [18], demonstrating that liposomal formulations were more efficient than any other vehicle that were tested for the delivery of carboxyfluorescein via the follicular route into the pilosebaceous units with the hamster ear model. Hoffman's group then developed liposome-mediated targeted delivery of melanin into hair follicles and the hair shaft itself in histocultured white-haired mouse skin [19]. These studies have many ramifications including targeted hair-growth modification and transfollicular transdermal delivery systems [20], which are further discussed below.

Hoffman and colleagues developed the technique of entrapping DNA in liposomes [21] and used DNA liposomes in order to target high molecular weight DNA to the hair follicle in histocultured skin, as a model of gene therapy of hair-growth processes [20].

These authors used DNA liposomes [22] to selectively target the *lacZ* reporter gene to the hair follicles in mice after topical application of the gene entrapped in liposomes. These results demonstrated that highly selective and safe gene therapy for the hair process is feasible. The fact that liposomes can selectively target the hair follicle for delivery has been confirmed in other laboratories [23].

Domashenko *et al.* [24] improved the transfection of human follicle cells after topical application of liposome-entrapped *lacZ* in mice as well as in a human scalp xenograft mouse model. They found that the liposome composition, timing of liposome application to the early anagen cycle and pretreatment with depilation and retinoic acid were important parameters for transfection efficiency, and that liposome application during anagen onset resulted in selective transfection of human hair follicle matrix cells. Depilation of the hair and the application of retinoic acid to the grafts before liposome application markedly increased the transfection efficiency.

These results demonstrated that genes can be selectively targeted by liposomes to the most important cells of the hair follicle. So far, this is the most selective targeting of a gene observed *in vivo* [24]. Future studies will include restoring hair pigment by the delivery and expression of the tyrosinase gene [25,26]. Hair growth is also a critical target for gene therapy. The highly selective nature of the topical application of liposome gene targeting is a very safe procedure.

2.3 Specific genes affecting hair colour and growth

Alexeev *et al.* used a chimeric RNA-DNA oligonucleotide to correct the albino point mutation in the mouse tyrosinase gene [27]. Chimeric oligonucleotides correct point mutations for homologous recombination and mismatch DNA repair. In this case, the correction restored tyrosinase activity and concomitant melanin synthesis in a heritable manner, which resulted in the production of pigmented hair shafts in albino mice. The chimeric oligonucleotide was delivered in liposomes as well as by intradermal injection in mice. These injections resulted in a more efficient, but less specific, delivery. The low efficiency observed may be a problem for the cosmetically successful restoration of hair pigment, as a minimum number of melanocytes located deep in the hair bulb must be converted to colour a single hair shaft.

A gene-gun method was developed for the transfer of human agouti signalling protein (ASP) cDNA to alter rat skin colour *in vivo*. Human ASP cDNA was cloned into a modified cytomegalovirus plasmid and delivered to the skin of Long-Evans rats by gene-gun bombardment. The control group was injected with plasmids encoding for green fluorescent protein. The treated skin showed a lighter skin colour after 3 days of *ASP* gene transfection. This depigmentation effect was the most prominent on day 14 and the skin gradually returned to its original pigmentation by day 28. Successful transfection of the *ASP* gene into the skin and hair follicles, as well as downregulation of melanocortin-1 receptor and tyrosinase expression following treatment, was confirmed using immunohistochemistry and western blot analysis. These observations demonstrate that gene transfer using the gene gun method can induce high cutaneous ASP production and facilitate a switch from dark to fair colour [28].

Hair pigmentation is a function of the tyrosinase gene. Lloyd *et al.* [29] found that the level of tyrosinase correlated with hair colour, with the lowest activity occurring in blond hair bulbs and the highest in red hair bulbs. However, most importantly, they found that the human tyrosinase antigen was absent in white hair bulbs [29]. Thus, the loss of tyrosinase seems to be the basis of hair turning white.

A recombinant retrovirus containing the *mel* locus of *Streptomyces antibioticus* was constructed. ORF-438, required for the transfer of copper to apotyrosinase, and tyrosinase genes were co-expressed in a single vector, pLmelSN. In order to evaluate the ability of the vector to restore pigment production in cells with a pigment disorder, albino-mouse skins were histocultured and then infected with the pLmelSN retrovirus. Melanin granules were observed in ~ 60% of albino-mouse hair follicles in the histocultured skin 6 days after infection. These results demonstrated that the *S. antibioticus mel* operon could express an active tyrosinase and produce melanin in the albino-mouse hair follicles. This novel gene-therapy approach, using a small and simple tyrosinase operon in a high-expression vector, has a potentially wide application for the therapy of pigment disorders in hair follicles [30].

Gene-therapy targeting of hair growth, on the other hand, may not be as demanding as restoring the pigment, as even partial restoration could potentially give satisfactory results. Sato *et al.* [31] showed that the sonic hedgehog (*shh*) gene, delivered with an adenovirus vector, stimulated anagen development and hair shaft production in the C57BL/6 mouse. The human hairless gene, which is responsible for alopecia universalis, has also been cloned [32] and could be used for hair follicle gene therapy.

2.4 *Ex vivo* approaches to increasing the efficacy of gene modification of the hair follicle

Hair follicle dermal sheath cells taken from the scalp of an adult human male could form new dermal papillae and hair follicles, which produce hair shafts when transplanted into the skin of an unrelated female [33]. The so-called immune privilege of hair follicle cells may have prevented their rejection. The results suggest the possible totipotency or inductive properties of the follicle dermal sheath cells [33], which may, therefore, be a possible *ex vivo* gene therapy target. The genetically altered cells may be transplanted to the donor, as well as to other patients.

Saito *et al.* have recently described a highly efficient genetic modification technique for hair follicles. It enables high transgene expression in growing hair shafts in mouse skin grafted onto nude mice [34]. Mouse anagen skin fragments were genetically modified *ex vivo* at a high efficiency with adenoviral-green fluorescent protein (GFP). The skin fragments were treated with collagenase, which made the hair follicles accessible to the adenoviral *GFP* gene, allowing high-efficiency transduction. The genetically modified skin fragments were subsequently grafted onto nude mice where GFP was readily visualised in as many as 75% of the hair follicles [34]. GFP fluorescence was visualised in the root sheath cells and matrix cells of the hair bulb. The majority of the follicles produced GFP-fluorescent growing hair shafts. GFP expression occurred to a much lesser extent in the upper epidermis and dermal fibroblasts. This novel technique has produced, for the first time, efficient genetic modification of the hair shaft.

2.5 The hair follicle genetically modified to produce therapeutic proteins

The hair follicle has a high capacity for manufacturing proteins. In a recent study, the topical application of both naked and liposome-entrapped plasmid vectors for the hepatitis surface antigen (HbsAg) resulted in antigen-specific immune responses [35]. The gene product may have been synthesised in the follicles.

3. Delivery of small molecules and melanin to the hair follicle

The use of three-dimensional histoculture in conjunction with confocal microscopy opened the opportunity to follow fine details of product-delivering liposome interactions with hair follicles at the cellular level. As a result, optimal liposome compositions would be established as well as the conditions for the delivery of the liposomal content into target cells. Mouse skin histocultures were incubated for 20 min with liposomes, or with a solution of free calcein dye at the same concentration as it was in the liposome preparation. After the tissues were thoroughly washed, the specimens were analysed with a Biorad MRC 600 laser confocal microscope. The histocultured skin incubated with free calcein solution exhibited a relatively low fluorescence with no preferential staining of any particular skin structure. Liposome-entrapped dye in contrast to free dye became associated with hair follicles. Liposomes were associated preferentially with hair follicles when liposomes were labelled with calcein, or when the liposomes themselves were labelled with fluorescent phosphatidylcholine [17,36].

Hoffman and colleagues have also reported liposome-mediated targeted delivery of melanin into hair follicles and the hair shaft in histocultured white-haired mouse skin. Melanin was entrapped into phosphatidylcholine liposomes by sonication. Pieces of outbred white-haired mouse skin that were derived from 1- to 2-week-old animals were harvested and then histocultured on collagen-gel supported sponges. Liposome interaction with the skin was initiated after 24 h of the histoculture. Mouse skin histocultures were incubated for 12 h with liposomes. A solution of free melanin, at the same concentration as was used in the liposome preparation, served as the control and was also incubated for 12 h with the histocultured skin. The skin histocultures were counter-stained with the dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), which is activated to fluorescence by nonspecific esterases that are only present in living cells [36]. Microscopically, the live tissues and cells fluoresced green so that the melanin that was localised in the tissues could be clearly identified. The majority of the melanin was seen to be localised around the hair follicles and the melanin was both observed at the periphery of the follicles and in the follicle cells, which were fluorescent due to the selective uptake of BCECF-AM. As a control, the skin histocultures were also incubated with free melanin at the same concentration that was used in preparing the liposomes. No free melanin was seen to be localised in the follicles. The liposome-entrapped melanin was transferred into the follicular cells and was delivered into the hair shaft to form the band-like melanin-distribution pattern in the terminally differentiated keratinocytes of the typical normal pigmented hair shaft. In the control, in which the skin histocultures have been incubated with the free melanin, no free melanin was observed either in the hair shafts or the hair follicular cells. Thus, it has been demonstrated that liposomes can specifically target an important molecule, in this case melanin, to the hair follicles in histocultured mouse skin and even enter the hair shaft in a normal pattern. These results demonstrate the great potential for liposome targeting of crucial substances to hair follicles in order to modify the hair follicle and hair shaft [19].

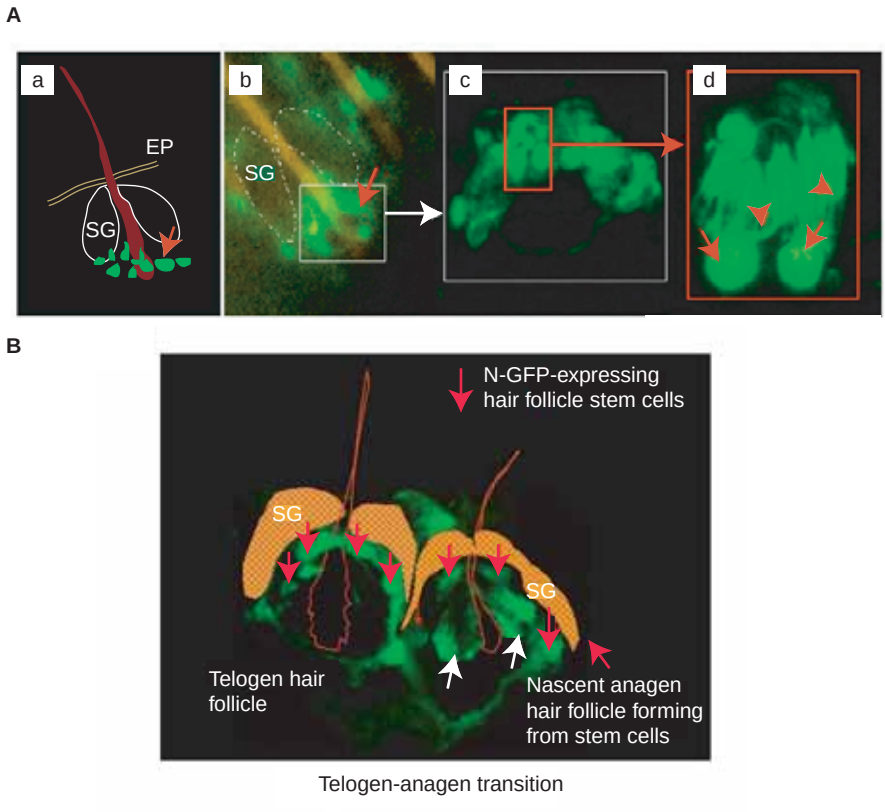

Figure 1. A) Hair follicle N-GFP-expressing cells in the telogen phase of N-GFP transgenic mouse skin. The skin sample was prepared freshly straight after excision from the back skin of a N-GFP transgenic mouse. It was then observed directly by fluorescence or confocal microscopy with the dermis side up after subcutaneous tissue was dissected out. a) Cartoon of telogen hair follicle showing position of N-GFP-expressing hair follicle stem cells. b) Low-magnification fluorescence microscopy image showing the ring of bulge N-GFP-expressing stem cells (small white box, see a). c) High-magnification confocal microscopy image corresponding to the small white box in b. Note the small round- or oval-shaped N-GFP-expressing cells in the bulge area of the hair follicle (small red box).
d) High-magnification fluorescence microscopy image showing two individual N-GFP-expressing stem cells corresponding to the red box in c. Note the unique morphology of the hair follicle stem cells and multiple dendrite-like structures of each cell. Red arrows indicate the cell body, and red arrowheads show the multiple dendritic structure of each cell. Original magnifications: b × 100; c × 400; d × 1600.
B) Telogen-anagen transition showing hair-follicle stem cells forming a nascent hair follicle. The image was taken by fluorescence microscopy 18 h after depilation. Note the bulge N-GFP-expressing hair follicle stem cells in the telogen phase (left hair follicle, red arrows) and the nascent anagen hair follicle directly formed from the bulge N-GFP-expressing stem cells (right hair follicle, white arrows). Original magnification: × 400. Reprinted from LI L, MIGNONE J, YANG M *et al.*: Nestin expression in hair follicle sheath progenitor cells. *Proc. Natl. Acad. Sci. USA* (2003) **100**(17):9958-9961 [37].
EP: Epidermis; N-GFP: Nestin-green fluorescent protein; SG: Sebaceous gland.

4. Pluripotent hair follicle stem cells as potential gene therapy targets

Nestin-expressing cells, marked by GFP fluorescence in nestin-linked GFP (N-GFP) transgenic mice, appear in the permanent upper hair follicle immediately below the sebaceous glands in the follicle bulge (Figure 1). These cells remarkably resemble neural stem cells, which also express nestin. Results of the immunohistochemical staining showed that nestin, GFP, keratin 5/8 and keratin 15 co-localise in the hair follicle bulge cells, outer root sheath cells and basal cells of the sebaceous glands. These data indicate that nestin-expressing cells, marked by GFP, in the hair follicle bulge are indeed progenitors of the follicle outer root sheath. The expression of the unique protein, nestin, in both neural and hair follicle stem cells suggests a possible relation [37].

N-GFP also labels developing skin blood vessels that seem to originate from hair follicles and form a follicle-linking network. This is seen most clearly by transplanting N-GFP-labelled vibrissa (whisker) hair follicles to unlabelled nude mice. New vessels grow from the transplanted follicle

and these vessels increase when the local recipient skin is wounded. The N-GFP-expressing structures are blood vessels because they display the characteristic endothelial-cell-specific markers $CD31^+$ and von Willebrand factor. This model displays very early events in skin angiogenesis and can serve for rapid antiangiogenesis drug screening [38].

N-GFP stem cells isolated from the hair-follicle bulge area that are negative for the keratinocyte marker keratin 15 can differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes *in vitro*. These pluripotent N-GFP stem cells are positive for the stem cell marker $CD34^+$, as well as keratin 15-negative, suggesting their relatively undifferentiated state. The apparent primitive state of the N-GFP stem cells is compatible with their pluripotency. Furthermore, Hoffman and colleagues showed that cells derived from N-GFP stem cells can differentiate into neurons after transplantation to the subcutis of nude mice [39]. These results suggest that hair follicle-bulge area N-GFP stem cells may provide an accessible, autologous source of undifferentiated multipotent stem cells for therapeutic application.

The sciatic nerve of C57BL/6 immunocompetent mice was severed and the hair-follicle stem cells that were isolated from the bulge were injected between the two severed regions of the sciatic or tibial nerve. The nerve was rejoined by the hair-follicle stem cells. Most of the GFP-expressing hair follicle cells differentiated into glial fibrillary acidic protein-positive Schwann cells with myelin sheaths in the sciatic nerve. The rejoined sciatic nerve contracted the gastrocnemius muscle following electrical stimulation. The ability to walk normally recovered after transplantation of hair-follicle stem cells between the fragments of the severed tibial nerve. These results suggest that hair-follicle stem cells promote the recovery of peripheral nerve injury; therefore, providing a potentially important accessible, autologous source of adult stem cells for regenerative medicine and a high relevant target for gene therapy [40].

5. Conclusions

The location and structure of the hair follicle offers unique therapeutic opportunities. As described above, Hoffman and colleagues and other scientists have shown that the follicle can be selectively targeted with functional genes. Two experimental strategies have been used: topical application of liposome-entrapped DNA and *ex vivo* targeting with adenoviral gene vectors. These highly specific methods for targeting the hair follicle offer the possibility of screening for genes that can correct defects in the hair follicle such as alopecia or loss of pigment, as well as taking advantage of the synthetic capacity of the hair follicle to manufacture therapeutic proteins for systemic diseases.

With the use of the N-GFP transgenic mice it was possible to visualise the hair follicle stem cells in real time as well as isolate and culture them. This capability gave rise to the possibility of selectively targeting the hair follicle stem cells for gene therapy, and the possibility of using the stem cells therapeutically for the growth of new hair follicles as well as for nerve regeneration.

6. Expert opinion

The hair follicle offers many targets for gene and drug therapy. This paper has reviewed liposomal delivery, which may target many types of drugs as well as genes to the follicle. This is a noninvasive and safe delivery system. *Ex vivo* approaches are very powerful but are somewhat invasive. Removal of hair-bearing skin or scalp fragments for collagenase treatment and adenoviral delivery is a very efficient means to deliver genes to the follicle that modify the properties of the hair shaft. The skin with the gene-modified follicles can then be regrafted. Perhaps the most exciting possibility is using the hair follicle stem cells as gene targets to modify not only hair but to treat other diseases. The gene-treated stem cells can then be used to regenerate nerves and other organs.

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