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Contribution to Revascularization by Reciprocal Random Skin Flaps

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Abstract

Background: The introduction of random skin flaps to plastic surgery has a major impact on wound closure and the reconstruction of normal and functional anatomical features, but the role of random skin flaps and the interactions between random skin flaps and recipients during random skin flap revascularization process remain to be fully elucidated.

Methods: The authors endeavored to establish a reciprocal random skin flap model between green fluorescent protein (GFP)-transgenic and wild-type (wt) C57BL/6 mice. Blood vessel origination, growth directions and anastomosis positions were detected by a multitude of detective methods at postoperative days 3, 7, 14 and 21.

Results: The results indicated that the reciprocal random skin flap model was successfully established between the two different mice; blood vessels originated from GFP-transgenic recipients grew into wt flaps, while blood vessels from GFP-transgenic flaps grew into wt recipients as early as day 3. It was promising and crucial to unveil vascular anastomoses between donor and recipient vascular networks occurring as early as day 3. Green fluorescent cell clusters increasingly augmented with significant differences among days 3, 7, and 14 ($P < 0.05$), but neither between days 14 and 21 ($P > 0.05$), nor between two separate vascular networks at each time point.

Conclusion: The authors harbored the idea that the random skin flaps played essential roles in promoting survival and were of vital significance to the ordered process of random skin flap revascularization by the outgrowth and by the ingrowths of capillary buds of skin flaps through a unique reciprocal random skin flap model.

Keywords: Transgenic mice; Random skin flap; Revascularization; Physiological mechanism; Therapeutic angiogenesis

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Introduction

Random skin flaps are often used in plastic and reconstructive surgery, head and neck surgery, orthopedic surgery, etc. to repair defects resulting from congenital abnormalities, trauma, or cancer treatment. However, the potential physiological mechanism of random skin flap revascularization has been uncertain for hundreds of years. With recent advances in molecular vascular biology, the fundamental mechanism of flap revascularization has gained increasing interest in the field of reconstructive surgery.

Early studies of skin graft revascularization mainly regarding free skin graft revascularization in the late 1800s suggested a direct connection between host and graft vessels, called inosculation. Before this, free skin graft survival was dependent on imbibition (fluid absorption), followed by a large number of anastomosis theories [1-4]. Besides inosculation/anastomosis, another principle of vascularization prevailed. Neovascularization, namely angiogenesis and vasculogenesis, was an ingrowth of newly formed blood vessels from the wound bed into the graft [4-6]. Concurrently, Zarem, Capla and Converse et al. [7-9] suggested that the process of vascularization of full-thickness skin grafts in mice was dominated by inosculation and vascular in growth from the recipient without

a contribution from donor derived cells.

However, few progresses in mechanistic studies have been reported about pedicle flaps or free flaps. In 1982, Young, using intravital dye, harbored the idea that the revascularization of pedicle skin flaps was evident at 3 to 4 days postoperatively at the distal hypoxic part of viable flaps, and that the whole flap had a collateral blood supply at 7 to 10 days after surgery [10]. This time frame of revascularization was similar to the records of wound healing in skin flaps [11,12]. According to the revascularization route by Tsur et al. [12] the paramount significance of early revascularization arose from both the wound bed and wound margin.

The interactions between random skin flaps and recipients remain to be elucidated, although the time frame about pedicle flap revascularization has been described. Furthermore, what is the role of random skin flaps in their own survival? The authors adopted a novel GFP-transgenic rodent model ideal for studying vessel formation and origination and easily distinguishing growth directions of newly formed vascular networks from those of preexisting vasculatures as well as identifying anastomosis sites and time points.

Materials and Methods

Reciprocal random skin flap model

All protocols were approved by the Sichuan University Medical Center Institutional Animal Care and Use Committee. 40 GFP-transgenic C57BL/6 mice (State Key Laboratory of Biotherapy, Sichuan University) and 40 wt C57BL/6 mice (Animal Center for Medical Experiments, Sichuan University), male, aged 10 to 20 weeks, were housed under specific pathogen-free conditions and treated according to NIH guidelines. Intraperitoneal anesthesia of chloral hydrate (3 mL/kg) was administered because of its long duration and low death rate. The random skin flap and recipient defect, approximately 1×1 cm in size, consisted of skin and subcutaneous fascia, including the panniculus carnosus as previously described [13]. To study the contribution of recipient-derived neovascularization, caudal recipient defects were brought out on GFP-transgenic mice, and caudally based random skin flaps were harvested on wt mice and transplanted to recipient sites. To determine the contribution of donor-derived neovascularization, cranial recipient sites were created on wt mice, and cranially based random skin flaps were produced on GFP-transgenic mice and transferred to recipient sites. All random skin flaps were sutured to recipient sites with interrupted 5-0 Ethilon (Ethicon, Inc., Somerville, NJ). Cyclosporine A (20mg/(kg.d); Novartis Pharma Stein AG, Switzerland) was used for intraperitoneal injection lasting for one week after surgery. And penicillin was utilized i.m. at the first three days postoperatively. To assess the flap survival, random skin flaps were assessed at postoperative days 3, 7, 14 and 21, respectively (n=10 pairs/time point). The mice were then euthanized by overdose of pentobarbital.

Vascular anastomosis detection

Blood supplies and vascular anastomoses were evaluated by fluorescent angiography at days 3, 7, 14, and 21 after surgery (n = 5 pairs/time point). Angiography was performed through caudal vein injection of fluorescite 10% injection (Cardinal Health Manufacturing Services B.V., Humacao, Puerto Rico) into the GFP-transgenic mice. Vasculatures of both donor flaps and recipients, and anastomoses between them were detected by fluorescent angiography using a Heidelberg retinal tomograph (Heidelberg Engineering GmbH, Heidelberg, Germany) with exposure conditions of 10 to 16 mA and 24 to 27 kV.

Assessment of origination and sites of neovascularization

To study the contribution of both donor-derived and recipient-derived neovascularization, tissues harvested from border areas of flaps and recipients were cut into 5-um thick frozen sections as well as paraffin sections at postoperative days 3, 7, 14 and 21 (n=5 pairs/time point). GFP fluorescence was inspected directly using of a Zeiss 20T fluorescent microscope after frozen sections were stained by 4',6-diamidino-2-phenylindole (DAPI). In order to further investigate origination, growth directions, and anastomosis sites of newly formed tissues including blood vessels, Hematoxylin & eosin (HE) staining was used in frozen sections too.

The 5-um thick serial paraffin sections were also used to examine the origination of blood vessels by immunohistochemistry analysis [13]. Briefly, the first serial section was stained with anti-GFP antibody (1:500, EMD Millipore, Germany) and the second one was stained with anti-CD31 monoclonal antibody (1:100, Santa Cruz, CA), followed by staining with a secondary antibody (Dako REALTM EnVision TM Detection System, Peroxidase/DAB+, Rabbit/Mouse, Denmark).

In addition, immunofluorescent staining was also exploited to verify the origin of blood vessels. Briefly, the frozen sections were stained with anti-CD31 antibody, followed by secondary antibody staining and inspection under fluorescent microscope. Blood vessels were quantified by a blinded surgeon as previously described [14].

Statistical analysis

All statistical data are expressed as mean ± standard deviation. Data was analyzed by One-Way ANOVA and values of $p< 0.05$ were considered statistically significant by means of IBM SPSS Statistics (version 19; SPSS, Inc.).

Results

The survival of reciprocal random skin flaps

To investigate the survival of reciprocal random skin flaps, all flaps were photographed by a digital camera at the same distance with the same focus at postoperative days 3, 7, 14, and 21, respectively. Almost all flaps survived well, except partial borders of two random skin flaps suffered from ischemia as early as day 3. Red scars softened gradually and thick hair grew slowly, but no defect or infection was detected (Figure 1).

Revascularization by the ingrowth of capillary of random skin flaps

Histological examination uncovered that blood vessel ingrowth from GFP-transgenic recipients into wt random skin flaps first appeared at day 3 and progressively increased through day 21. The recipient cells including blood vessels appeared not only at the periphery, but also in the center of random skin flaps. These cells structured as sprout-like or vessel-like cell clusters as shown by DAPI staining (Figure 2A), which was further confirmed by HE staining (Figure 2B). There were significant differences as to relative ratios of GFP-positive areas to 40 um × 40 μm areas among days 3, 7 and 14, ($P< 0.01$), but not between days 14 and 21 ($P >0.05$, Figure 2C). Furthermore, same results from serial paraffin sections of immunohistochemistry analysis demonstrated that recipients-derived blood vessels (GFP+/CD31+) increased in wt flaps as time went by (Figure 3).

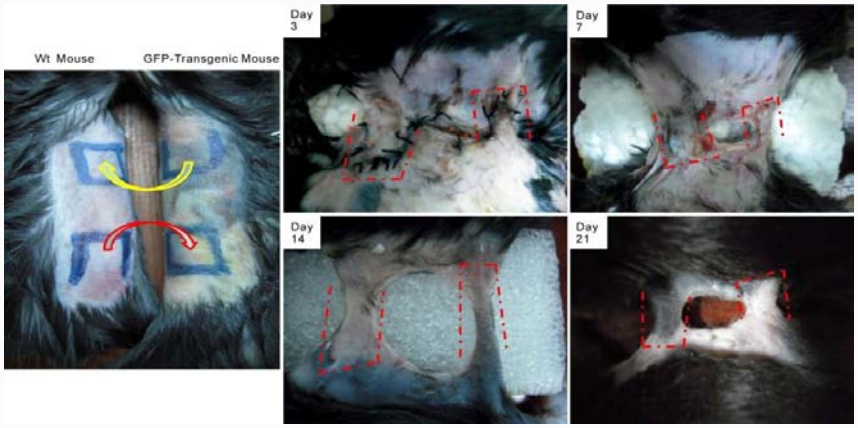

Figure 1: The design and survival of the reciprocal random skin flap model between GFP-transgenic and wt C57BL/6 mice. Arrows point to the transplantation direction of random skin flaps. Dotted frames outline the shape and position of the random skin flaps after transplantation at different time points.

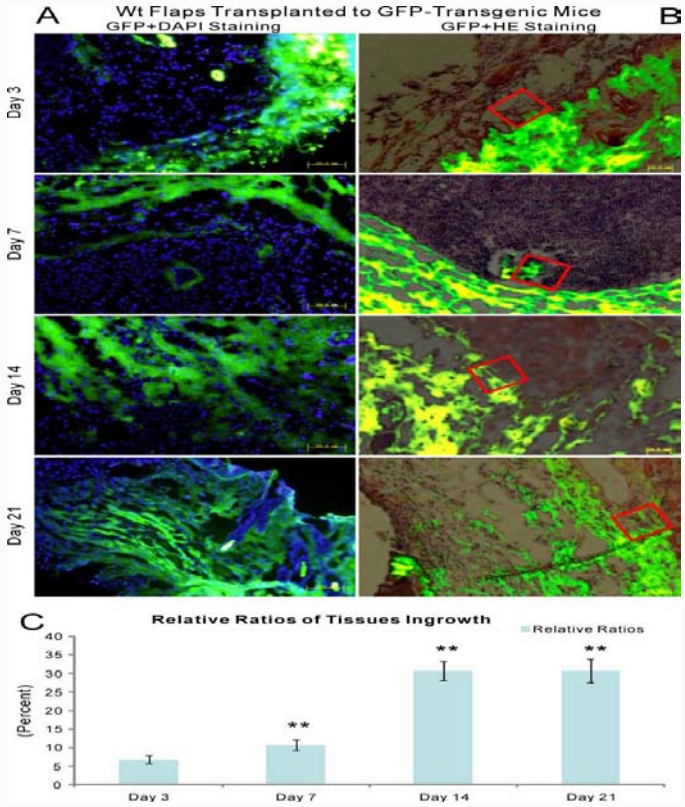

Figure 2: The model of wt random skin flaps transplanted to GFP-transgenic recipients with a combination of green fluorescence and DAPI staining as well as green fluorescence and HE staining. **A**: green fluorescence and DAPI staining, **B**: green fluorescence and HE staining, **C**: relative ratios of tissue ingrowths. The red box outlined one point at the border area between random skin flaps and recipients. Newly generated capillary sprouts, blood vessels and other cells grew into random skin flaps from recipients as early as day 3 and progressively increased through day 21.

Revascularization by the outgrowth of capillary of random skin flaps

Histological examination of GFP expression revealed that newly formed blood vessels from random skin flaps into wt recipients emerged as early as day 3 and gradually accumulated through day 21. Donor cells including blood vessels appeared not only at the periphery, but also in the center of recipients, taking on capillary sprout-like or vessel-like cell clusters by DAPI staining (Figure 4A). This phenomenon was further manifested by HE staining (Figure 4B). There were significant differences regarding the relative ratios of GFP-positive areas to 40 um × 40 μm areas among days 3, 7, and 14 ($P < 0.01$), but not between days 14 and 21 ($P > 0.05$, Figure 4C). There were, however, no significant differences between two separate vascular networks at each time point ($P > 0.05$). GFP-positive tissues (GFP+/CD31-) including blood vessels (GFP+/CD31+) progressively increased in recipients over time on serial sections (Figure 3). Immunofluorescent staining further approved GFP+/CD31+ blood vessels were donor (flap)-derived vessels (Figure 5).

Vascular anastomoses

Vascular anastomoses of flap-derived and recipient-derived blood vessels were first noticed at day 3 in both fluorescent angiography and histological examination. Blood vessels full of fluorescent blood flow

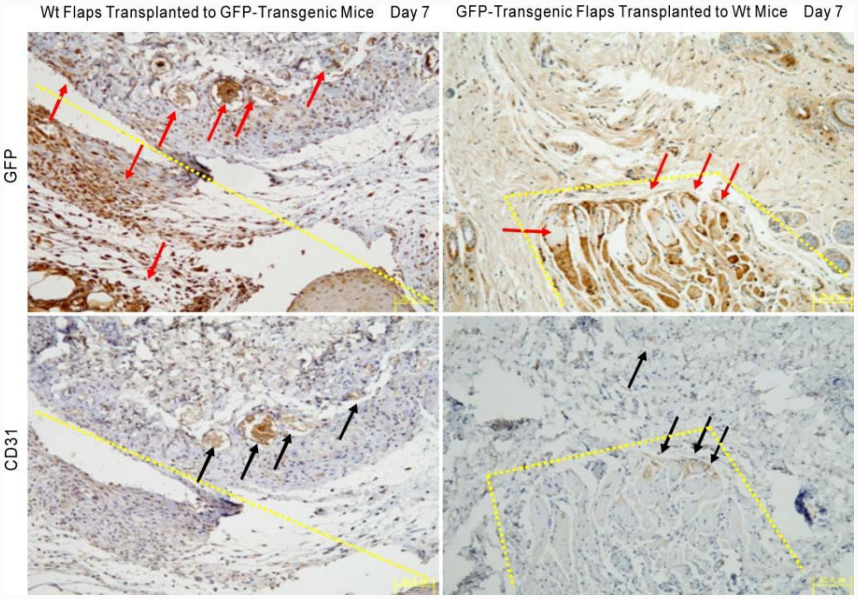

Figure 3: Immunohistochemistry analysis of neovascularization in serial paraffin sections. The dotted lines and frames distinguished random skin flaps from recipients. Red arrows pointed to GFP-positive tissues, and black arrows indicated CD31-positive blood vessels. GFP+/CD31+ blood vessels initially appeared at day 3 and gradually increased in wt flaps as time went by.

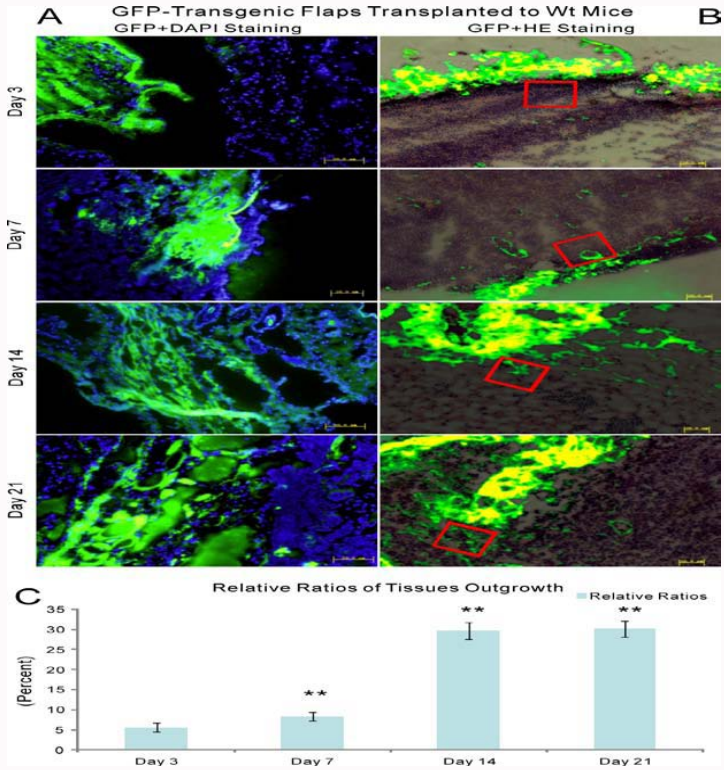

Figure 4: The combination of green fluorescence and DAPI staining as well as green fluorescence and HE staining in the model of GFP-transgenic random skin flaps transplanted to wt recipients. **A**: green fluorescence and DAPI staining, **B**: green fluorescence and HE staining, **C**: relative ratios of tissue in growth. Red box outlined one point at the border area between random skin flaps and recipients. Newly generated blood vessels and other cells grew from random skin flaps into recipients as early as day 3 and remarkably augmented through day 21.

signals could be directly observed at day 3 in the wt C57BL/6 random skin flaps transplanted to GFP-transgenic mice model, and they progressively augmented through day 21 by fluorescent angiography. There were significant differences between day 3 and other time points ($P < 0.01$), but not between days 7, 14 and 21 ($P > 0.05$, (Figure 6)).

Discussion

The authors demonstrated that random skin flap vascularization is an ordered process of vascular ingrowth, blood vessels outgrowth, followed by anastomoses in both recipients and donor flaps by using a reciprocal random skin flap model. Through this rodent model,

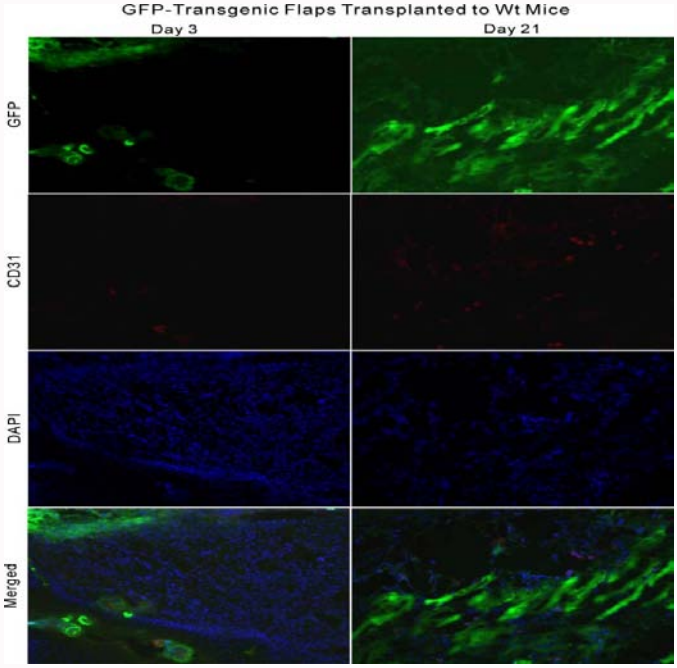

Figure 5: The combination of green fluorescence, DAPI and CD31 staining in the model of GFP-transgenic random skin flaps transplanted to wt recipients. The CD31+/GFP+ blood vessels increasingly grew from GFP-transgenic flaps to wt recipients as time went by.

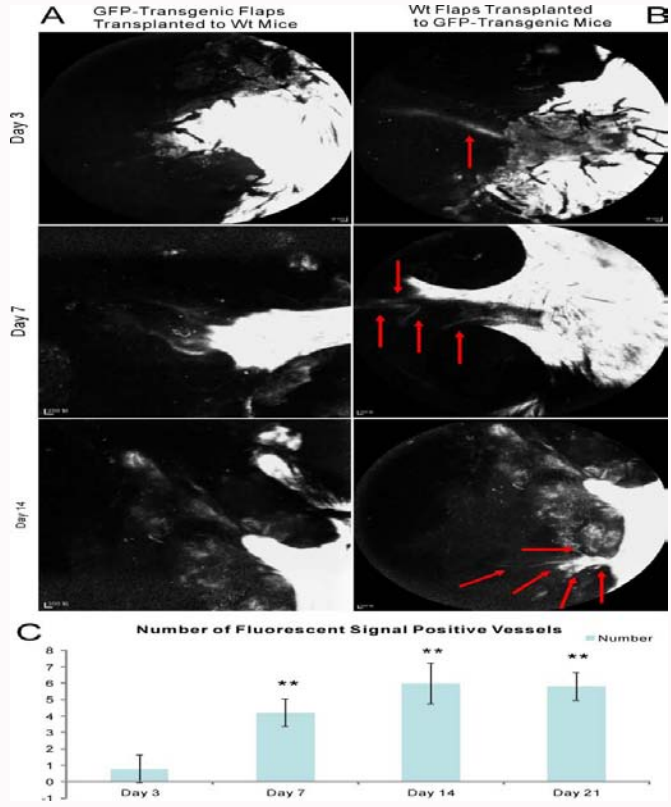

Figure 6: Vascular anastomoses were detected by fluorescent angiography. **A:** the model of GFP-transgenic random skin flaps transplanted to wt recipients, **B**: the model of wt random skin flaps transplanted to GFP-transgenic recipients, **C**: statistic result of the number of fluorescent signal-positive blood vessels. Blood vessels filling with fluorescent signal could be directly observed at day 3 in the model of wt random skin flaps transplanted to GFP-transgenic recipients. Blood vessels enhanced gradually as time went by and might reach a platform stage at day 14 and 21.

the authors were able to mark and trace recipient-derived tissues including blood vessels as well as flap-derived tissues throughout the process of revascularization in vivo, pinpointing times and locations of all critical steps.

Through this homogenous model, the authors could easily differentiate recipient tissues with donor structures including blood vessels without the interference of immunologic rejection. The authors held the idea that mutual vascular growth played a critical

role in the random skin flap revascularization process. In other words, donor endothelia outgrowth were coincident with endothelial cell ingrowth from surrounding hosts (Figure 2,4). Vascular ingrowth from GFP-transgenic recipients into wt random skin flaps emerged at the periphery of transplanted flaps as early as day 3 and progressively augmented through day 21 (Figure 2). Mirroring this process, in the reciprocal random skin flap model, blood vessels grew from GFP-transgenic random skin flaps into peripheral wt recipients as early as day 3 (Figure 4).

Vascular anastomoses between the preexisting vasculatures and newly formed blood vessels might be a crucial strategy in vascularization process. The GFP-positive tissues including blood vessels were located at the periphery and in the center of both flaps and recipients by histological examination (Figure 2-6), indicating that vascular anastomoses happened as early as day 3, consistent with free skin graft revascularization by O'Ceallaigh et al. [3]. After recipient-derived blood vessels had almost anastomosed with donor-derived vascular networks in both recipients and random skin flaps, it was reasonable to account for GFP-positive tissues including blood vessels increased significantly more at day 7 than day 3 (Figure 2,4). These had lent support to vessel anastomoses supplying constituent cells that could act as building blocks for new blood vessels [15].

However, in terms of GFP-positive cell clusters, there were no obvious differences between days 14 and 21. A possible explanation was that blood flow resumed and flap tissues became reoxygenated after vascular anastomoses occurred between recipient vasculatures and flap vessels. Thus, potential hypoxic signaling pathways stimulating revascularization no longer existed and the revascularization process ceased [8]. Furthermore, GFP-positive cell clusters had not been found in liver or kidney (data not provided). These data suggested that recipient-derived endothelial progenitor cells migrated into the periphery and central area of random skin flaps [3], and vice versa because of ischemia, but not anywhere such as liver or kidney. In addition, a multitude of tissues besides vascular endothelial progenitor cells showed exceedingly great growth potential under ischemia, greatly promoting flap survival and wound coverage.

These results hinted that preexisting vascular networks might be potential channels for the immigration of endothelial progenitor cells and other cells into the center of random skin flaps, which corresponded with other researchers [8]. They might also shed light on the fact that mesenchymal stem cells or vascular endothelial progenitor cells were these migrated cells and played an important role in random skin flap survival as described [13,16].

These results could be easily extended to free flaps, bone grafts and tissue-engineered prefabrications, which emphasize vascular/tubular anastomoses between recipient vessels and donor vascular networks or tissue-engineered construct tubular systems. One prerequisite of tissue engineering was the necessity to acquire sufficient neovascularization to support central cell survival. Therefore, therapeutic angiogenesis, using various growth regents such as vascular endothelial growth factor, basic fibroblast growth factors, deferoxamine and dimethyloxalylglycine, had yielded an amazing increase in flap survival and differentiation of endothelial progenitor cells into vascular networks [17,20]. But one extremely important prerequisite was that therapeutic angiogenesis gave priority to a systemic way. The revascularization potential in a systemic way has been verified by our previous job as well as other researchers' work [13].

Conclusion

In conclusion, the authors initially manifested that the random skin flaps played essential roles in promoting survival and the mechanism of random skin flap revascularization occurred by means of an ordered process by the in growth and by the outgrowth of capillary buds of skin flaps, eventually vascular anastomoses between two separate vascular networks, and finally restoration of circulatory continuity through a unique reciprocal random skin flap model.

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