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Nestin-Expressing Interfollicular Blood Vessel Network Contributes to Skin Transplant Survival and Wound Healing

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ABSTRACT

Using nestin-driven green fluorescent protein (ND-GFP) transgenic mice, we previously demonstrated an inter-hair-follicle blood vessel network that expresses ND-GFP and appears to originate from ND-GFP expressing hair-follicle stem cells. We report here that angiogenesis of transplanted skin or healing wounds originates from this ND-GFP-expressing microvasculature network. ND-GFP-expressing blood vessels were visualized growing from the ND-GFP-expressing hair-follicle stem cell area and re-establishing the dermal microvasculature network after skin transplantation or wound healing. When the ND-GFP stem cell area from the vibrissa (whisker) from ND-GFP mice was transplanted to transgenic mice ubiquitously expressing RFP, we observed chimeric ND-GFP-RFP blood vessels, suggesting the joining of inter-follicular blood vessel networks from the transplant and host. These observations suggest that the inter-hair-follicle blood-vessel network contributes to skin transplant survival and wound healing. J. Cell. Biochem. 110: 80–86, 2010.

KEY WORDS: GFP; NESTIN; HAIR FOLLICLE; INTERFOLLICULAR BLOOD VESSEL NETWORK; SKIN TRANSPLANT; SKIN WOUND HEALING

We have previously reported that nestin, a marker for neural progenitor cells, is also selectively expressed in hair-follicle stem cells [Li et al., 2003]. Nestin-driven green fluorescent protein (ND-GFP)-expressing cell, from the hair-follicle bulge area in ND-GFP transgenic mice, behave as stem cells, differentiating to form much of the hair-follicle each hair growth cycle [Li et al., 2003].

Subsequently, we demonstrated that ND-GFP stem cells isolated from the hair-follicle bulge area can differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro. These pluripotent ND-GFP stem cells are positive for the stem cell marker CD34, as well as negative for keratin 15, suggesting their relatively undifferentiated state. The apparent primitive state of the ND-GFP hair follicle stem cells is compatible with their pluripotency [Amoh et al., 2005a].

We then showed that nestin-expressing hair follicle stem cells transplanted into the gap region of severed sciatic nerves greatly enhance the rate of nerve regeneration and the restoration of nerve function in mice. The nestin-expressing follicle cells transdifferentiated largely into Schwann cells, which are known to support neuron regrowth [Amoh et al., 2005b].

We then severed the thoracic spinal cord of C57BL/6 immunocompetent mice and transplanted GFP-expressing hair follicle stem cells to the injury site. Most of the transplanted cells also differentiated into Schwann cells that apparently facilitated repair of the severed spinal cord. The rejoined spinal cord reestablished extensive hind-limb locomotor performance [Amoh et al., 2008].

We also previously showed in the ND-GFP transgenic mice that ND-GFP also labels developing skin blood vessels that originate from the hair follicle stem cell area and form a follicle-linking network. This is seen most clearly by transplanting ND-GFP-labeled vibrissa (whisker) hair follicles to unlabeled nude mice. New vessels grow from the transplanted follicle, and these vessels increase when the local recipient skin is wounded. The ND-GFP-expressing structures are blood vessels, since they display the characteristic endothelial-cell-specific markers CD31 and von Willebrand factor [Amoh et al., 2004].

In the present study using transgenic mice expressing red fluorescent protein (RFP) as hosts, we demonstrate that ND-GFP-expressing blood vessels from skin transplants join the host dermal microvasculature network and also play a role in wound healing.

MATERIALS AND METHODS

ND-GFP TRANSGENIC MICE

The regulatory elements of the nestin gene were used to generate transgenic mice in which nestin-expressing cells also express GFP.

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These mice are termed nestin-driven (ND)-GFP mice. The GFP-positive cells, therefore, reflect the distribution of nestin-positive cells. In the adult brain, nestin-GFP cells are approximately 1,400-fold more efficient in generating neurospheres than are GFP-negative cells. Nestin-GFP-expressing cells encompass the majority of the neural stem cells in the adult brain [Mignone et al., 2004]. We subsequently showed that ND-GFP is expressed in hair follicle stem cells and in an interfollicular blood vessel network in ND-GFP mice [Li et al., 2003; Amoh et al., 2004].

TRANSPLANTATION OF ND-GFP-EXPRESSING SKIN INTO NUDE MICE

ND-GFP transgenic mice, 6–8 weeks old, with almost exclusively telogen hair follicles, were used as donors in skin transplantation experiments. The mice were anesthetized with a mixture of ketamine (0.04 mg/g body weight), xylazine (0.03 mg/g body weight), and acepromazine (0.02 μg/g body weight) in phosphate buffered saline (PBS). The mice were depilated by a hot mixture of rosin and beeswax. Skin samples for transplant (2 cm × 2 cm) were dissected from the dorsal skin at the level of the subcutis. The subcutis was removed, and the skin was rinsed in sterile calcium- and magnesium-free PBS (Cellgro, Herndon, VA). The ND-GFP-expressing donor skin was transplanted into the dorsal skin of 6–8-week-old recipient *nu/nu* mice (Harlan, San Diego, CA) under anesthesia. The donor skin was transplanted with nylon sutures (6-0). After 10, 18, and 28 days, the skin samples of the transplanted mice were excised under anesthesia and directly observed by fluorescence microscopy from the dermis side in the vertical thick sections (0.5 mm). Skin samples were also used for frozen sections. The skin samples used for frozen sections were embedded in tissue-freezing embedding medium (Triangle Biomedical Sciences, Durham, NC) and frozen at −80°C overnight. Frozen sections of 5 μm thickness were cut with a Leica CM1850 cryostat and air-dried. The sections were directly observed by fluorescence microscopy; after that, they were used for the immunohistochemical staining of CD31.

WOUND FORMATION IN ND-GFP MICE

ND-GFP transgenic mice, 6–8 weeks old, with almost exclusively telogen hair follicles, were used for wound formation, as described above. The mice were anesthetized with the mixture of ketamine, xylazine, and acepromazine in PBS. The mice were depilated by a hot mixture of rosin and beeswax. The full-thickness skin was folded, and two neighboring, full-thickness wounds, ≈15 mm apart, were made with a 2-mm biopsy punch. Skin was excised at 15 days after wounding under anesthesia. The skin samples were directly observed by fluorescence microscopy from both the epidermis and dermis side in vertical thick sections (0.5 mm).

FLUORESCENCE MICROSCOPY

The ND-GFP skin samples were directly observed under an Olympus IX-71 inverted microscope equipped with a mercury lamp power supply. The microscope had a GFP filter set (Chroma Technology, Brattleboro, VT). The samples of dorsal skin were directly observed.

IMMUNOHISTOCHEMICAL STAINING

CD31 in the frozen sections from ND-GFP transgenic mice was detected with the anti-rat immunoglobulin horseradish peroxidase detection kit (BD Pharmingen, San Diego, CA) following instructions from the manufacturer. The primary antibody used was CD31 mAb (1:50). Substrate–chromogen 3,3′-diaminobenzidine staining was used for antigen staining. CD31 mAb (CBL1337) was purchased from Chemicon (Temecula, CA).

TRANSGENIC RED FLUORESCENT PROTEIN NUDE MICE

The RFP nude mouse was obtained by crossing non-transgenic nude (nu/nu) mice with the transgenic C57/B6 mouse in which the beta-actin promoter drives RFP (DsRed2) expression in essentially all tissues [Vintersten et al., 2004]. In crosses between nu/nu RFP male mice and nu/+ RFP female mice, the embryos fluoresced red. Approximately 50% of the offspring of these mice were RFP nude mice. In the RFP nude mouse, all the organs brightly express RFP, including the heart, lungs, spleen, pancreas, esophagus, stomach, duodenum, the male and female reproductive systems; brain and spinal cord; and the circulatory system, including the heart, and major arteries and veins. The skinned skeleton highly expressed RFP. The bone marrow and spleen cells were also RFP-positive [Yang et al., 2009].

TRANSPLANTATION OF ND-GFP-EXPRESSING HAIR FOLLICLE STEM CELLS INTO THE SUBCUTIS AND UNDERNEATH THE KIDNEY CAPSULE OF RFP TRANSGENIC NUDE MICE

Male or female ND-GFP mice, 8–12 weeks old, were used as the source of ND-GFP hair follicle stem cells. Mice were anesthetized with the mixture of ketamine, xylazine, and acepromazine in PBS as described above. All surgical procedures were performed under a sterile environment. The upper lip containing the vibrissal pad was cut and its inner surface was exposed. The vibrissae were dissected under a binocular microscope and plucked from the pad by pulling them gently by the neck with fine forceps as described [Kobayashi et al., 1993]. Collagen capsules of each follicle were removed with a 27G needle (BD Syringe). The ND-GFP-expressing hair follicle stem cell area, whose location was easily identified by the presence of the nerve endings, was then amputated from the upper-part of the follicles using a fine needle. All samples were then incubated at 37°C in DMEM-F12 (GIBCO/BRL), containing B-27 (GIBCO/BRL) and 1% penicillin/streptomycin (GIBCO/BRL). After 48 h, the ND-GFP hair follicle stem cell area was transplanted into the subcutis or underneath the kidney capsule of RFP transgenic nude mice. The incision was closed with polypropylene sutures (6-0, Ethicon). After 5, 10, and 14 days, the subcutis of the transplanted mice was observed directly with the IV100 Scanning Laser Microscope (Olympus Corp., Tokyo, Japan) in a skin flap.

IMAGING

The Olympus IV100 intravital scanning laser microscope (Olympus Corp.) was used for in vivo imaging experiments [Yang et al., 2007].

RESULTS AND DISCUSSION

GFP IMAGING OF BLOOD VESSEL NETWORK AFTER SKIN TRANSPLANTATION

At day 10 after transplantation of ND-GFP-expressing skin into nude mice, skin samples were directly observed in vertical thick sections (0.5 mm). ND-GFP-expressing blood vessels were found in the transplanted ND-GFP-expressing skin (Fig. 1a). At day 18, the ND-GFP-expressing blood vessels were associated with anagen hair follicles in the transplanted ND-GFP-expressing skin. The ND-GFP-expressing blood vessels were associated with the ND-GFP-expressing hair follicle stem cells (Fig. 1b). Results of immunohistochemical staining showed that ND-GFP-expressing vessels were CD31-positive (Fig. 1c).

At day 18, the skin samples were directly observed with epidermis up and dermis down. ND-GFP-expressing blood vessels were growing in the transplanted ND-GFP-expressing skin. The ND-GFP-expressing blood vessels formed a network with the skin blood

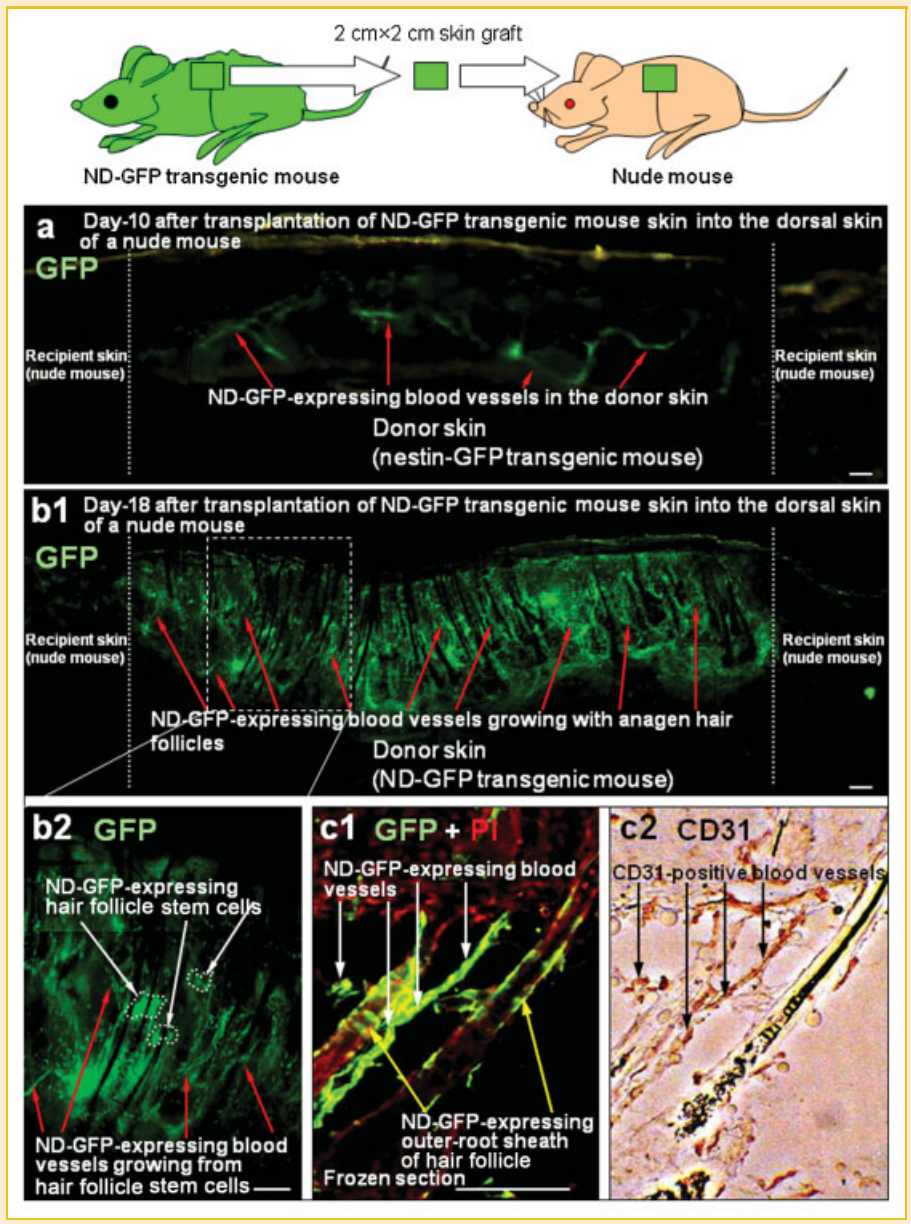

Fig. 1. a: On day 10 after ND-GFP-expressing skin transplantation into nude mice, skin samples were directly observed in vertical thick sections (0.5 mm). After ND-GFP-expressing skin transplantation into non-transgenic nude mice, the ND-GFP-expressing blood vessels (red arrows) were found in the transplanted ND-GFP-expressing skin. b: At day 18 after ND-GFP-expressing skin transplantation into nude mice, ND-GFP-expressing blood vessels (red arrows) were associated with anagen hair follicles in the transplanted ND-GFP-expressing skin. b2: Higher magnification of area of (b1) indicated by the white dashed box. The ND-GFP-expressing blood vessels (red arrows) were connected to the ND-GFP-expressing hair follicle pluripotent stem cells (white arrows, white dashed areas) [Amoh et al., 2009]. c: Fluorescence (c1) and immunohistochemical staining (c2) showed that ND-GFP-expressing vessels (white arrows) (c1) were CD31-positive (black arrows) (c2). Counter-staining with propidium iodide. Scale bars, 100 μm.

vessels of the recipient nude mice. ND-GFP-expressing blood vessels had blood flow (Fig. 2a,b). At day 28, similar observations were made (Fig. 2c,d).

GFP IMAGING OF BLOOD VESSEL NETWORK AFTER WOUNDING

At day 15 after wounding, the skin samples were directly observed from both the epidermis side and dermis side in ND-GFP transgenic mice. The ND-GFP-expressing blood vessels were connected to ND-GFP-expressing hair follicle stem cells. At day 15, the skin samples were directly observed in vertical thick sections (0.5 mm) in ND-GFP transgenic mice. The ND-GFP-expressing blood vessels were growing into the wound. The ND-GFP-expressing blood vessels were associated with ND-GFP-expressing hair follicle stem cells (Fig. 3c1–c3).

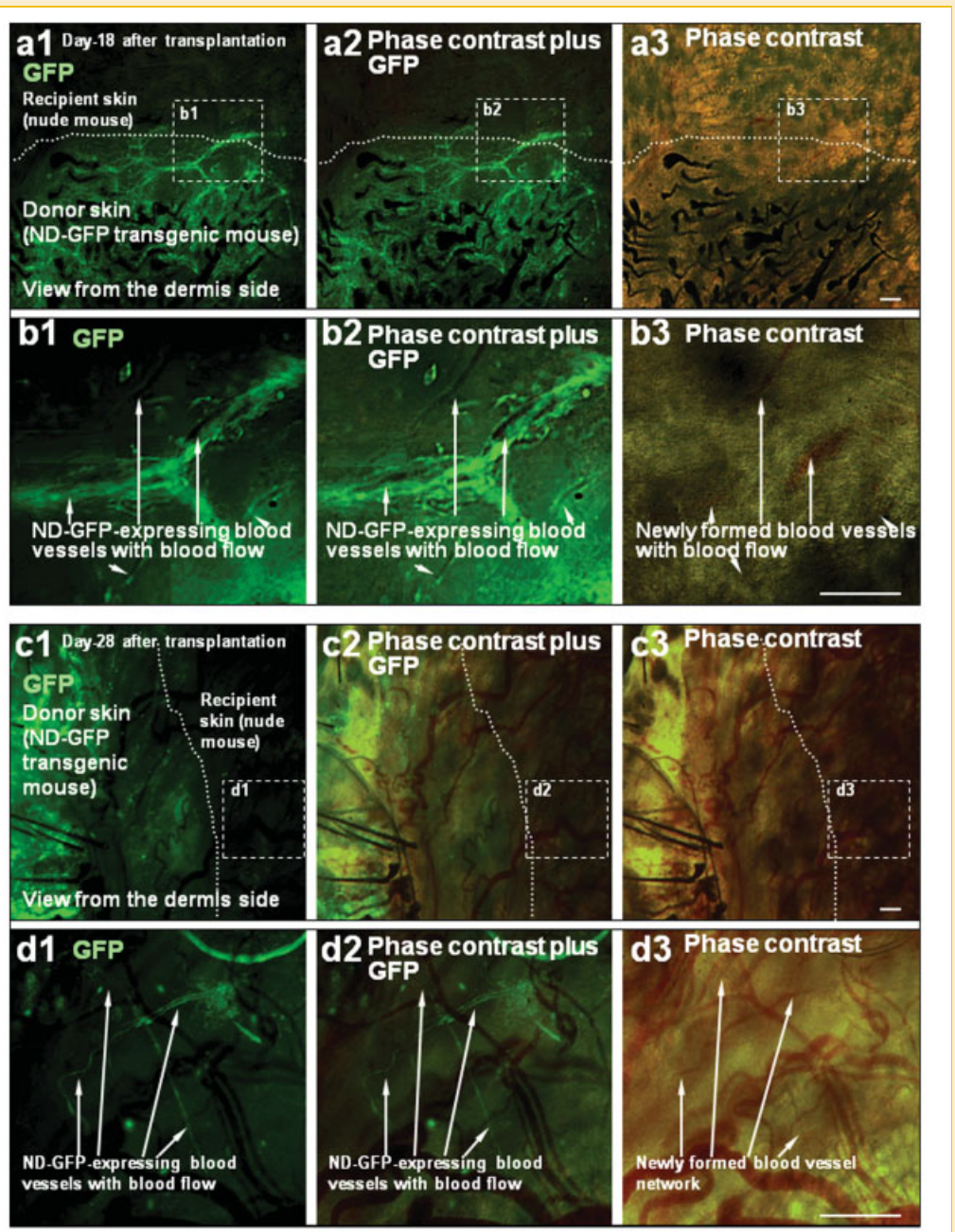

Fig. 2. a,b: On day 18 after ND-GFP-expressing skin transplantation into nude mice, the skin samples were directly observed with epidermis up and dermis down. ND-GFP-expressing blood vessels (white arrows) were growing in the transplanted ND-GFP-expressing skin. b: Higher magnification of area of (a) indicated by the white dashed box. The ND-GFP-expressing blood vessels formed a network with the blood vessels of the recipient nude mice. The ND-GFP-expressing blood vessels had blood flow. c,d: On day 28 after ND-GFP-expressing skin transplantation into nude mice, the skin samples were directly observed with epidermis up and dermis down. The ND-GFP-expressing blood vessels (white arrows) were growing in the transplanted ND-GFP-expressing skin. d: Higher magnification of areas of (c) indicated by the white dashed boxes. The ND-GFP-expressing blood vessels formed a network with blood vessels from the recipient nude mice. The ND-GFP-expressing blood vessels had blood flow. Scale bars, 100 μm.

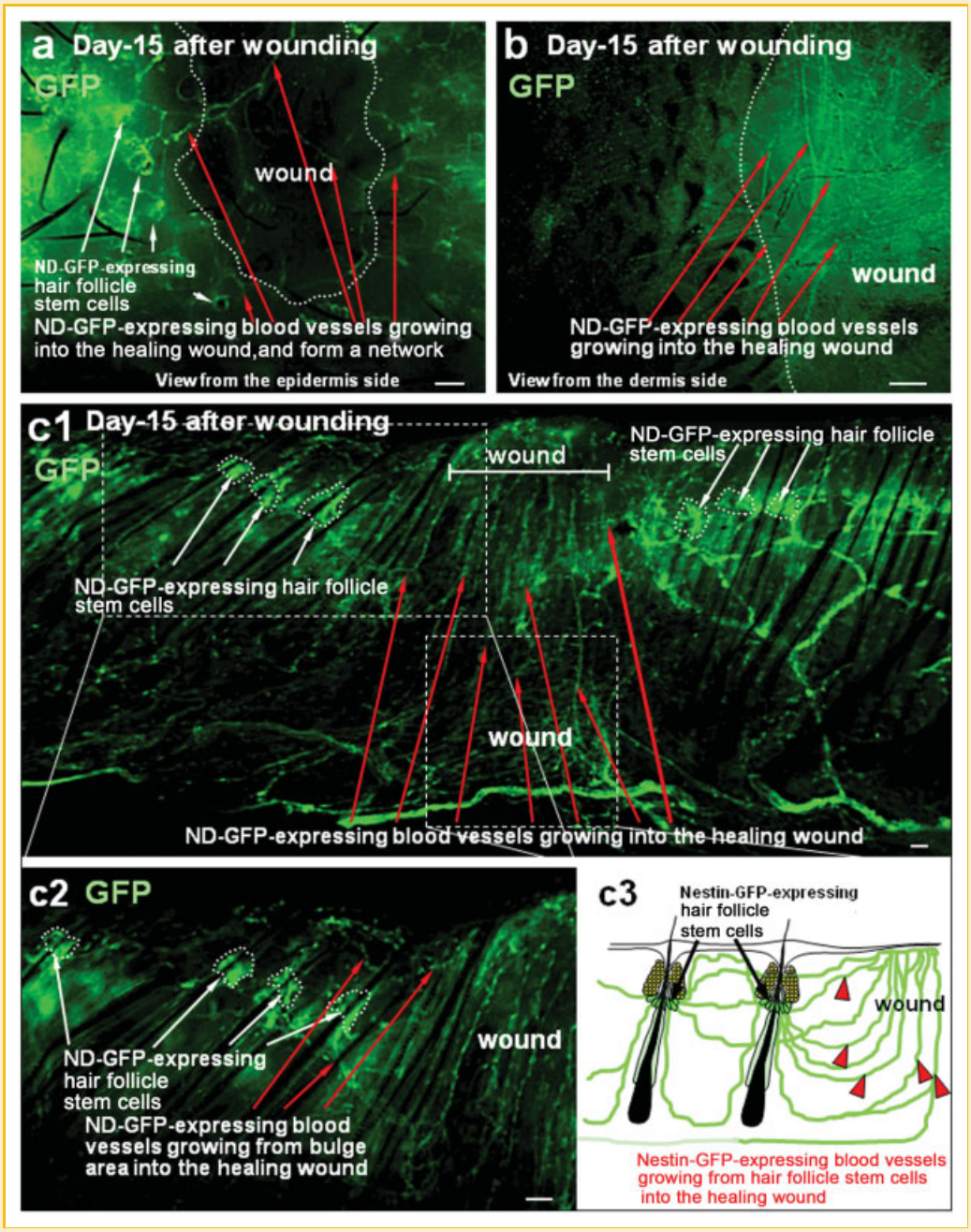

Fig. 3. a,b: On day 15 after wounding, the skin samples were directly observed from epidermis side (a) and dermis side (b) in ND-GFP transgenic mice. a: ND-GFP-expressing blood vessels (red arrows) were connected to the ND-GFP-expressing hair follicle stem cell area (white arrows). a,b: The ND-GFP-expressing blood vessels reformed the dermal microvasculature network in the wound (red arrow). The ND-GFP-expressing blood vessels were growing into the wound (red arrows). c: On day 15 after wounding, the skin samples were directly observed in vertical thick sections (0.5 mm) in ND-GFP transgenic mice. The ND-GFP-expressing blood vessels were growing into the wound (red arrows). The ND-GFP-expressing blood vessels (red arrows) were connected to the ND-GFP-expressing stem cell area (white arrows, white dashed areas). c2: Higher magnification of areas of (c1) indicated by the white dashed box. c3: The schema of (c2). Scale bars, 100 μm.

COLOR-CODED IMAGING OF CHIMERIC VESSELS FORMED AFTER SKIN TRANSPLANTATION FROM ND-GFP MICE TO RFP MICE

At day 5 after ND-GFP hair follicle stem cells transplantation into RFP transgenic nude mice, the skin was directly observed from the dermis side (Fig. 4a). The immature dermal microvasculature network surrounding the RFP-expressing hair follicles was composed of ND-GFP and RFP cells (Fig. 4a2). Blood vessels were observed that had both ND-GFP-expressing cells and RFP-expressing vessels (Fig. 4a3). At day 10, the skin was directly observed from the dermis side (Fig. 4b). The ND-GFP-expressing and RFP-expressing blood vessels formed a dermal microvasculature network (Fig. 4b2,b3). At day 14, the skin was directly observed from the dermis side (Fig. 4c). A vasculature network composed of GFP and RFP chimeric vessels was observed (Fig. 4c2,c3).

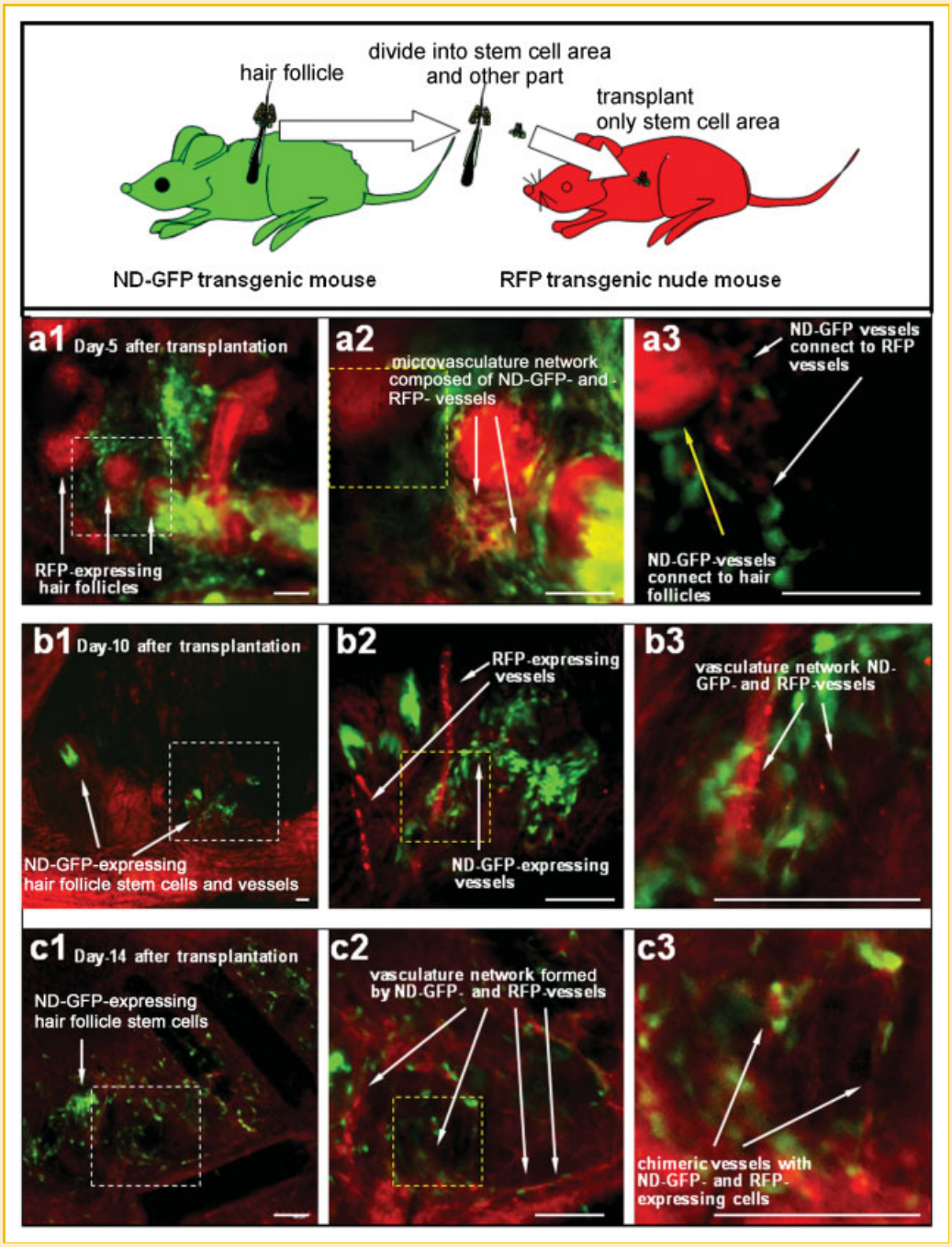

Fig. 4. a1–a3: On day 5 after ND-GFP hair follicle stem cell area transplantation into RFP transgenic nude mice, the skin was directly observed from the dermis side. a1: Hair follicles of RFP-transgenic mouse expressed RFP (white arrows). a2: ND-GFP-expressing cells formed a dermal immature microvasculature network which surrounded RFP-expressing hair follicles (white arrows). a2: Higher magnification of areas of (a1) indicated by the white dashed box. a3: ND-GFP-expressing vessels were connected to the RFP-expressing hair follicle (yellow arrow) and RFP-expressing vessels (white arrows). a3: The higher magnification of areas of (a2) indicated by the yellow dashed box. Scale bars, 100 μm. b1–b3: On day 10 after ND-GFP-expressing skin transplantation into RFP nude mice, the skin was directly observed from the dermis side. b1: The ND-GFP-expressing stem cell area and surrounding blood vessels (white arrows) were growing in the recipient RFP-expressing skin. b2: RFP-expressing vessels are clearly shown. ND-GFP-expression decreases because these blood vessels are mature. b2: The higher magnification of areas of (b1) indicated by the white dashed box. b3: ND-GFP-expressing blood vessels and RFP-expressing blood vessels are in the dermal microvasculature network. b3: The higher magnification of areas of (b2) indicated by the yellow dashed box. Scale bars, 100 μm. c1–c3: On day 14 after ND-GFP-expressing skin transplantation to RFP nude mice, the skin was directly observed from the dermis side. c2: The vasculature network was formed from GFP- and RFP-vessels. c2: The higher magnification of areas of (c1) indicated by the white dashed box. c3: Parts of vasculature network had chimeric vessels composed of ND-GFP- and RFP- expressing cells. c3: The higher magnification of areas of (c2) indicated by the yellow dashed box. Scale bars, 100 μm.

We reported earlier that many of the newly formed nestin-expressing vessels in the skin of ND-GFP transgenic mice originate from hair-follicle stem cells during the anagen phase [Amoh et al., 2004]. The vessels are labeled in transgenic mice by ND-GFP. The ND-GFP vessels originating from the follicles vascularize the dermis. Their follicular origin was most evident when transplanting ND-GFP-labeled follicles to unlabeled nude mice, where fluorescent new blood vessels originated only from the labeled follicles. The vessels from the transplanted ND-GFP follicles also responded to presumptive angiogenic signals from healing wounds [Amoh et al., 2005a].

Using the ND-GFP mouse to image the hair-follicle stem cells and associated blood vessels, we previously demonstrated that doxorubicin, a widely used cancer chemotherapy agent, caused inhibition of the ND-GFP-expressing vessels and dystrophy of the hair follicles. The hair-follicle stem cells appear not to be affected by doxorubicin. These studies suggested an important role of hair-follicle-associated blood vessels in maintaining normal hair-follicle structure and function and in chemotherapy-induced alopecia [Amoh et al., 2007].

The results of the present study demonstrate the importance of the interfollicular blood vessel network in skin transplantation and wound healing. This is vividly observed when skin with an ND-GFP-expressing blood vessel network was transplanted to RFP mice and chimeric interfollicular blood vessels were formed expressing both GFP and RFP.

The interfollicular blood vessel network of transplanted skin has a remarkable capability to link up with the skin blood vessel network of a recipient animal. These results have important clinical ramifications.

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