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Title:
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Date:
2016-07-01

Citation:
Asgari, A. Z., Rufaut, N. W., Morrison, W. A., Dilley, R. J., Knudsen, R., Jones, L. N. & Sinclair, R. D. (2016). Hair transplantation in mice: Challenges and solutions. Wound Repair and Regeneration, 24 (4), pp.679-685. https://doi.org/10.1111/wrr.12435.

Persistent Link:
https://hdl.handle.net/11343/291396

Hair Transplantation in Mice: Challenges and Solutions

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Word count: 3,021 (not including abstract, references or figure legends)

Number of display items: 2

Funding sources: National Health and Medical Research Council of Australia (NHMRC) Medical Postgraduate Scholarship (567168), Australasian College of Dermatologists/Fred Bauer Research Grant and a CASS Foundation Science and Medicine Grant.

Conflict of interest: Non declared.

Abstract

Hair follicle cells contribute to wound healing, skin circulation, and skin diseases including skin cancer and hair transplantation is a useful technique to study the participation of hair follicle cells in skin homeostasis and wound healing,

Although hair follicle transplantation is a well-established human hair restoration procedure; follicular transplantation techniques in animals have a number of shortcomings and have not been well described or optimised.

To facilitate the study of follicular stem and progenitor cells and their interaction with surrounding skin, we have established a new murine transplantation model, similar to follicular unit transplantation in humans. Vibrissae from GFP transgenic mice were harvested, flip-side micro-dissected and implanted individually into needle hole incisions in the back skin of immune-deficient nude mice. Grafts were evaluated histologically and the growth of transplanted vibrissae was observed.

Transplanted follicles cycled spontaneously and newly formed hair shafts emerged from the skin after two weeks. Ninety percent of grafted vibrissae produced a hair shaft at 6 weeks. After pluck-induced follicle cycling, growth rates were equivalent to un-grafted vibrissae. Transplanted vibrissae with GFP positive cells were easily identified in histological sections.

We established a follicular vibrissa transplantation method that recapitulates human follicular unit transplantation. This method has several advantages over current protocols for animal hair transplantation. The method requires no suturing and minimizes the damage to donor follicles and recipient skin. Vibrissae are easier to micro-dissect and transplant than pelage follicles and once transplanted, are readily distinguished from host pelage hair. This facilitates measurement

of hair growth. Flip-side hair follicle microdissection precisely separates donor follicular tissue from inter-follicular tissue and donor cells remain confined to hair follicles. This makes it possible to differentiate migration of hair follicle cells from inter-follicular epidermis in lineage tracing wound experiments using genetically labelled donor follicles.

Key words: mouse model, hair follicle, follicular unit transplant, vibrissa follicle transplant, wound healing

Introduction

Hair follicle cells contribute to wound healing, skin circulation, and skin diseases including non-melanoma skin cancers such as basal cell skin cancer. Hair follicle transplantation is used in hair biology research to investigate the role of hair follicle cells in normal homeostasis of skin, in wound healing and in skin disease. Cells originating from hair follicles can be distinguished from inter-follicular epidermis by transplanting follicles from genetically-labeled donors. The effects of manipulating gene expression specifically in follicles can be investigated by transplanting follicles from transgenic mice.

Follicular unit hair transplantation is an established procedure for hair restoration in humans. The method involves harvesting hairy skin from the occipital scalp, microdissection into individual follicular units and implantation of these follicular units into bald scalp in naturally occurring units of 1 to 4 hairs (1, 2). Donor skin is harvested, either by 1 mm punch biopsies or by removing a strip of occipital scalp skin, and then micro-dissected into follicular units with the aid of a stereo-microscope. Each follicular unit is inserted into the bald scalp using either a micro-blade or a 18-21 gauge needle. Minimal damage of the recipient site together with high graft survival rates are advantages of this technique.

Since the initial descriptions of hair transplantation by Okeda in 1939 (3) and Oreitrich in 1959 (4), the techniques have been constantly refined to improve cosmetic outcomes, graft survival and minimize damage to recipient skin. However, there is no detailed description of an animal

hair transplantation protocol in the dermatology literature that incorporates these refinements or has been optimised for animal recipients.

Previously published hair induction studies in animals have employed recombination and implantation of dermal or epidermal parts of hair follicles (5-8) or cultured cells (6, 9-11). In these experiments, there was damage to the transplanted tissue, with either the hair follicle separated into different parts or dissociated cells being cultured prior to implantation. Damage to the transplanted follicles could potentially alter the behaviour of the follicles. In another published study isolated vibrissae were transplanted into an incisional wound and sutured (12). This procedure is time-consuming, and damage to the recipient skin induces a wound healing response that could alter experimental outcomes.

Sato *et al.* (13) micro-dissected pelage hair follicles and transplanted them into nude mice. We selected vibrissa rather than pelage donor follicles, as they are easier to harvest, micro-dissect and transplant. Measurement of hair growth and hair cycling is also simpler in transplanted vibrissae rather than pelage follicles.

We present a vibrissa transplantation protocol in nude mice based on the human follicular unit transplantation technique. The normal anatomical relationship of the hair follicle and surrounding tissue as well as the minimal damage to transplanted follicles and recipient skin make this a suitable model for studying the normal physiological function of hair follicles and skin. This technique has the advantage of being simpler and quicker than other protocols for animal hair transplantation.

Method

Animals

All animal experiments were performed with the approval of St Vincent's Health (Melbourne) Animal Ethics Committee (AEC number 45/10). Animals were maintained in a PC2 animal housing room with a 12-hour light/dark cycle. Mouse feed and water was available *ad libitum*. Male athymic nude mice (BALB/c-Foxn1nu/Arc) were purchased from the Animal Resources Centre, Western Australia. Green fluorescent protein (GFP) transgenic mice, C57BL6/Tg14 (act-EGFP) OsbY01, were obtained from The Ludwig Institute for Cancer Research, Australia. This transgenic mouse line was originally created by The Genome Information Research Center, Osaka University, Osaka, Japan (14)..

Aseptic technique and anesthesia methods

All surgical instruments where sterilized at 125°C for 15 minutes. Mice were anaesthetized by inhalation of Isoflurane (Baxter Healthcare Ltd. , Deerfield, Illinois US) 2% (v/v) in oxygen and the depth of anaesthesia as well as the respiration rate was monitored throughout the procedures. Each animal was secured on the operating table by taping of all limbs. All surgical procedures were carried out in a sterile environment.

Hair follicle microdissection

Vibrissae from GFP transgenic mice were used as donor hair follicles for transplantation into nude mice. Donor mice were killed by cervical dislocation at 4 to 6 weeks of age. Hair follicle flip-side microdissection was performed as previously described (12). In brief, the upper lip containing the vibrissa pad was excised, and fixed onto sterile gauze on a foam pad using 25G needles, with the dermal surface exposed. Drops of Dulbecco's Modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA, USA) containing 1% (v/v) antibiotic-antimycotic, (penicillin, streptomycin, and Fungizone, Life Technologies) were added as necessary to prevent the tissue from dehydrating.

With the aid of a stereomicroscope, the follicles were teased away from surrounding tissue using fine watchmakers' forceps (Fig. 1a, b). They were then plucked from the pad by pulling them gently by the neck with fine forceps (Fig. 1c). Approximately 25 to 30 hair follicles were micro-dissected from each GFP transgenic mouse. All follicles were kept in DMEM containing the antibiotics, in a 10-cm petri dish, and kept cold on ice.

Follicular unit hair transplantation

Microdessected follicles from GFP transgenic mice were transplanted into the dorsal skin of nude mice. Recipient mice were anaesthetized by inhalation of 2% (v/v) Isoflurane. A hole was made in the recipient skin at an approximate angle of 30° using a 25 gauge needle (Fig. 1d). The needle was then removed and the dissected hair follicle was placed in the hole (Fig. 1e, f) using No. 5 watchmakers' forceps. The hair follicles were placed into the skin at an angle parallel to the host pelage. All needles were fitted on 3 ml syringes for ease of use. Hair follicles were

transplanted 2–3 mm apart. To keep them in place, Vaseline gauze and a non-adherent absorbent dressing (Multigate Medical Products Pty Ltd NSW Australia) were placed over the transplanted follicles and secured with an adhesive bandage (Elastoband light adhesive bandage 75 mm, Beiersdorf Australia Ltd NSW Australia) (Fig. 1g).

When awake, the animals were put in individual cages with easily accessed food during the first 3 days after transplantation. Enrofloxacin (Baytril 25mg/mL from Bayer, Leverkusen, Germany) was added to their drinking water at 200 mg/l for 5 days after hair transplantation to prevent infection. The dressings were removed under general anaesthesia after three days.

There were two groups of experimental animals. In the "Low Follicle Density" group, each nude mouse received less than 5 whisker follicles (n=5). In the "High Follicle Density" group, the mice received 25-27 follicles (n=7). Hair fibre growth was measured in Low Follicle Density mice. The success rate of the transplantation technique, anatomy of transplanted follicles and migration of GFP positive cells into the inter-follicular epidermis in intact skin (n=5) and incisional wounds (n=2) were studied in High Follicle Density mice.

Measurement of hair growth after hair plucking

Hair lengths were measured in Low Follicle Density animals to make it easier to identify individual fibres in trichogram images. Hair fibres were plucked 6 weeks after hair transplantation to induce a new hair cycle. New hair growth was measured 15 days after plucking

by measuring the length of the new hair shaft emerging from the skin. Phototrichograms (Nikon SL-1 Macro Cool-Light and Cannon Powershot A520 cameras) were taken under general anaesthesia. The length of hair shafts emerging from the skin was measured in trichograms using an image analysis program (ImageJ, NIH, Bethesda MD) and calibrated against an internal scale marker.

Incisional wound experiment

Six weeks after hair follicle transplantation, under general anaesthesia (Isoforane inhalation), a linear wound was applied within 1mm and parallel to transplanted follicles in the upper back of two animals, using a No. 15 blade. Animals were reviewed after one hour and then every day. Wounds were harvested after 4 days.

Histological analysis

Mice were sacrificed by cervical dislocation 6 weeks after transplantation (n=5), or 4 days after incisional wound (n=2), and skin containing the transplanted follicles was collected with a minimum margin of 5 mm; this was prepared for frozen and paraffin embedded tissue sectioning. Specimens containing incisional wounds were cut in half (at a 90° angle to the liner wounds) and embedded in frozen blocks with the section side face-down to allow vertical sectioning of skin.

To prepare the frozen tissue, specimens were fixed in 10% (w/v) paraformaldehyde (PFA) for 6 hours followed by 15% (w/v) sucrose for 24 hours at 4°C. Specimens were then placed in

optimal cutting temperature compound (O. C. T). (Sakura Finetek CA USA) and frozen with liquid nitrogen. Frozen specimens were cut in 18 μm thick sections (Reichert-Jung Cryocut 1800), dried at room temperature overnight and stained the next day, or stored at –80°C. Slides with frozen sections were washed in distilled water and counterstained with diamidino-2-phenylindole (DAPI) (1 μg/mL) for 1 minute, then washed in PBS and mounted.

For paraffin embedded tissue, specimens were fixed in 10% PFA for 24 hours and then were stored in phosphate-buffered saline (PBS) at 4°C. Specimens were processed by dehydrating them through a graded alcohol series followed by infiltration with paraffin using an automated tissue processor (Shandon Hypercenter XP). Specimens were embedded in paraffin blocks and then 5 μm thick sections were prepared.

To assess innervation of transplanted hair follicles, a neuronal marker, PGP9. 5 antibody (Abcam Ab10404, Cambridge, UK) was used for immunohistochemical analysis of formalin-fixed paraffin-embedded samples. Sections were deparaffinized and rehydrated in baths of graded alcohol to water. Sections were treated with hydrogen peroxide and then incubated in a blocking reagent (Ultra V block, Thermo Fisher Scientific, Waltham, MA USA) for 30 minutes. Anti-PGP9. 5 antibody was applied at 1/1500 dilution for one hour. Immunoperoxidase staining was performed using peroxidase labeled polymer (EnVision plus, DAKO, Denmark), according to the manufacturer's instructions. All histology slides were observed using an Olympus BX-61 microscope.

To assess participation of transplanted follicles in normal homeostasis and wound re-epithelialization, migration of GFP positive cells from transplanted follicles into the inter-

follicular epidermis was examined in animals without a wound (5 animals) and animals with an incisional wound (2 animals). For each animal, the location of all GFP-positive cells was examined in approximately 50 frozen sections. Each animal was scored as having GFP-positive cells either present or absent in the inter-follicular epidermis. The difference between the proportions of wounded and unwounded animals with GFP-positive cells in the epidermis was analysed using a Chi-Square test.

Results

Transplanted follicle cycling and graft success rate

Transplantation success rates were studied in a total of 7 mice transplanted with 168 hair follicles (20–27 follicles each, Fig. 1h). The mice appeared healthy and were able to move with the dressings. Shedding hair shafts were detected on day 3 when the dressing was removed, suggesting that transplantation induced hair cycling and entry to telogen and exogen. Even when the hair shaft was shed, the transplanted follicle was still visible as a small lump under the skin. The new hair fibres were fine and appeared around day 14 (white arrow, Fig. 1j), suggesting that the follicles had re-entered anagen, the growth phase of the follicle cycle.

Most transplanted follicles contained a new hair shaft by 6 weeks (Fig. 1k). Transplant success rates were calculated, as the number of visible vibrissa hair shafts on each mouse at 6 weeks,

expressed as a percentage of the number of originally transplanted follicles. The success rate was 89% ±8% (mean ± SD)

The Low Follicle Density mice (5 mice, 2-5 follicles per mouse) were used to measure hair shaft growth rates. Follicles containing a newly-growing hair shaft were plucked 6 weeks after transplantation. A total of 16 follicles in 5 mice were plucked (*i. e.* 2-5 follicles per mouse). The length of hair shaft emerging from the skin on Day 15 was 6. 5 ±2. 0 mm (mean ± SD).

Histological analysis

Histology examination 6 weeks after transplantation in 5 animals showed normal anatomy of transplanted whisker follicles. The cavernous vessel around the transplanted follicles contained blood cells (Fig. 2b, black arrowhead), which suggests that the transplanted hair follicle was connected to the host′s circulatory system. Thick nerves were also visible around the transplanted follicles, suggesting that they had also been innervated (Fig. 2a, b, black arrow).

Fluorescent GFP-positive cells could readily be detected in cryosections of grafted follicles. Examination of skin with transplanted follicles in 5 animals with no wound showed no GFP-positive cells in the inter-follicular epidermis. Approximately 50 (47–58) frozen sections were evaluated for each animal, incorporating a total of 21 transplanted follicles presence in the histology slides. All observed GFP-positive cells stayed in the hair follicles and had not moved into the surrounding tissue (Fig. 2c–f), suggesting that the implanted follicles did not participate in homeostasis of the surrounding skin.

However, in 2 animals receiving an incisional wound within 1mm and parallel to transplanted follicles, histology examination on day 4 showed that GFP positive cells had migrated into the inter-follicular epidermis (Fig. 2g, h, white arrow). Approximately 50 (46-53) frozen sections were evaluated for each animal, incorporating a total of 6 transplanted follicles present close to the incisional wounds in the histology slides. Migrating GFP-positive cells were seen in both animals, suggesting that follicular cells migrate into healing epithelium in adjacent wounds to participate in wound reepithelialization (Fig. 2g, h).

These data suggest that cells originating from transplanted follicles migrate to inter-follicular epidermis in the presence of a skin wound (n=2) but not during the homeostasis of unwounded skin (n=5). The difference between the proportions of wounded and unwounded animals with GFP-positive cells in the epidermis was statistically significant (P<0. 01, Chi-square test, Table 1).

Conclusions

In this study, we describe a refined hair follicle transplantation protocol that uses vibrissae from GFP transgenic mice for transplantation into the back skin of recipient nude mice. The graft survival rate was similar to human scalp hair restoration surgery (about 90%). GFP-positive follicles were readily identified in histological sections and integrated normally into GFP-negative skin. Transplanted follicles were innervated and their cavernous blood vessels appeared to be connected to the host circulation system. On histology examination of transplanted follicles

in 5 nude mice, no GFP-positive cells were detected in the inter-follicular epidermis and GFP-positive dermal and epidermal cells remained confined to the site of implantation and did not expand within the host tissue suggesting that the follicle cells did not participate in homeostasis of the inter-follicular epidermis. However in the presence of a skin wound, GFP-positive cells originating from transplanted follicles did participate in healing epithelium as shown in the histology examination of two nude mice on day 4 after incisional wounds (Fig. 2g, h). This latter result was anticipated, as Ito et al. have previously shown that hair follicle cells contribute in wound re-epithelialization but do not participate in homeostasis of skin (15).

Vibrissa follicles enter telogen after transplantation, and then spontaneously re-enter anagen. Similar follicle cycling is seen after human hair transplantation and after transplantation of skin patches in mice (16). Similar to normal hair follicles, the transplanted vibrissae can be induced to cycle by plucking of their hair shafts. The growth rate of normal vibrissae in mice is about 1mm/day during the anagen phase (17). After plucking a growing whisker, a delay of 8-11 days is seen before a new hair shaft appears (17). The growth of transplanted vibrissae, 6. 5 ±2. 0 mm at 15 days after plucking, is consistent with these values.

Vibrissae at different positions in the mystacial pad have different patterns of hair growth (17). The posterior vibrissae are larger with a longer hair shaft compared to anterior follicles. The vibrissae appeared to maintain these different characteristics after transplantation, with the larger follicles producing longer and thicker hair shafts. Overall, the grafted vibrissae have normal anatomy and behave as normal hair follicles.

The hair transplantation protocol we describe has several advantages over alternative methodologies. The hairless phenotype of the nude mouse hosts is beneficial, making it easy to secure transplanted hair follicles with dressings, and to identify the transplanted follicles under the skin. Our technique minimizes damage to recipient skin and no suturing is required. Vibrissae are easy to isolate and micro-dissect with the flip side technique, besides, vibrissa follicles produce large hair fibres which are easier to detect and measure than transplanted smaller pelage hair follicles (13).

Furthermore, as shown in Figure 2, c and f, transplanted vibrissa follicles did not contain any surrounding donor inter-follicular epidermis. This makes it possible to differentiate migration of hair follicle cells from inter-follicular epidermis in lineage tracing experiments using genetically labelled donor follicles. In contrast it would be very difficult to exclude surrounding inter-follicular epidermis from the hair follicles when microdissecting pelage hair follicles. This was seen in the transplantation experiments performed by Sato *et al.* where GFP positive inter-follicular epidermis was also transplanted into the nude mice (13).

Follicle transplantation models have several applications in skin and hair biology research. Variations to the methods we describe would be possible using donor follicles from humans, transgenic mice and alternative recipients. Murine models of human skin disease could be used to investigate the role of follicle cells in these diseases. Migration of specific cell populations from hair follicles could be studied by using transgenic mouse donor follicles with cell populations that are fluorescently or luminescently labelled. Furthermore, there are cell populations in hair follicles and skin which are phenotypically similar but which nevertheless

may behave differently. For example several molecular markers such as K15 (15) are common to stem cells in hair follicles and inter-follicular skin. Transplantation of hair follicles from a genetically-labeled donor into a non-labeled host would allow follicular and inter-follicular cells to be distinguished and their behaviour to be evaluated *in vivo*.

Grafting follicles from knock-out or knock-in mice would enable studies of genetically modified follicles in the context of wild-type skin. This approach allows investigation of signalling pathways and genes of interest, specifically in follicle cells. The advantage over simply looking at the phenotype of the transgenic mice is that the indirect effects of the gene in other tissues would be removed. Thus follicle transplantation models could be used to explore the migration of hair follicle cells and their contribution to homeostasis, wound healing, skin circulation, and diseases such as melanoma and non-melanoma skin cancer.

Acknowledgements

We thank M. Okabe (Osaka University, Japan) for permission to use the C57BL/6-TgN (act-EGFP) OsbC14-Y01-FM131 mice and Dr. Maree Faux (Ludwig Institute for Cancer Research, Australia) for providing them. This work has been funded by Australasian College of Dermatologists/Fred Bauer Research Grant and a CASS Foundation Science and Medicine Grant. A. Z. Asgari has a National Health and Medical Research Council of Australia (NHMRC) Medical Postgraduate Scholarship.

Table 1

	Total number of animals	*Number of animals with GFP+ cells in epidermis*	*Number of animals without GFP+ cells in epidermis*	*p (Chi-square test)*
Unwounded	5	0	5	0. 0082
Wounded	2	2	0	

Table 1: Frequency of intra epithelial migration of GFP+ hair follicle cells in animals with and without an incisional wound.

Figure legends

Figure 1. Vibrissa follicle microdissection and transplantation in nude mice. (a–c) Hair follicle flip-side microdissection technique: the follicles are separated from surrounding tissue using fine forceps (a, b); the hair follicle is plucked from the mystacial pad by pulling it gently by the neck (c). (d–f) Vibrissa transplantation in nude mice. First a hole is made by a 25G needle (d). Then a micro-dissected follicle is placed in the hole (e, f). (g) Dressing after transplantation. (h–j) Apparent hair cycling in transplanted follicles: hair follicles lose their hair shaft between days 0 (h) and 7 (i). New hair shafts (white arrows) appear around day 14 (j). (k) Most transplanted follicles produce vibrissa-like hair shafts by 6 weeks.

Figure 2. Histology examination of transplanted vibrissae after 6 weeks in intact skin (a–f) and on day 4 of an incisional wound (g, h). (a, b) Immunohistochemical staining for PGP9.5 (a pan-neural marker). Stained nerve bundles (black arrows) can be seen around the transplanted hair follicles (white asterisks). Red blood cells (black arrowhead) can be seen in the cavernous vessel around the transplanted hair follicles. (c–f) GFP positive cells were confined in the transplanted hair follicle and were not found in the inter-follicular epidermis. (g, h) Migrating GFP positive cells into the interfollicular epidermis (white arrow) 4 days after an incisional wound. The dotted line roughly indicates the border between transplanted hair follicle and surrounding tissue and red arrows show the edge of the transplanted follicle in epidermis. White asterisk: transplanted follicle, red asterisk: autofluorescent granulation tissue. Scale bar: 100 μm.

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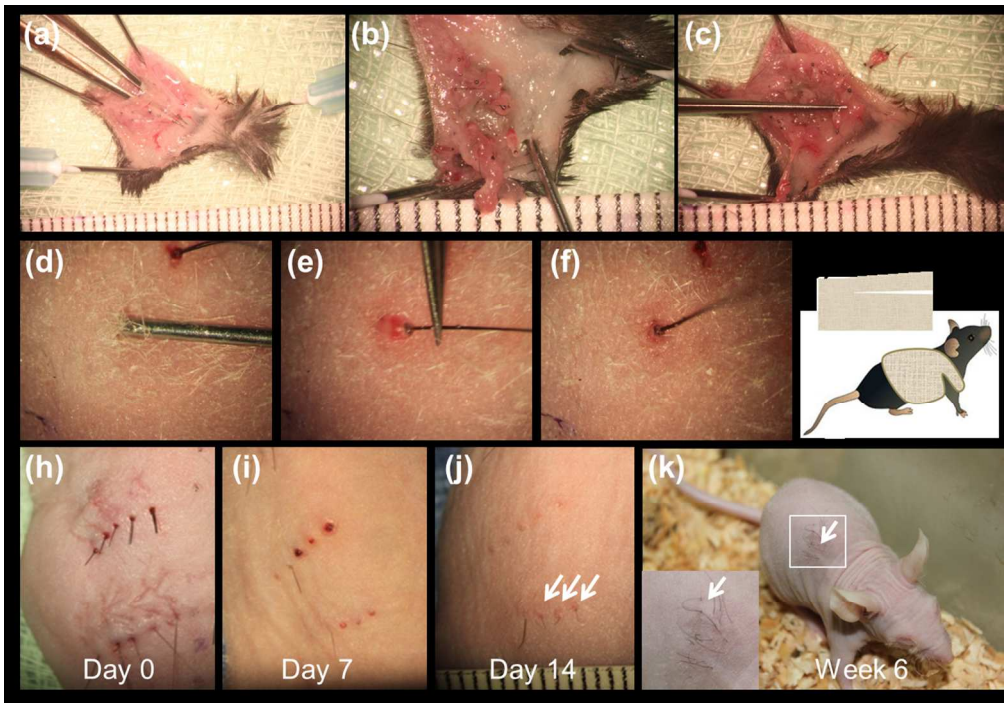

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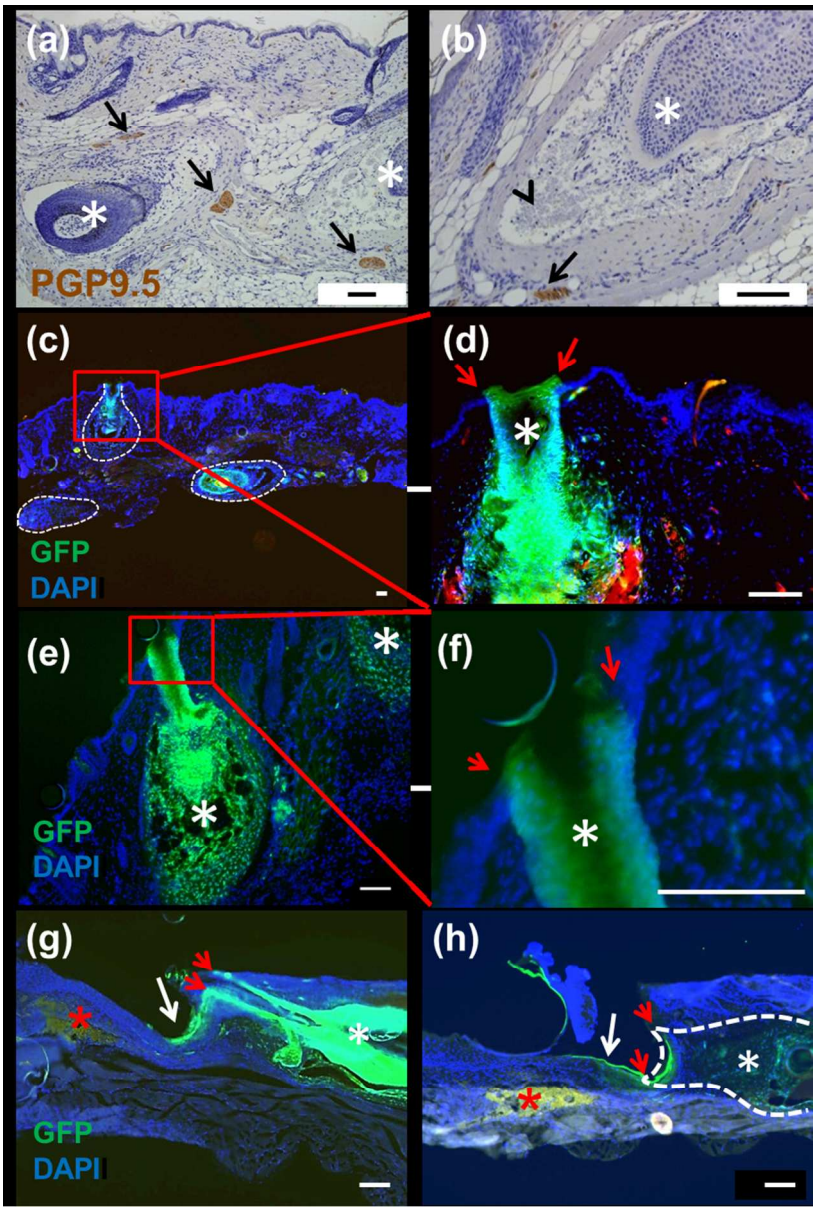

Figure 2. Histology examination of transplanted vibrissae after 6 weeks in intact skin (a–f) and on day 4 of an incisional wound (g, h). (a, b) Immunohistochemical staining for PGP9.5 (a pan-neural marker). Stained nerve bundles (black arrows) can be seen around the transplanted hair follicles (white asterisks). Red blood cells (black arrowhead) can be seen in the cavernous vessel around the transplanted hair follicles. (c–f) GFP positive cells were confined in the transplanted hair follicle and were not found in the inter-follicular epidermis. (g, h) Migrating GFP positive cells into the interfollicular epidermis (white arrow) 4 days after an incisional wound. The dotted line roughly indicates the border between transplanted hair follicle and surrounding tissue and red arrows show the edge of the transplanted follicle in epidermis. White asterisk: transplanted follicle, red asterisk: autofluorescent granulation tissue. Scale bar: 100μm.
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