# Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells

**Yasuyuki Amoh*†‡, Lingna Li*, Meng Yang*, A. R. Moossa‡, Kensei Katsuoka†, Sheldon Penman§, and Robert M. Hoffman*‡¶**

*AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111; †Department of Dermatology, Kitasato University School of Medicine, Sagamihara 228-8555, Japan; ‡Department of Surgery, University of California, San Diego, CA 92103; and §Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139-4307

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**Besides forming hair shafts, the highly organized, metabolically vigorous hair follicle plays several crucial roles in skin architecture. The follicle contains a distinct population of presumptive follicular stem cells that express nestin, also a marker for neural stem cells. These nestin-expressing follicle cells are located principally in the follicular bulge region. Nestin-driven GFP (ND-GFP), transfected into mice, principally labels cells in the bulge region, which is consistent with the cells' being the stem cells of the hair follicle. We report here that ND-GFP also labels developing skin blood vessels that appear to originate from hair follicles and form a follicle-linking network. This is seen most clearly by transplanting 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, 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.**

GFP | skin angiogenesis | interfollicle network | wound healing | stem cells

Hair growth is a unique cyclic regeneration phenomenon. The hair follicle undergoes repeated cycles of periods of growth (anagen), regression (catagen), and rest (telogen) throughout the life of mammals (1). The progenitor or stem cells for the outer-root sheath and possibly other structures of the hair follicle appear to reside in a permanent upper portion of the hair follicle, the so-called bulge area (2, 3). This region has been shown to contain the slow-cycling cells or label-retaining cells that mark a stem cell population. Taylor *et al.* (4) reported that, during the follicle growth cycle, bulge stem cells differentiate into the various cell types of the hair follicle and can, in addition, form a variety of epidermal cells. A similar result was obtained by Fuchs and coworkers (5), who engineered transgenic mice to express histone H2B-GFP controlled by a tetracycline-responsive regulatory element as well as a keratin-5 promoter. Bulge cells behaved as label-retaining cells, consistent with a stem cell role. During anagen, newly formed GFP-positive populations, derived from the bulge stem cells, form the outer-root sheath hair matrix cells, hair, and inner-root sheath. Also, in response to wounding, some GFP-labeled stem cells exited the bulge, migrated, and proliferated to repopulate the infundibulum and epidermis (5). Other experiments (2) have shown that, in addition to the bulge area, the upper outer-root sheath of vibrissa (whisker) follicles of adult mice may contain stem cells. These can differentiate into hair-follicle matrix cells, sebaceous gland basal cells, and epidermis. Morris *et al.* (6) used the keratin-15 promoter to drive GFP in the hair-follicle bulge cells. They showed that bulge cells in adult mice generate all epithelial cell types within the intact follicle and hair during normal hair-follicle cycling. After isolation, adult keratin-15-GFP-positive cells could reconstitute the cutaneous epithelium.

We have recently reported that nestin, a marker for neural progenitor cells, is also selectively expressed in cells of the hair-follicle bulge (7). Follicle bulge cells, labeled with nestin-driven GFP (ND-GFP), behave as stem cells, differentiating to form much of the hair follicle each hair growth cycle. Nestin also occurs in new perifollicular blood vessels (8), which are formed in response to follicular angiogenic signals during the anagen growth phase (9).

We report here that many of the newly formed nestin-expressing vessels in the skin originate from hair-follicle cells during the anagen phase. These are labeled in transgenic mice by ND-GFP. The ND-GFP vessels emerging from follicles vascularize the dermis. Their follicular origin is most evident when transplanting ND-GFP-labeled follicles to unlabeled nude mice. Here, fluorescent new blood vessels originate only from the labeled follicles. The vessels from the transplanted ND-GFP follicles responded to presumptive angiogenic signals from healing wounds. The ability to form new blood vessels must be added to the pluripotency of hair-follicle stem cells.

## Materials and Methods

**ND-GFP Transgenic Mice.** Nestin is an intermediate filament gene that is a marker for CNS progenitor cells and neuroepithelial stem cells (10). Transgenic mice carrying GFP under the control of the nestin second-intron enhancer were used for studying and visualizing the self-renewal and multipotency of CNS stem cells (10–12). Hair-follicle stem cells strongly express nestin, as evidenced by nestin-regulated GFP expression (7).

**Visualization of Nestin Expression in Anagen Mouse Skin.** ND-GFP transgenic mice (from G. Enikolopov, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), 6–8 weeks old with almost exclusively telogen (resting) hair follicles, were anesthetized with tribromoethanol (i.p. injection of 0.2 ml per 10 g of body weight of a 1.2% solution). The mice were depilated with a hot mixture of rosin and beeswax to induce anagen. Samples were excised from dorsal skin under anesthesia before depilation and at 48 and 72 h after depilation, when the hair follicles were in early anagen. The skin samples were divided into three parts, one for fluorescence microscopy and the others for frozen sections or air-dried fragments. The samples for frozen sections were embedded in tissue-freezing embedding medium (DAKO) and frozen at −80°C overnight. Frozen sections 5 $\mu$m thick were cut with a CM1850 cryostat (Leica, Deerfield, IL) and were air-dried.

**Transplantation of ND-GFP Vibrissa Follicles to Nude Mice.** ND-GFP transgenic mice were anesthetized with tribromoethanol, and the vibrissa follicles were excised. All surgical procedures were carried out in a sterile environment. The upper lip containing the

Abbreviations: ND-GFP, nestin-driven GFP; VWF, von Willebrand factor.

¶To whom correspondence should be sent at the * address. E-mail: all@anticancer.com.

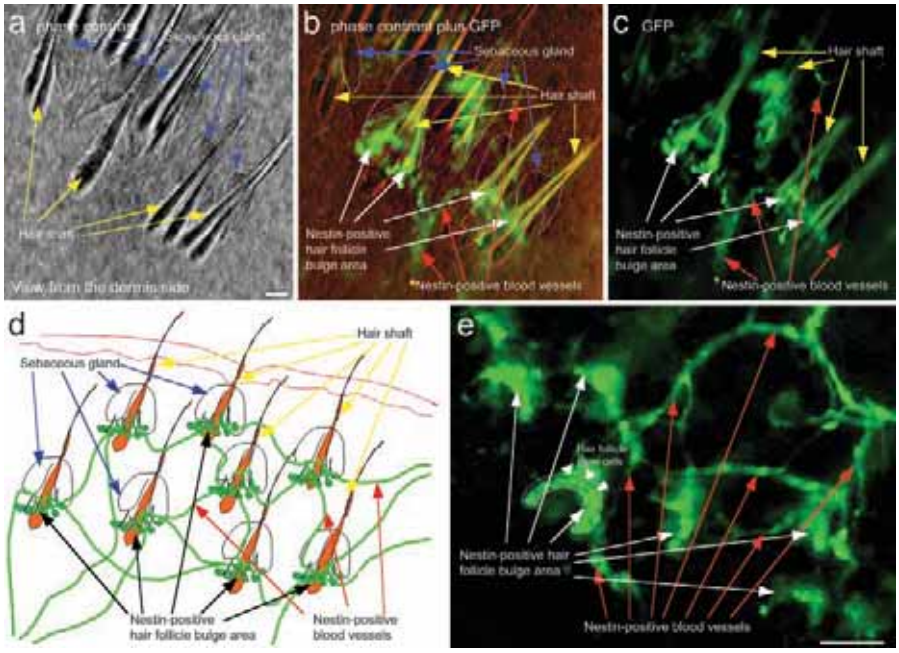


**Fig. 1.** View from the dermis side of the dorsal skin in ND-GFP transgenic mice. (*a*) Phase-contrast microscopy. Sebaceous glands (blue arrows) are located around the hair shaft (yellow arrows). (*b*) Phase-contrast microscopy plus GFP fluorescence. ND-GFP cells are visualized in the follicular bulge area (white arrows) and blood vessels (red arrows). The follicular bulge area is located beneath the sebaceous gland. (*c*) GFP fluorescence. The ND-GFP blood vessels (red arrows) are connected to ND-GFP hair follicles (white arrows). (*d*) Schematic of telogen hair follicle showing position of ND-GFP hair-follicle bulge areas (black arrows) and blood vessel network (red arrows). (*e*) GFP fluorescence. The ND-GFP blood vessels (red arrows) are associated with ND-GFP hair-follicle bulge areas (white arrows). (Scale bars, 100 $\mu$m.)

vibrissal pad was cut, and its inner surface was exposed. The follicles were dissected under a binocular microscope and plucked from the pad by pulling them gently by the neck with fine forceps. All follicles were then kept in DMEM/F-12 medium containing B-27 supplement (GIBCO/BRL). Isolated vibrissa follicles were transplanted s.c. in 6- to 8-week-old *nu/nu* mice (AntiCancer, San Diego). The incision was closed with nylon sutures (6-0). Samples of subcutis of the transplanted mice were subsequently excised and directly observed by fluorescence microscopy and air-dried or prepared for frozen sections for immunohistochemical staining.

**Transplantation of ND-GFP Vibrissa Follicles to Nude-Mouse Wounded Skin.** Nude mice were anesthetized with tribromoethanol as described above. The full-thickness skin was folded, and two neighboring, full-thickness wounds, ≈15 mm apart, were made with a 2-mm biopsy punch. ND-GFP vibrissa follicles were transplanted as described above. The mice were anesthetized with tribromoethanol, and wounded skin samples were excised at day 10 after wounding for analysis as described above.

**Transplantation of ND-GFP Vibrissa Follicles Under the Kidney Capsule of Nude Mice.** Vibrissa follicles were harvested as described above. All follicles were then kept on ice in DMEM/F-12 medium containing B-27 supplement until they were transplanted underneath the kidney capsule of 6- to 8-week-old *nu/nu* mice, which were anesthetized as described above. An incision was made on the left flank of the recipient mouse, and the kidney was exposed. Two follicles were inserted beneath the kidney capsule. The kidney was then brought back into place, and the incision was closed with nylon sutures (6-0). On day 14 the kidney capsule of each transplant mouse was excised and directly observed by fluorescence microscopy.

**Fluorescence and Confocal Microscopy.** Fluorescence microscopy was carried out by using an Olympus IMT-2 inverted microscope (Melville, NY) equipped with a mercury lamp power supply. The microscope had a GFP filter set (Chroma Technology, Rockingham, VT). An MRC-600 confocal imaging system (Bio-Rad) mounted on a Nikon Optiphot with a Plan Apo 10× objective was also used to directly observe skin tissue with GFP expression.

**Immunohistochemical Staining.** CD31 and von Willebrand factor (VWF) in air-dried skin and frozen sections were detected with the anti-rat Ig horseradish peroxidase (HRP) detection kit (BD Biosciences) for CD31 or anti-rabbit Ig HRP detection kit (BD Biosciences) for VWF, following the manufacturer's instructions. CD31 mAb (CBL1337) was purchased from Chemicon. VWF polyclonal antibody (A0082) was purchased from DAKO. Substrate-chromogen 3,3′-diaminobenzidine staining was used for detection.

## Results

**ND-GFP-Labeled Vessel Network Interconnects Hair Follicles.** The nestin-expressing hair follicles are interconnected by an ND-GFP-labeled dermal vascular network visualized in ND-GFP transgenic mice (Fig. 1). Immunohistochemical staining showed that the network vessels display CD31 antigen and VWF, indicating that they are blood vessels.

**Transplanted ND-GFP Vibrissa Follicles Give Rise to Blood Vessels.** Vibrissa follicles expressing ND-GFP were isolated from transgenic mice and transplanted into the s.c. tissues of unlabeled nude mice. ND-GFP vessels were detected growing from the transplanted ND-GFP hair follicle in nude-mouse skin by day 3. By day 28, the nestin-GFP-expressing vessels had developed into an extensively branched network and appeared to anastomose with existing vessels in the recipient nude mice (Fig. 2). Immu-

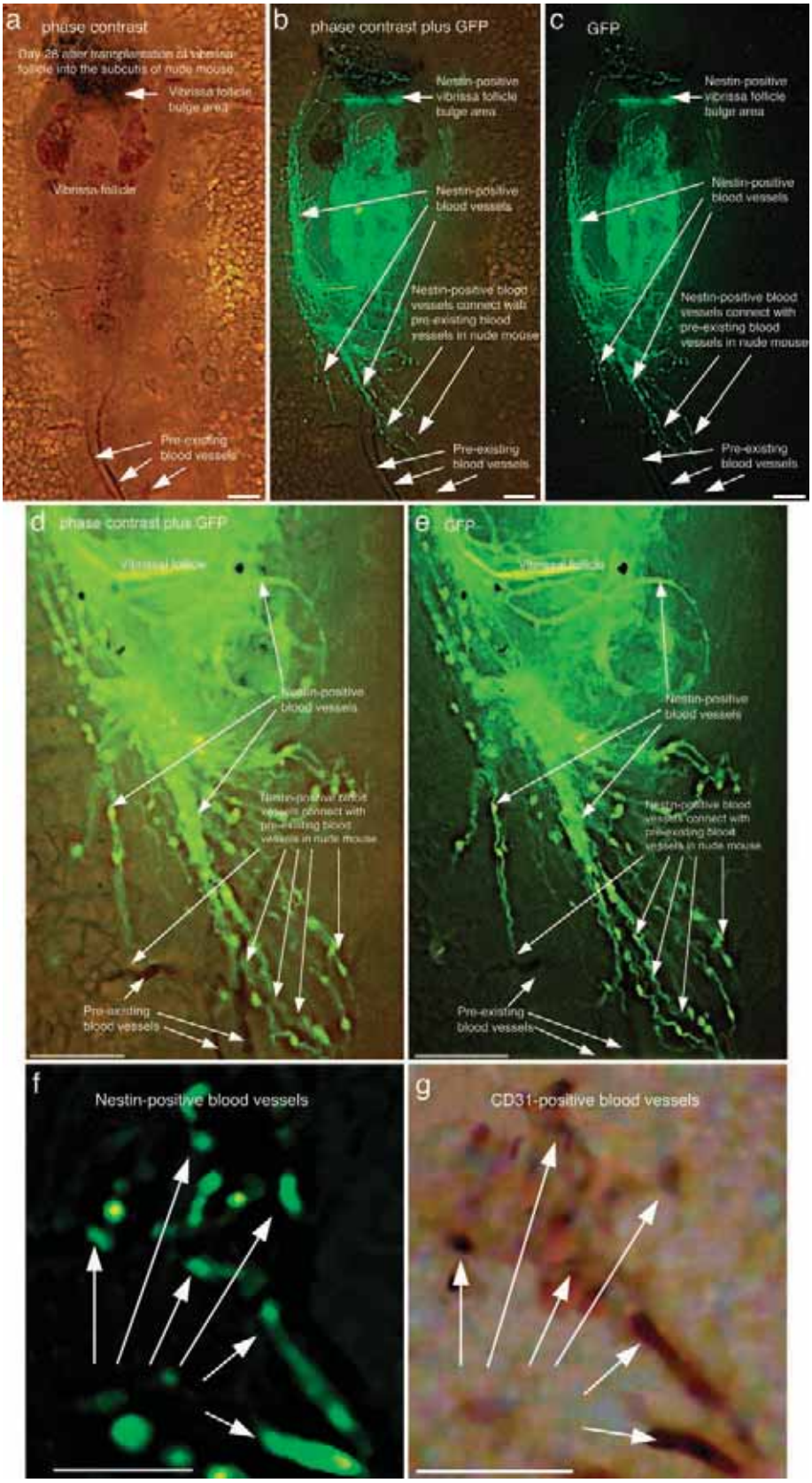


**Fig. 2.** Transplantation of an ND-GFP vibrissa follicle into the subcutis of a nude mouse. (*a*) Phase-contrast micrograph of follicle 28 days after transplantation. (*b*) Phase-contrast micrograph plus GFP fluorescence. (*c*) GFP fluorescence. In *b* and *c*, ND-GFP blood vessels (arrows) are seen growing from the transplanted ND-GFP hair follicle and associating with preexisting blood vessels in the nude-mouse skin. (*d* and *e*) Higher magnification of the ND-GFP vessels of *b* and *c*, respectively. (*f* and *g*) Colocalization of GFP and the endothelial cell marker CD31 (arrows). *f* is a fluorescent image, and *g* shows the same field air-dried and immunohistochemically stained with CD31. (Scale bars, 100 $\mu$m.)

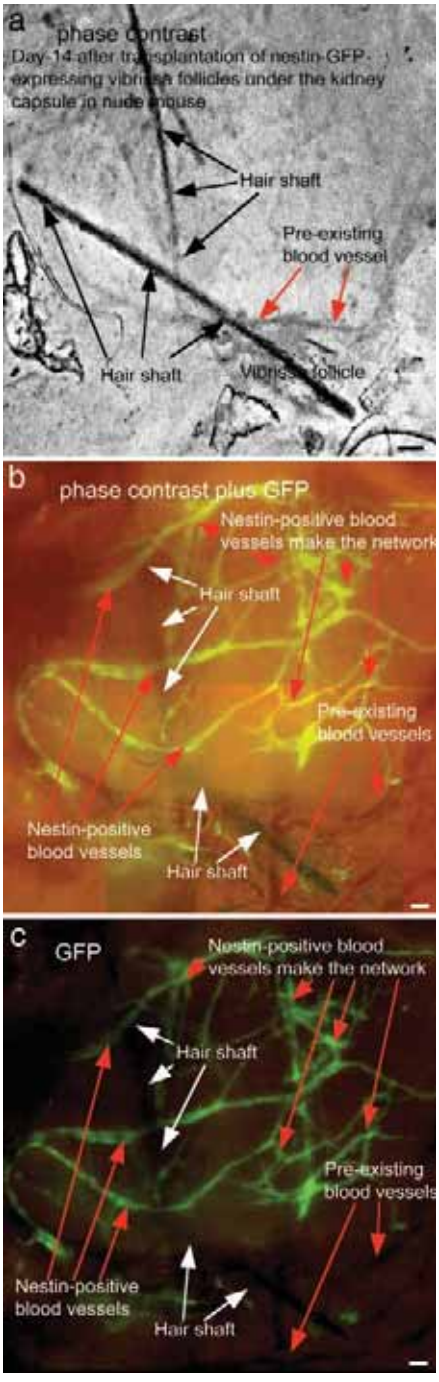


**Fig. 3.** Transplantation of ND-GFP vibrissa follicles under the kidney capsule of a nude mouse. The ND-GFP vessels are visualized to form a network at day 14 after transplantation. (*a*) Phase-contrast micrograph. (*b*) Phase-contrast micrograph plus GFP fluorescence. (*c*) GFP fluorescence. (Scale bars, 100 $\mu$m.)

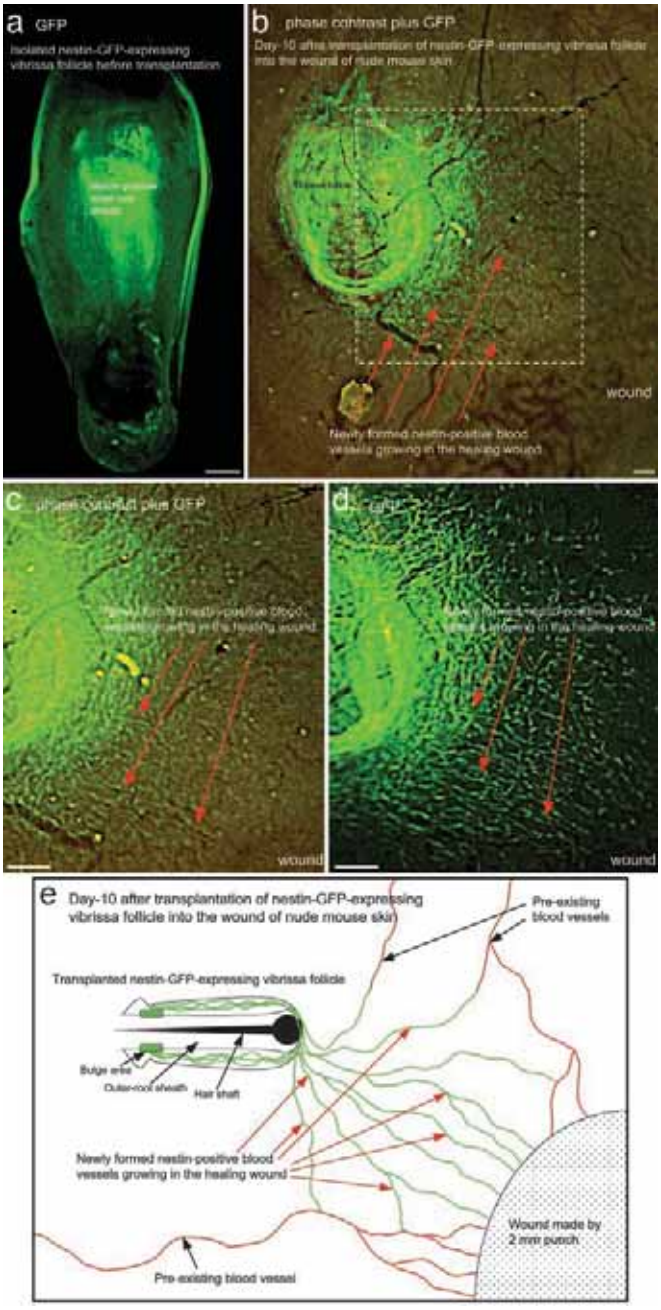


**Fig. 4.** Transplantation of an isolated ND-GFP vibrissa follicle into wounded nude-mouse skin. (*a*) The isolated ND-GFP vibrissa follicle before transplantation. (*b*) Day 10 after transplantation of the ND-GFP vibrissa follicle into wounded nude-mouse skin. The ND-GFP vessels (arrows) are growing from the ND-GFP vibrissa follicle toward the healing wound. (*c* and *d*) Higher magnification of the area in *b* indicated by the white dashed box. (*e*) Schematic of transplantation of the ND-GFP vibrissa follicle into wounded nude-mouse skin. (Scale bars, 100 $\mu$m.)

nohistochemical staining showed that CD31 antigen and GFP fluorescence colocalized in nascent vessels.

**Transplanted ND-GFP Vibrissa Follicles also Give Rise to Blood Vessels in the Kidney of Nude Mice.** After transplantation of ND-GFP vibrissa follicles under the kidney capsule in a nude mouse, an ND-GFP blood vessel network around the transplanted follicles was observed on day 14 (Fig. 3). The ND-GFP vessels appeared to anastomose with preexisting blood vessels.

**Enhanced Growth of ND-GFP Vessels from Transplanted Hair Follicles in Wounded Skin.** Wounded skin samples containing transplanted ND-GFP vibrissa hair follicles were harvested for fluorescence microscopy. The images showed that ND-GFP vessels grew from the hair follicles toward the wound (Fig. 4). The presence of a wound in the vicinity of the transplanted follicle significantly enhanced vessel outgrowth. Apparently, vessels originating in the follicle responded to angiogenic signals arising from the wound vicinity. Immunohistochemical staining showed that

CD31 was expressed in the ND-GFP-expressing vessels growing into the wound.

## Discussion

Angiogenesis, the highly active growth and destruction of capillary blood vessels, has come to occupy an increasingly important role in understanding tissue maintenance, wound repair, and the growth of malignancies (13). Identifying the source of the cells for new blood vessels has become increasingly important both scientifically and for therapeutic design. There have been numerous recent reports of endothelial cells arising from bone-marrow-derived stem cells (14). There is also evidence that endothelial stem cells can be derived from adipose tissue (15). However, these previously identified sources of endothelial stem cells may not be able to supply blood vessels in the skin because of skin's unique structure. The results presented here indicate that an important and previously unrecognized function of hair-follicle stem cells is to supply endothelial cells that can form blood vessels in the skin.

The repertoire of hair-follicle stem cell potential may be even broader than reported here. A number of investigators (5, 16) found that multipotent adult stem cells isolated from mammalian skin dermis, termed skin-derived precursors, can proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells, and adipocytes. However, the exact location of these stem cells in skin was unknown, and their functions were unclear. The present report suggests that the hair follicle is an important source of stem cells for dermal blood vessels and very likely for other tissues as well. These results support reports that follicle cells contribute to wound repair as well as to skin transplant survival (5, 6).

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