ORIGINAL

Analysis of skin graft survival using green fluorescent protein transgenic mice

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Abstract : Skin grafting has become a basic and established operation technique ; however, it is not clear how skin grafts adapt to recipient beds and replace their functions. In this study, we analyzed the origin of cells in adapted transplants by using green fluorescent protein (GFP) transgenic mice, which emits green fluorescence in the whole body. The dorsal skins of GFP transgenic mice were transplanted to the back of wild-type mice. Similarly, wild-type skins were transplanted to the back of GFP transgenic mice. Since transplantation with full thickness back skin was not successful due to severe immunorejection, tail skins, which contain fewer epidermal Langerhans cells, were used for the experiments. Six months after transplantation, immunohistochemical analysis of the grafts revealed that tissues derived from ectodermal origin such as the epidermis, hair follicles, and sebaceous glands survived in transplanted grafts, but that other tissues such as the dermis, nerves and blood vessels are partly replaced by tissues from recipient beds. Our results further demonstrated that transplantation analyses with GFP transgenic mice could be a useful approach to study the origin of cells in transplants. J. Med. Invest. 54 : 267-275, August, 2007

Keywords **:** *skin graft, transplantation, GFP (green fluorescent protein), stem cells, hair follicle*

INTRODUCTION

Skin grafting has become a routine procedure in plastic surgery ; however, basic studies concerning the functional anatomy of transplanted skin are still fairly limited. An undesirable appearance with contraction, hypertrophic scars and pigmentation are sometimes observed in skin grafts ; for example, it is well known that thin skin grafts show graft contraction more often than thick skin grafts, although it is unclear why the contraction differs by the thickness of skin grafts (1).

Another example is that skin grafts excised from the abdomen often cause problems especially in Mongoloids, such as aberrant pigmentation, heterotopic hair growth, and different skin texture, when transplanted onto the palm. As the palm has glabrous skin and few melanin, grafted skin with pigmentation and hair result in an undesirable appearance. It has been reported that the sweat glands and sebaceous glands of grafts have the same anatomical structure as before transplantation (1). These observations suggest that some characteristics of the skin graft are conserved after transplantation ; however, emotional sweating is observed in skin grafts transferred to the palmar and plantar (2, 3). Similarly, skin grafts acquire the sensory pattern of the recipient beds (3, 4). These findings suggest that regenerated nerves derived from the recipient side can ingrow to skin grafts. Furthermore, the survival of skin grafts is dependent on the rapid re-establishment of an adequate blood circulation ; how-

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ever, it is unknown whether the vessels for new blood circulation originate in the old capillaries surviving in the grafts or whether new capillaries ingrew from the recipient tissues (5-10).

In this study, we asked whether grafted skin survives on recipient tissues or whether grafts are eventually replaced by recipient cells when engraftment has succeeded.

Skin contains various complex elements, such as the epidermis, dermis, hair follicles, sebaceous glands, nerves, and blood vessels. To distinguish the grafts and recipient tissues, we took advantage of GFP transgenic mice and immunohistochemicaly analyzed how transplanted skin contributes to regenerated tissues in host mice.

MATERIALS AND METHODS

Mouse strains

GFP transgenic mice (C57BL6 strain) that express enhanced GFP (EGFP) cDNA under the control of a chicken β-actin promoter and cytomegalovirus virus enhancer were kind gifts from Dr M. Okabe (Osaka University). As non-GFP control mice, C57BL6 strain mice were used. The mice were maintained under pathogen-free conditions and were handled in accordance with the Guidelines for Animal Experiments of Tokushima University Faculty of Medicine. The experiments were initiated when the mice were 8-16 weeks of age.

Transplant Surgical Techniques

The mice were anesthetized by intraperitoneal injection of sodium pentobarbitone (0.1 mg/g body weight). The back skin (1.5×1.5 cm) or the tail skin (1.5×1.0 cm) was grafted in the center of the dorsal region of the anesthetized recipient mouse (Fig. 1). The bandages were removed 7 days after surgery, and the grafts were observed weekly. Photographs were obtained with a KODAK DC260 digital camera. Images were processed using Adobe Photoshop 5.0 (Adobe Systems).

Immunohistochemistry

Grafted skins dissected from GFP transgenic mice (n=69) or non-GFP control mice (n=66) were fixed in 5% buffered formaldehyde for 24 hr at room temperature, and embedded in paraffin using automatic embedding equipment (Sakura Rotary, Sakura Seiki). Sections (2.5 μm thickness) were mounted on poly-L-lysine-coated glass slides, deparaffinized in xylene, and dehydrated in a descending series of ethanol. To inactivate endogenous peroxidase, the dehydrated sections were treated with 0.3% hydrogen peroxide in methanol for 30 min and rinsed in tap water. The slides were then blocked with 1.5% bovine serum albumin (BSA) for 1 hr at room temperature followed by incubation with rabbit polyclonal anti-GFP antibody (Santa Cruz Biotechnology) at a final concentration of 1 : 200 in PBS overnight at 4°C. After washing with PBS, slides were incubated with anti-rabbit HRP (Cell Signaling Technology) at a final concentration of 1 : 200 for 1 hr at room temperature. After washing with PBS, slides were developed for 2 min at room temperature in 0.1% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.03% hydrogen peroxide to visualize GFP-positive cells. The reaction was stopped by washing slides in distilled water for 5 min. The sections were also counterstained with Mayer's Hematoxylin (Muto Pure Chemical), dehydrated, and mounted in Enthellan New (Merck). (11)

RESULTS

To analyze the survival of skin grafts and the replacement of grafts by recipient tissues, we used GFP transgenic mice that express EGFP protein in all tissues. Initially we tried full thickness skin obtained from the center back of mice for transplantation experiments ; however, the survival rate of grafts was not satisfactory, and the grafted skins were often severely contracted and rejected within two weeks. To overcome this problem, we next tested tail skin for transplantation experiments. Transplantation was remarkably improved with tail skin and the grafts successfully survived on the back of mice. Although the survival rate of the full thickness dorsal skin grafts for two weeks after transplantation was 15/41 (36%), the tail skin showed a much improved survival rate, 126/135 (93%). Thus, we decided to use the tail skin for the later study. As shown in Fig. 1, full thickness tail skin grafts obtained from GFP mice were transplanted to the dorsal region of wild-type mice (Fig. 1. left). We also performed all other combinations including transplantation of the wild-type skin grafts to the back of GFP mice (Fig. 1. right, Table 1) ; however, when GFP skin grafts were transplanted to wild-type mice, we found that GFP grafts in the wild-type mice were gradually contracted. As shown in Fig. 2, we often observed cicatricial contracture of GFP skin grafts that progressed

over several months. Table 1 summarizes the results of transplantation experiments. When tail skin grafts of GFP transgenic mice in wild-type mice were analyzed, we observed that most of the grafts began to contract after about two weeks and were slowly rejected. Only 12% (8/66) of GFP grafts were successfully engrafted over 6 months. In contrast, wild-type mouse skin grafts transplanted to GFP transgenic mice were well engrafted, and the rate of successful transplantation was 51% (35/69).

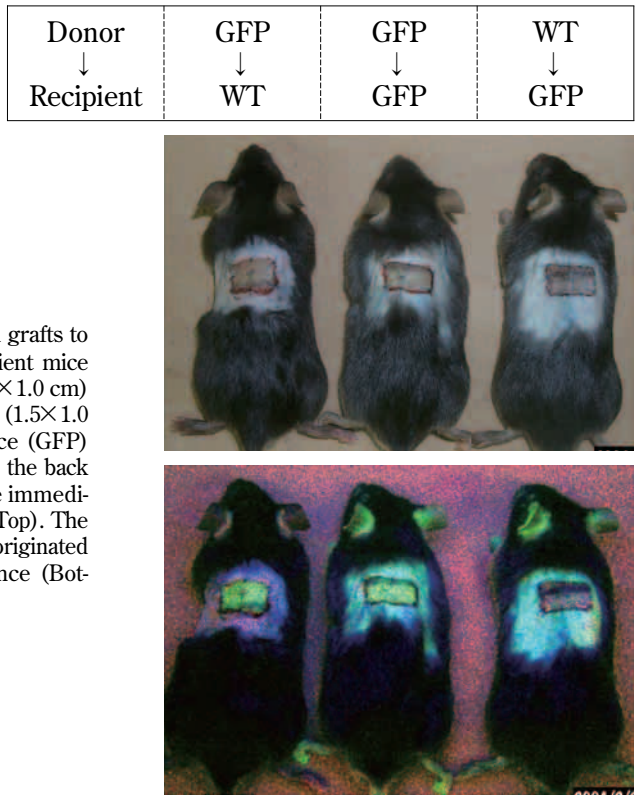

Fig. 1. Transplantation of full thickness skin tail grafts to the back of mice. Hairs on the back of the recipient mice were clipped and a full thickness skin wound (1.5×1.0 cm) was prepared. The same size of full thickness skin (1.5×1.0 cm) was obtained from GFP transgenic donor mice (GFP) or wild-type donor mice (WT), and transplanted to the back of the recipient mice. GFP mice and wild-type mice immediately after surgical operation under normal light (Top). The same mice illuminated under excitation light. Skin originated from GFP transgenic mice emits green fluorescence (Bottom).

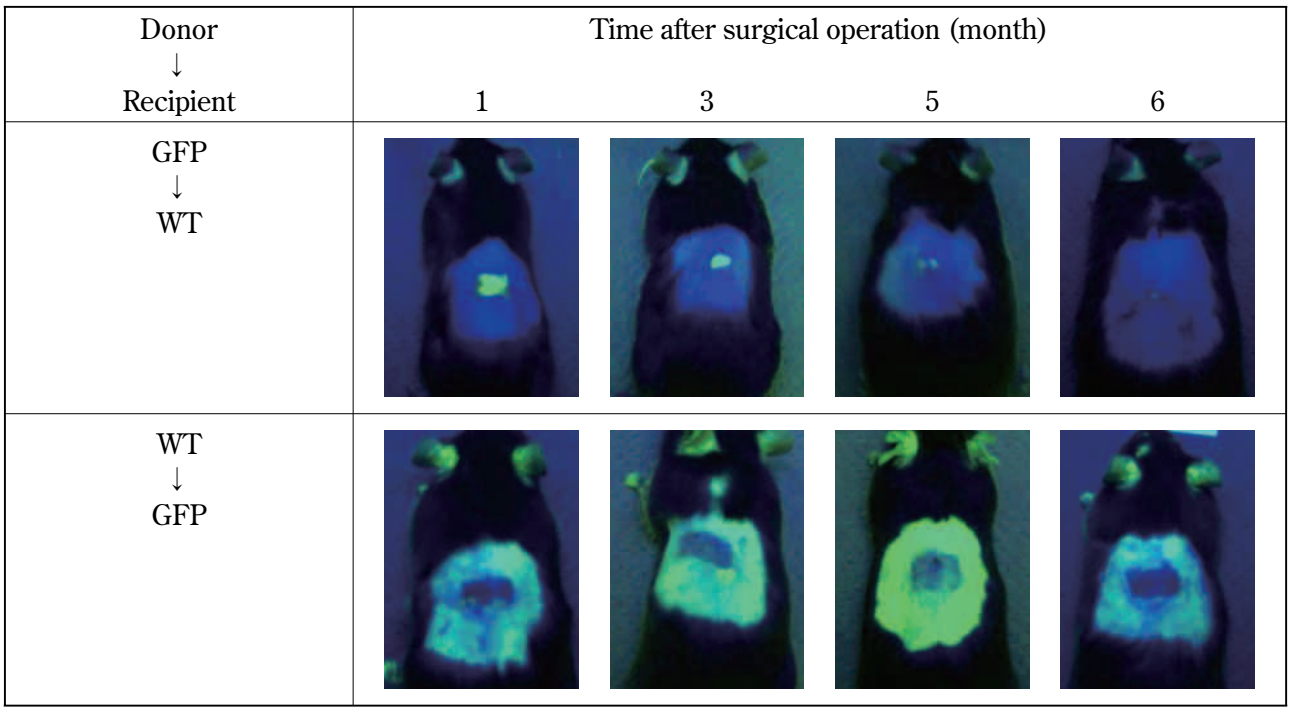

Fig. 2. The skin grafts of GFP mice were gradually rejected by wild-type recipient mice. The time course analysis of grafted skins was 6 months. Tail skin of the GFP transgenic mice was transplanted to wild-type mice (Top). GFP tail skin was green under ultraviolet rays. The size of the GFP skin graft gradually decreased due to contracture of the graft. Tail skin of wild-type mouse was transplanted to GFP transgenic mice (Bottom). Wild-type skin grafts do not emit green fluorescence, and the grafted skin took successfully for 6 months without contracture.

In this experiment, the rate of slow rejection of skin grafts was significantly decreased to 45% (31/69). To assess the contribution of grafts and recipient tissues for skin regeneration, localization of GFP-positive cells in the skin grafts was analyzed. In formaldehyde-fixed and paraffin-embedded tissues, GFP was severely denatured and no autofluorescence of GFP was observed. To visualize the localization of GFP-positive cells, we performed immunohistochemical analyses with anti-GFP antibody. As shown in Fig. 3A and C, when wild-type grafts were transplanted to GFP mice, most of the tissues including the epidermis, hair follicles, and sebaceous glands were GFP-negative. Interestingly, GFP-positive cells were found in the dermis in a punctuated manner. Observation at high magnification revealed that GFP-positive cells were detected in some nerves, blood vessels, and the dermis (Fig. 4A, C, E). Similarly, GFP grafts transplanted to wild-type mice were analyzed (Fig. 3B, D and Fig. 4B, D, F). Most of the graft cells were GFP-positive, and many nerves and blood vessels were also GFP positive. These results suggest that the epidermis, hair follicles, and sebaceous glands survive in transplanted tissues, and that blood vessels and nerves were partially replaced by those derived from recipient tissues.

During the course of experiments, we noticed that skin grafts slowly contracting over several months did not have any hair follicles (83/134, 62%) (Fig. 5). In contrast, successfully engrafted transplants always maintained hair follicles (51/134, 38%).

Table 1. Summary of transplantation experiments using tail skin grafts. Full thickness tail skins were transplanted to the back of recipient mice as shown in Fig. 1. We tested all four combinations with donor tail skin grafts and recipient mice, and the results were analyzed 6 months after operation.

Donor	Recipient	Successful transplantation for 6 months	Slow rejection	Rejection within 1 week
GFP	WT	12% (8/66)	79% (52/66)	9% (6/66)
GFP	GFP	100% (3/3)	0% (0/3)	0% (0/3)
WT	WT	100% (5/5)	0% (0/5)	0% (0/5)
WT	GFP	51% (35/69)	45% (31/69)	4% (3/69)

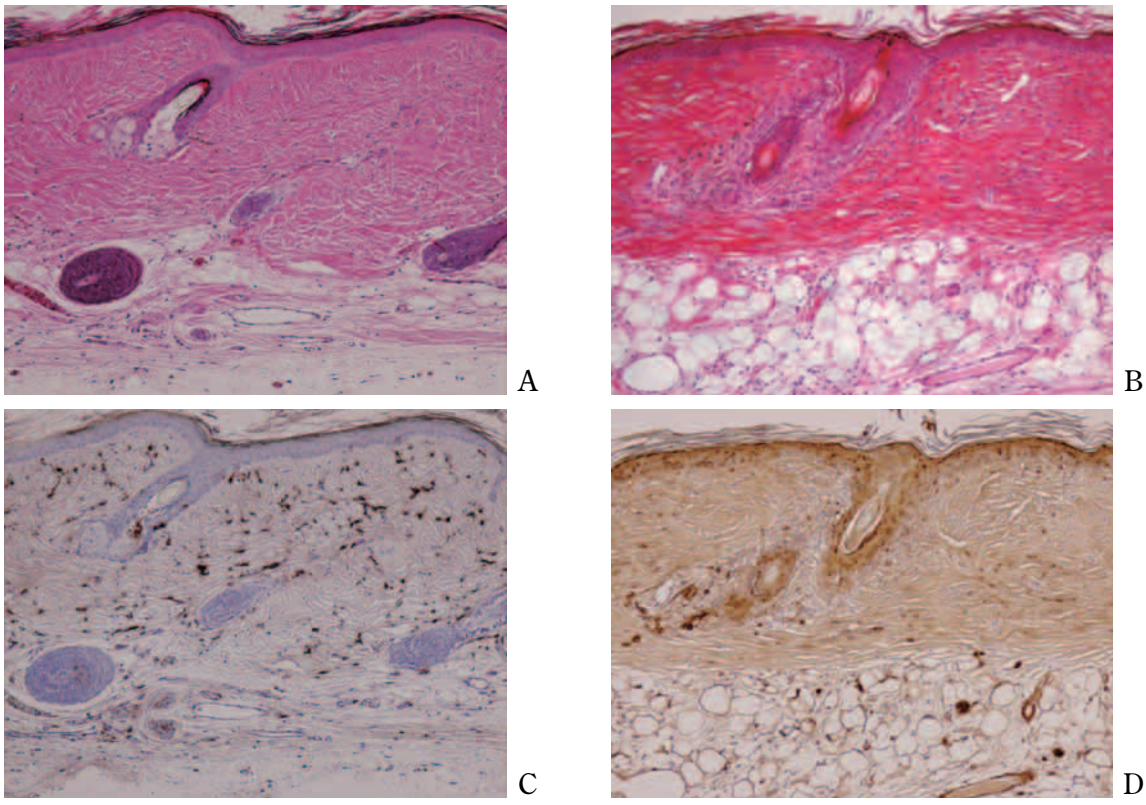

Fig. 3. Immunohistochemical analysis of the grafted skin 147 or 180 days after transplantation (low magnification, ×100). Tail skin of wild-type mice was transplanted to the back of GFP transgenic mice (A, C). Tail skin of GFP transgenic mice was transplanted to the back of wild-type mice (B, D). Sections were stained with H.E. (A, B). Successive sections were stained with anti GFP-antibody (C, D). GFP-positive cells were detected with DAB staining (brown). Epidermis, hair follicles, and sebaceous glands survived in the grafts. The dermis of skin grafts partially survived.

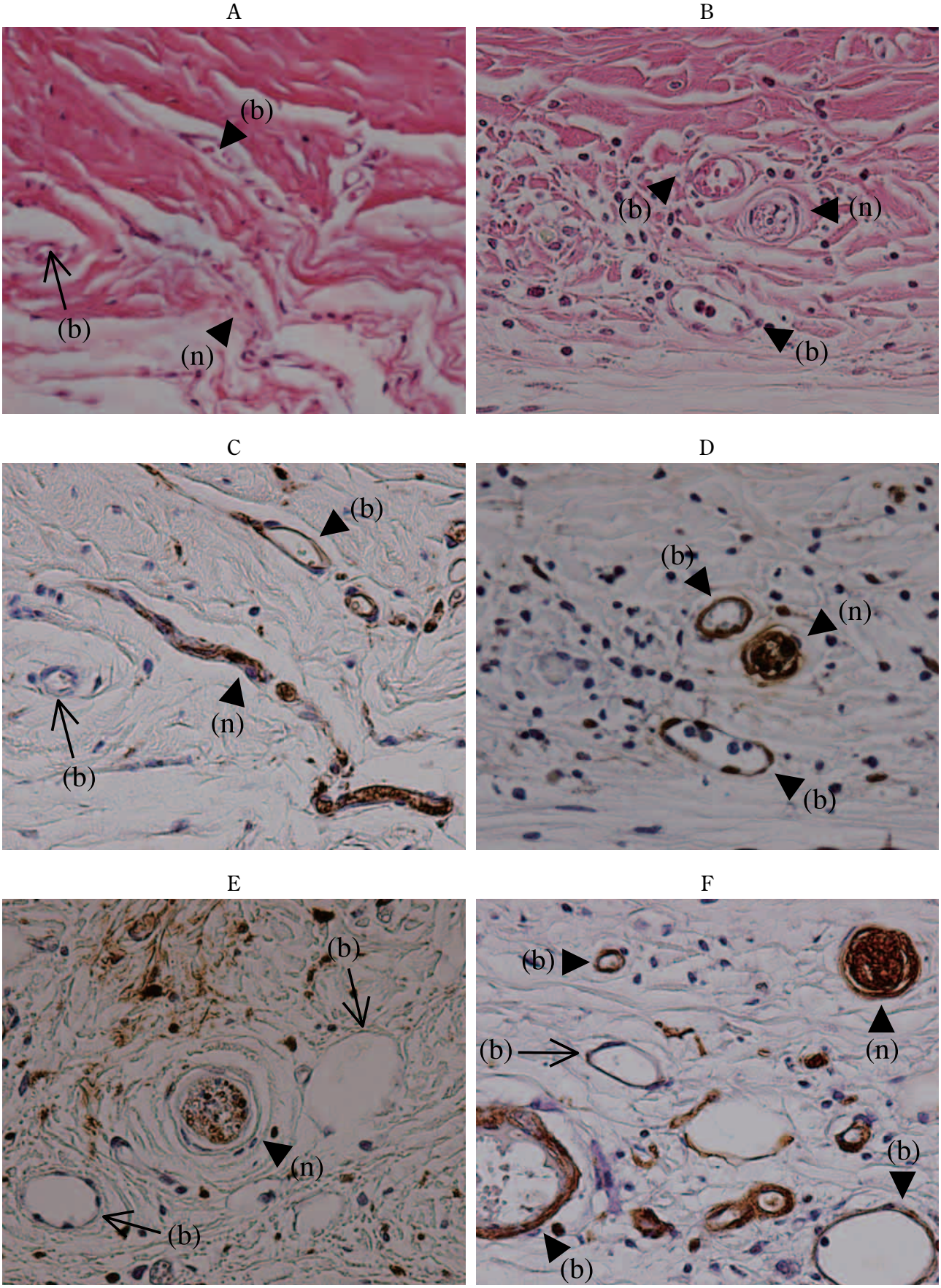

Fig. 4. Immunohistochemical analysis of grafted skin at high magnification. Sections obtained from skin grafts 147 or 180 days after transplantation were immunohistochemicaly analyzed with GFP antibody at high magnification (×400). Wild- type tail skin transplanted to GFP transgenic mice (A, C, E). GFP tail skin transplanted to wild-type mice (B, D, F). Blood vessels (b) and nerves (n) are shown. Arrows and arrow heads indicate GFP-positive and GFP-negative cells, respectively.

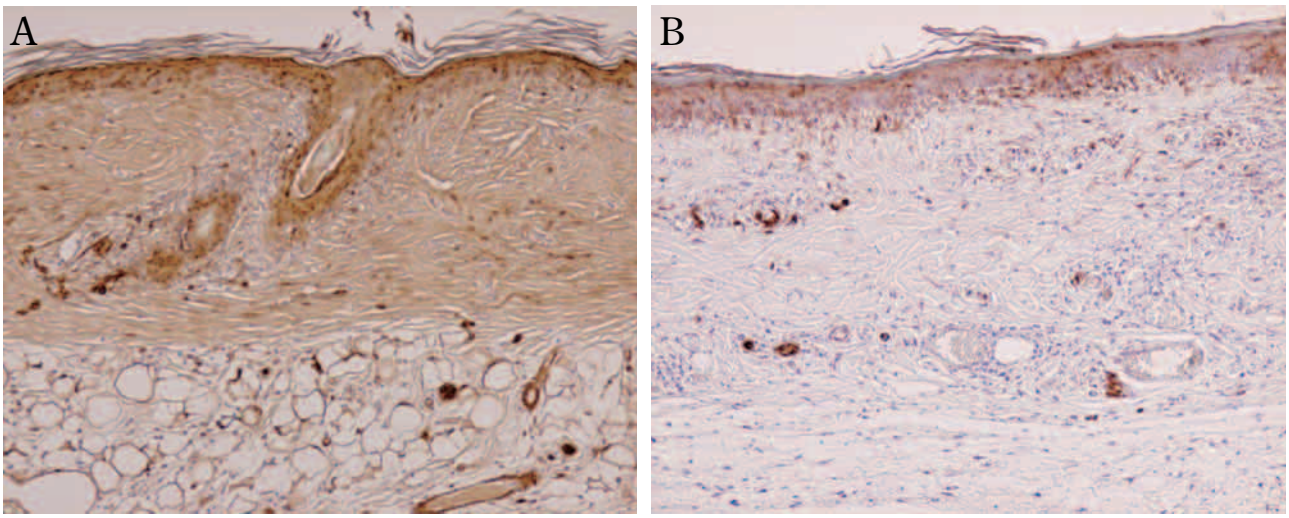

Fig. 5. Histological analysis of transplants with or without contracture. The tail skin graft obtained from GFP transgenic mice was transplanted to wild-type mice. Successful skin grafts without contracture after 180 days posttransplantation (A). Grafted skin with contracture after 84 days posttransplantation. Contracted skin grafts lost hair follicles (B).

DISCUSSION

In this paper, we analyzed the tissue element specificity of skin grafts for survival on recipient tissues. So far, various methods have been reported to distinguish transplanted tissues. Y-chromosomes were used to probe transplanted male tissues from female recipients (12). Human antigens were used to detect human grafts transplanted to nude mice (5, 6). LacZ was also useful to identify ROSA26 mice transplants in wild-type mice (13, 14) ; however, these methods required multiple procedures including fixation of tissue samples, and did not allow analyses of live animals.

In this study, we used GFP transgenic mice to distinguish grafts and recipient tissues. This system allows us to visualize the process of engraftments and to distinguish grafts from recipient tissues simply by illuminating under excitation light. Although the expression of GFP in transgenic mice is not ideally homogeneous, and the protein expression level was especially high in cardiac, skeletal, and smooth muscles, GFP is also expressed in the skin (15-17).

Unexpectedly, we had difficulty transplanting mouse dorsal skins to others. Skin is an integral part of the immune system, in which epidermal Langerhans cells (LCs) function as specialized antigen-presenting cells. Thus, the immunogenecity of skin allograft directly correlates with the density of LCs. For example, skin grafts derived from the trunk and ear express the highest density of LCs and are most rapidly rejected, whereas tail skin with relatively few LCs shows a much milder response (18). According to this information, we tested the transplantation of tail skins, and found that more than 90% of tail skin grafts survived over 100 days on the back of mice. Interestingly, we observed that GFP tail skin grafts transferred to wild-type mice were gradually rejected within 6 months. In contrast, wild-type grafts transplanted to GFP mice showed much less rejection (Table 1). Although most of the published literature describes successful transplantations with GFP-expressing tissues or cells, they were rather short-term observations (19). Taking this into account, our results suggested that exogenous GFP could cause weak but significant immunoreactivity that results in this slow rejection of GFP-expressing cells, and gives apparently lower survival rates of GFP grafts than wild-type skins during long-term engraftment.

Histological analyses of GFP mice skin grafts that survived for more than 6 months in wild-type mice revealed that GFP-positive grafted cells surviving for a long time were the epidermis, hair follicles, and sebaceous glands. In contrast, only part of the dermis, blood vessels, and nerves was GFP-positive. When wild-type skin was transplanted to GFP transgenic mice, we observed that GFP-positive cells, presumably lymph cells derived from host mice, infiltrated into the periphery of hair follicles in wild-type skin grafts for 1 week after transplantation (data not shown). After 6 months, however, the number of GFP-positive cells was decreased and only a limited number of GFP-positive cells were sparsely detected throughout the dermis. GFP-positive cells were not detected in the epidermis, hair follicles, and sebaceous glands. A part of the dermis, blood vessels, and nerves was GFP-positive.

Our results suggested that epidermal tissues survived in host animals for a long time but dermal tissues are gradually replaced by host tissues.

Survival of grafts in host animals depends on cell types ; for example, grafted bones and arteries survived for the initial period after transplantation. However, they rather function as the framework of grafts, and are gradually replaced by host osteoblasts or host endothelial cells, respectively. Then, new bones or blood vessels take the place of grafted tissues (2). In the case of skin grafts, survival of the epidermis of skin grafts seems apparent, because abdominal skin transplanted to the palm maintain their original appearance ; therefore, Peer proposed the cell survival theory, in which the great majority of autogenous tissue grafts survive and are not replaced by host tissue cells (2). On the other hand, Roger, *et al.* claimed that a large part of blood vessels on grafted skin was replaced by recipient vessels (20). They also claimed that grafted dermal tissues were partially replaced ; however, a large part of the epidermis, sweat glands, hair follicles, collagen fiber and elastic fiber of the grafted skin survived (20). The results obtained in our study using GFP transgenic mice rather support Roger's model.

According to Oshima, *et al*., stem cells of the epidermis are localized in the bulge region of hair follicles, and these stem cells have been shown to differentiate into the epidermis and hair follicles (14). In their experiments, they amputated the bulge region of vibrissal follicles of wild-type adult mice and transferred the identical bulge region obtained from Rosa 26 adult mice. As a result, they demonstrated that transplanted cells contribute to form all epithelial lineages including hair follicles, the outer root sheath, inner root sheath, and sebaceous gland (14). In our experiment, when grafted skins slowly contracted over several months, the loss of hair follicles in grafted tissues was always observed (Fig. 5), and the epidermis without hair follicles was eventually replaced by host cells. These results suggest that epidermal stem cells localized in the bulge region play important roles in the successful engraftment of skin. Survival of grafted tissues for a long time suggests the presence of stem cells in the grafted tissues. Similarly, failure of skin graft survival could be explained by insufficient stem cells to maintain the grafted tissues.

In this study, we also observed that the dermis of the skin graft was gradually replaced by host tissues. Nerves stained with anti-S100 antibody were also partially replaced. Although stem cells of the dermis have not been identified yet, unidentified stem cells in the dermis or mesenchymal stem cells derived from bone marrow of host animals may be cooperatively involved in successful engraftment.

Cultured epidermal allografts have been considered to promote rapid healing and to survive as epidermis ; however, recent reports rather suggest that cultured allografts accelerate the healing processes by temporary coverage of the wound region, and are finally replaced by host epithelium (21). To improve skin transplantation methods, tissue-engineered hybrid skin layered on an acellular dermal matrix has been developed for a therapeutic option in clinical treatment. Takami, *et al.* developed a hybrid version of a skin graft. They used skins obtained from a skin bank, and prepared dermal tissues by washing away the epidermis layers (22). The dermal tissues were sandwiched with cultured epidermal cells and fibroblasts derived from recipients. The hybrid skins were then transferred to female patients with severe burns. As a result, the hybrid grafts were successfully engrafted for 45 days after transplantation. Since epidermal LCs in xenografts cause immunorejection and necrosis of the grafts when skin grafts are transferred to other patients, tissue-engineered skins composed of cultured autologous epidermal cells and fibroblasts with acellular alogenic dermal matrix as a scaffold seem a superior method to avoid severe rejection after transplantation (22).

Although various transplantation techniques has been developed for clinical treatment, it has not been well elucidated whether the grafted skin survives or is replaced in recipient tissues. In this study, we demonstrated that epidermal cells basically survived, and that dermal tissues were gradually replaced by host cells. Our data also suggested the importance of stem cells in bulge regions for successful engraftment. Hybrid-type tissue-engineered skins containing epidermal cells and acellular dermal matrix, however, do not have blood vessels, nerves and other adnexa, such as hair follicles and sebaceous glands and therefore have difficulty surviving permanently in recipient tissues without stem cells in the bulge region. Further clinical cases are required for the proper evaluation of these tissue-engineered skins in the future. Very recently, Capla, *et al.* demonstrated that replacement of the donor graft vasculature by endothelial and endothelial progenitor cells from the recipient along preexisting channels is the predominant mechanism for skin graft revascularization (13). The mechanisms they

proposed for skin graft vascularization seem quite interesting. Further analyses of the regeneration of neural cells in skin grafts may also provide crucial knowledge for more improved clinical treatment.

CONCLUSION

Taking advantage of GFP transgenic mice, we analyzed the tissue specificity of cell survival in skin grafts. Although GFP causes weak immunoreactions in recipient animals during long-term observation, histological analysis revealed that the ectodermal layers including the epidermis, hair follicle, and sebaceous glands of grafted tissues survived in the host tissues. Dermis including nerves and blood vessels of the grafts, however, were partially replaced by host tissues. Our data also raised the possibility that epidermal stem cells in the epidermis play important roles in the successful skin engraftment.

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