



The Fate of Nonvascularized Fat Grafts: Histological and Bioluminescent Study

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Background: Nonvascularized fat grafting has become one of the most popular options for breast contouring. However, the survival process of the grafts remains to be elucidated. In this study, we tracked the fate of nonvascularized fat grafts with in vivo bioluminescence and immunohistochemistry.

Methods: Nonvascularized fat grafts or vascularized adiposal flaps from luciferase transgenic rats were transplanted to Lewis rats. The bioluminescent signals from the grafts were monitored longitudinally. In addition, nonvascularized fat grafts from Lewis rats were engrafted to Lewis rats and the viability of the adipocytes in the grafts was evaluated with immunohistochemical staining for perilipin at postoperative week 1, 2, 3, 4, and 6.

Results: The bioluminescent signals from the nonvascularized fat grafts increased drastically from postoperative day 3 to 7, stayed flat from day 7 to 12, and declined from day 12 to 17, whereas those from the vascularized fat flaps remained throughout the entire postoperative period. Immunohistochemistry revealed that the survival zones with large adipocytes were decreased within 2 weeks and the regenerating zones with small adipocytes appeared after 3 weeks.

Conclusions: Our study showed the process of survival and regeneration of nonvascularized fat grafts and suggested that graft-derived stromal cells proliferated within 7 days after transplantation and differentiated into adipocytes after postoperative week 3. (*Plast Reconstr Surg Glob Open* 2013;1:e40; doi: 10.1097/GOX.0b013e3182a7e827; Published online 13 September 2013)

Free autologous fat transplantation, which was first attempted by Neuber¹ in 1893, has been used for soft-tissue augmentation in a diverse range of plastic, reconstructive, and aesthetic surgeries. Moreover, the advent of liposuction techniques

in the 1980s led to widespread clinical utility of nonvascularized fat grafts (nVFGs). Clinical outcomes of nonvascularized fat grafting are variable and depend on the technique of harvest and transplantation.

To obtain the satisfactory clinical result, it is essential to understand the survival process of nVFG. Many researchers have examined the survival process or the viability of nVFG with histological findings based on hematoxylin and eosin staining²⁻⁷ and have supported the “cell survival theory.”⁸ However,

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living adipocytes are distinguishable from dead ones not with morphological observation but with immunohistochemistry for perilipin,⁹ which is a protein that coats lipid droplets in adipocytes.^{10,11} Recently, Eto et al¹² showed the fate of the nVFG using immunohistochemical staining for perilipin in early phase after transplantation. They demonstrated that most adipocytes in the nVFG began to die on day 1 and the number of proliferating cells increased from day 3. However, the long-term survival and regenerative process of nVFG remains to be elucidated.

In vivo bioluminescence imaging (BLI) is an innovative method that can quantify the real-time viability of the luciferase-expressing cells noninvasively and repeatedly.¹³ The luminescent intensity is in proportion to cell numbers.¹⁴ Therefore, BLI has been used to track tumor, immune, and stem cells.^{14–17}

In this study, we used in vivo BLI with luciferase transgenic rats and immunohistochemical staining for perilipin to clarify the fate of the nVFG within 6 weeks after transplantation.

METHODS

Animals

Adult male Lewis rats weighing 240–260 g were obtained from Charles River Japan (Yokohama, Japan) and used as recipients or donors. Inbred (Lewis) transgenic rats with firefly luciferase (Luc-Tg rats), which express the marker gene ubiquitously in all organs and tissues,¹⁸ were used as donors. All experiments in this study were performed in accordance with the Jichi Medical University Guide for Laboratory Animals.

Vascularized Adiposal Flap Transfer Using Luc-Tg Rats as Donors (Group 1)

As a control study, 5 vascularized adiposal flaps (VAFs) were harvested from the inguinal fat pads of Luc-Tg rats with the associated vascular pedicles (consisting of the femoral and superficial inferior epigastric arteries and veins) and transferred to the inguinal regions of 5 recipient rats. The pedicles were anastomosed to the femoral artery and veins with 11-0 nylon microsutures under an operating microscope. Each graft was trimmed to the same weight and volume, 1.35 g and 1.5 cm³, before transplantation. At the end of the study, the rats were anesthetized, and the patency of the anastomosed vessels was confirmed under the microscope.

Nonvascularized Fat Transplantation Using Luc-Tg Rats as Donors (Group 2)

Nine nVFGs were harvested from the epididymal fat pads of Luc-Tg rats and were transplanted to the dorsal subcutaneous regions of 9 recipient rats using the core

fat grafting technique as Guyuron and Majzoub¹⁹ have described previously. Briefly, a 5-mm incision was made at the dorsal region, and the graft was injected into the subcutaneous layer through the incision using a 1-cm³ syringe whose tips were trimmed in an oblique fashion. Each graft was trimmed to the same weight and volume, 0.27 g and 0.3 cm³, before transplantation.

Nonvascularized Fat Transplantation Using Wild Lewis Rats as Donors (Group 3)

Fifteen nVFGs were harvested from the epididymal fat pads of Luc-Tg rats and were grafted to the dorsal subcutaneous regions of 15 recipient rats in the same way as in group 2.

In Vivo BLI

In vivo optical imaging of the grafts was obtained using the noninvasive bioimaging system IVIS (Xenogen, Alameda, CA) and was analyzed using the Igor (WaveMetrics, Lake Oswego, OR) and IVIS Living Image (Xenogen) software packages. To detect photons from luciferase-labeled cells, we injected D-luciferin (potassium salt; Biosynth, Postfach, Switzerland), the luciferase substrate, intravenously into the penile vein of the rats (7.5 mg) anesthetized with isoflurane. The integration time was fixed at 10 seconds duration for each image, and serial images were acquired every 1 minute till 10 minutes in group 1 and 30 minutes in group 2 after the administration of D-luciferin with the field of view set at 25 cm. The signal intensity was quantified as photons flux in units of photons/s/cm²/steradian in the region of interest, and the maximum of signal intensity was recorded each time. Bioluminescent signals were measured in recipient rats for up to 6 weeks (group 1: postoperative week 0, 1, 2, 3, 4, and 6 and group 2: daily for 3 wk and postoperative week 4, 5, and 6).

Histological Study

At the end of the study, the grafts of the group 3 were harvested, fixed in 10% buffered formalin, embedded in paraffin, and were sectioned at 4 μm. Immunohistochemical staining was performed using anti-perilipin rabbit monoclonal antibody (Cell Signaling: #9349) and polymer-based detection system (EnVision+ system, Dako). Moreover, we performed in situ end labeling using TUNEL (terminal deoxynucleotidyl transferase-mediated biotin nick end labeling) staining to detect cells undergoing apoptosis in the grafts.

RESULTS

In Vivo Tracking of the Grafts

To track the graft viability, we used luciferase transgenic rats as the donors of the grafts.

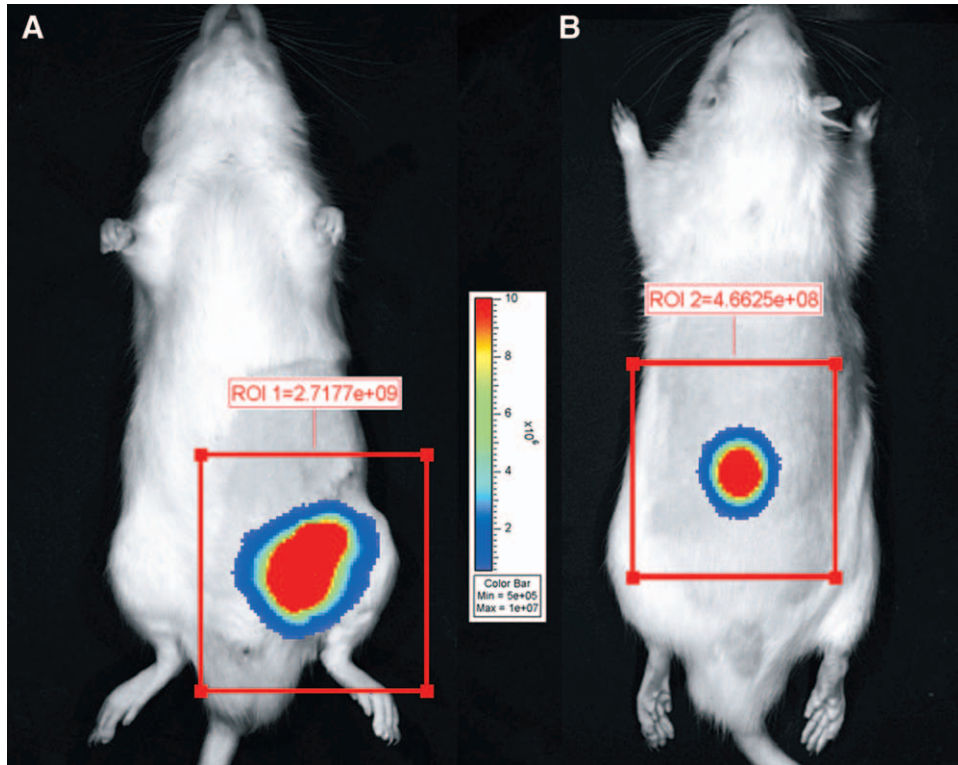


Fig. 1. In vivo optical bioluminescence imaging of representative rats on day 7. The transplanted VAF (A) and nVFG (B) were detectable and quantifiable.

Bioluminescent signals from all grafts were easily detectable and quantifiable over time (Fig. 1). In group 1, the VAF maintained their signal intensity throughout the entire period (Fig. 2A). The maximum intensity was obtained at 2 or 3 minutes after the administration of D-luciferin (Fig. 3A).

In group 2, signal intensity of the nVFG increased drastically from day 3 through day 7, remained from

day 7 through day 12, and decreased from day 12 through day 17 after transplantation. Afterward, relatively stable bioluminescent signals were observed through the following periods (Fig. 2B). Until postoperative day 3, the signal intensity increased gradually after the administration of D-luciferin, suggesting insufficient blood flow. After postoperative day 4, the signal intensity peaked at 2 or 3 minutes, suggesting

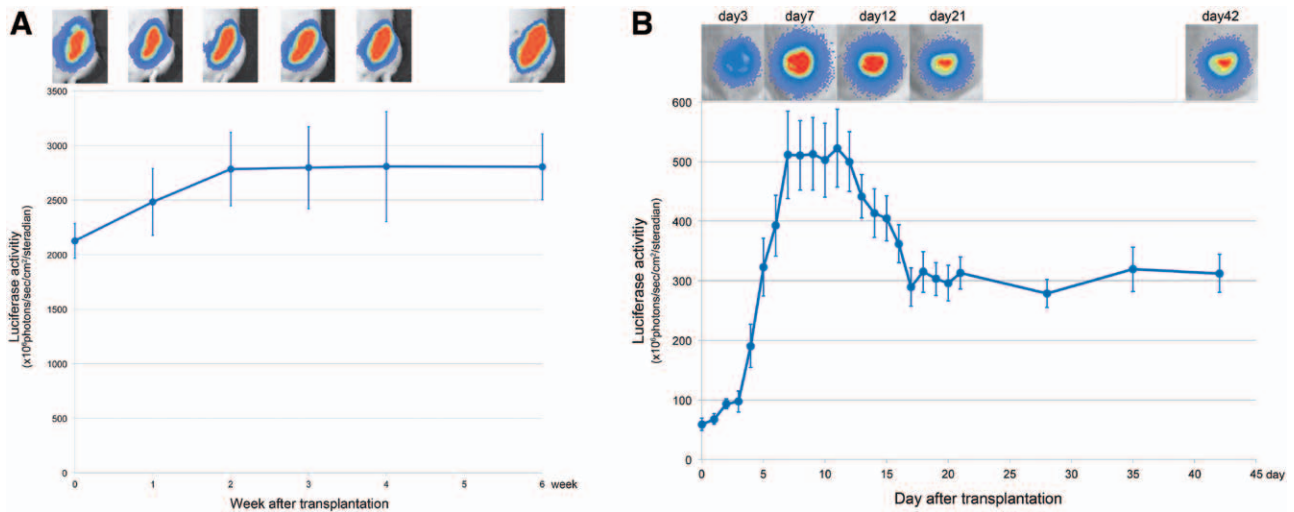


Fig. 2. The maximum signal intensity from VAF (A) and nVFG (B). VAF maintained their signal intensity throughout the entire period. On the other hand, the maximum signal intensity of nVFG increased drastically from postoperative day 3 to 7 and declined from day 12 to 17. Data presented as means \pm SEM.

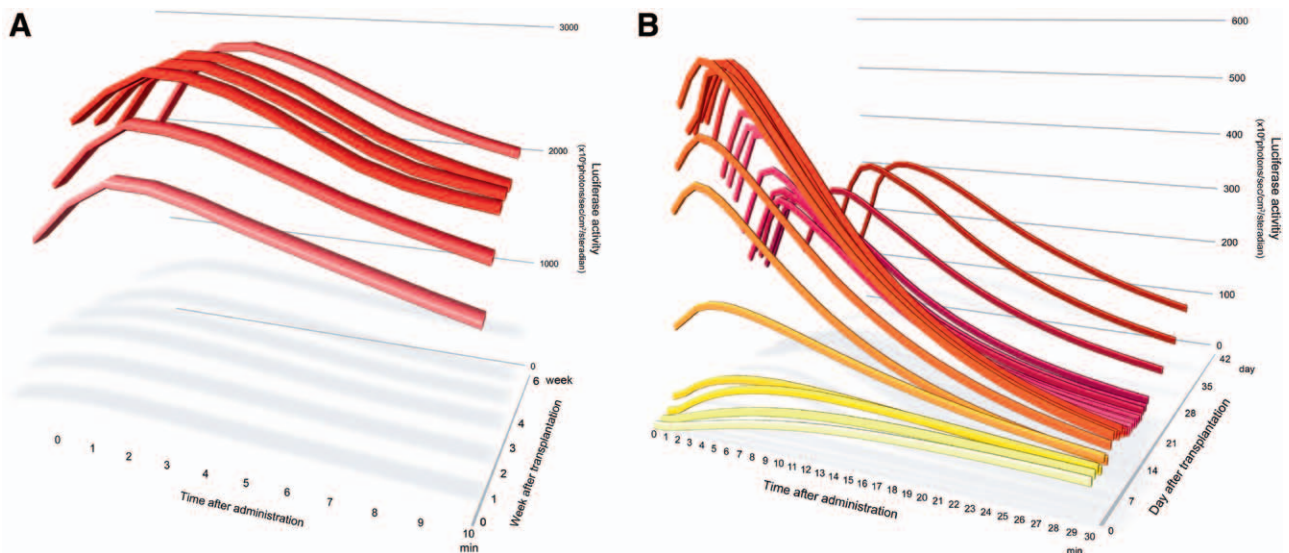


Fig. 3. The time course of bioluminescent signal in VAF (A) and nVFG (B) after administration of d-luciferin. The maximum intensity of VAF was obtained at 2 or 3 min after administration at all testing points. On the other hand, the signal intensity of nVFG showed gradual increase after administration until postoperative day 3, but after postoperative day 4, it showed a peak at 2 or 3 min after administration.

the restoration of blood flow (Fig. 3B). There was no significant difference in the signal intensity per the initial volume at postoperative week 1 and 2 between the 2 groups (Fig. 4).

Histological Findings of the nVFGs

To evaluate the viability of adipocytes after grafting, we used immunohistochemical staining for perilipin,

which could distinguish living adipocytes from dead adipocytes. At week 1 and 2, perilipin-positive adipocytes were detected only at the margins of the grafts, and the cells in the center appeared to be living adipocytes morphologically but were perilipin-negative. After week 3, perilipin-positive small adipocytes appeared at the margins of the perilipin-negative areas and the perivascular regions. At week 6, the 3 zones,

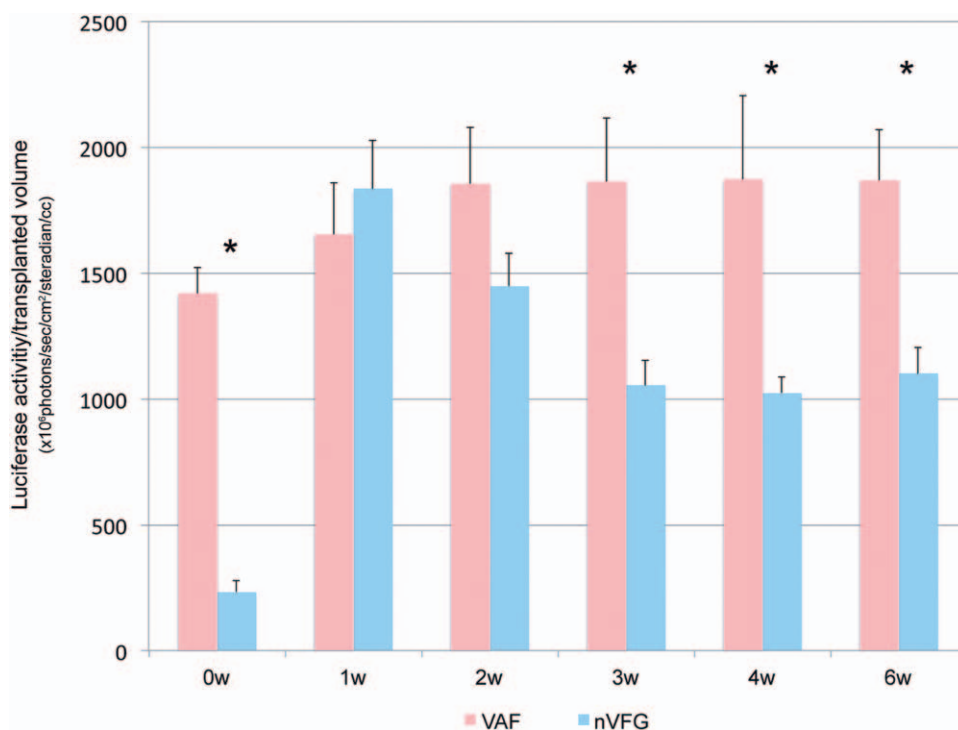


Fig. 4. The bioluminescent signal intensity per transplanted volume of VAF and nVFG (**P* < 0.05).

which Eto et al¹² have described, namely, the surviving area with large perilipin-positive adipocytes, the regenerating area with small perilipin-positive adipocytes, and the necrotic area with no perilipin-positive cells were easily detectable (Fig. 5).

To detect the cells that decreased from day 12 through day 17, TUNEL staining was performed at week 2. TUNEL-positive cells were detected at the stroma (Fig. 6).

DISCUSSION

In this study, we used *in vivo* BLI and immunohistochemistry to examine the time course of the viability of the transplanted nVFG longitudinally. Our approach revealed the dynamic process of survival and regeneration in the nVFG.

The bioluminescent signal intensity is in proportion to cell numbers according to previous studies¹⁴;

however, the light may not be emitted in proportion to the amount of luciferase expressed in the anaerobic environment because luciferase are oxygenases.¹³ Therefore, during the early postoperative period when the blood flow is insufficient, it is thought that the signal intensity of nVFG mainly reflects blood flow, and after enough revascularization, it represents the viability of the grafts.

In group 1, the maximum signal intensity from the VAF was maintained throughout the entire postoperative period, which suggested that immediate vascular reconstruction maintained the viability of transplanted adipose tissues. These findings correspond to the previous reports based on histological analysis.^{20,21} On the other hand, in group 2, the maximum signal intensity from the nVFG increased drastically from postoperative day 3 through day 7. Moreover, the interval till the signals reached the

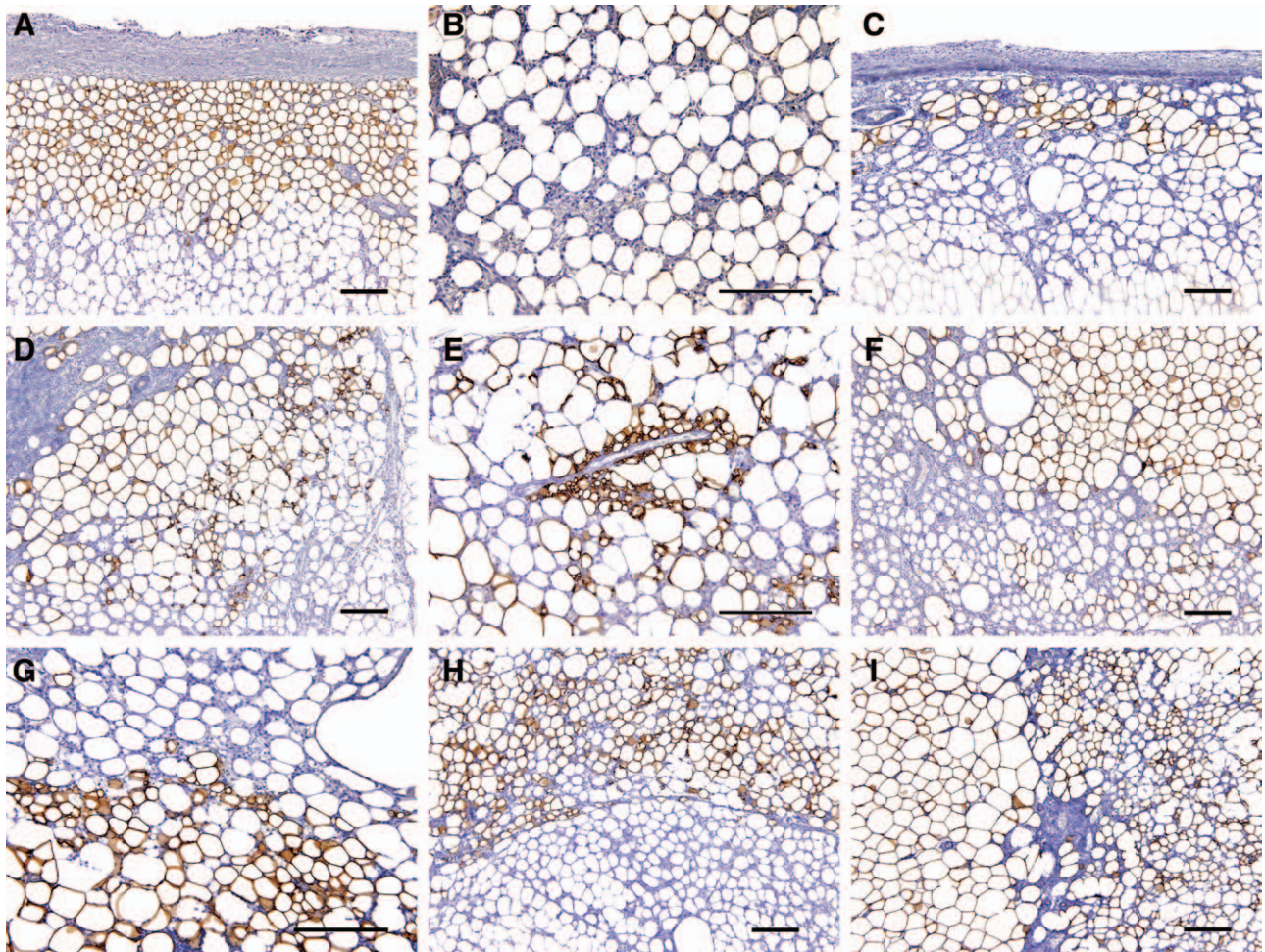


Fig. 5. Immunohistochemical staining for perlipin in the nonvascularized fat grafts. Perlipin-positive adipocytes in the border of the grafts at week 1 (A) and week 2 (C). Adipocyte-like but perlipin-negative cells in the center of the grafts at week 2 (B). Perlipin-positive small adipocytes in the border of the perlipin-negative area at week 3 (D) and the perivascular region at week 4 (E). The perlipin-positive areas were easy to distinguish from the perlipin-negative areas at week 4 (F) and week 6 (G and H).

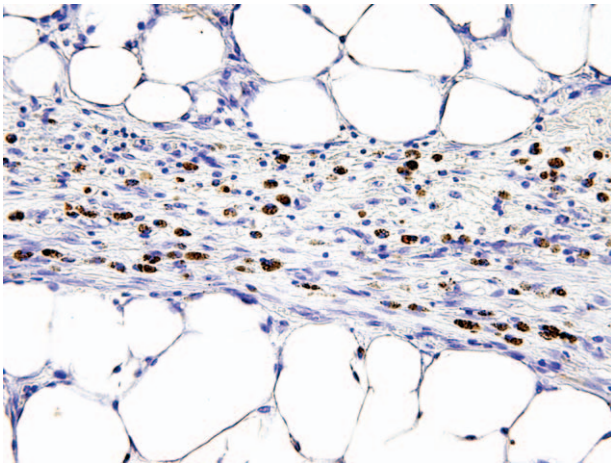


Fig. 6. TUNEL staining in the nonvascularized fat grafts at week 2. TUNEL-positive cells were detected in the stroma.

maximum shortened from day 3 through day 7. These findings suggest that the blood flow in nVFG had been restored from postoperative day 3 to 7 and are mostly in accordance with the reports based on histological findings of small grafts such as fat grafts,⁸ islet grafts,²² and parathyroid grafts.²³

The maximum signal intensity in nVFG was maintained stably from day 7 to 12, and the signal intensity per the initial volume of the nVFG was almost equal to that of VAF at postoperative week 1 and 2. However, immunohistochemical findings demonstrated that perilipin-positive adipocytes had drastically decreased at week 1 and 2. These findings

suggested that many adipocytes were dead and the donor-derived stromal cells were proliferated compensatorily during the period.

From day 12 through day 17 after transplantation, the maximum signal intensity from nVFG declined. Moreover, TUNEL-positive cells were detected in the stroma at week 2. These findings suggested that a part of the proliferated stromal cells, probably adipose-derived stem/stromal cells (ADSCs), underwent apoptosis. Further investigations are required to reveal the mechanism of apoptosis in fat grafts.

After day 17, relatively stable bioluminescent signals were observed. Moreover, perilipin-positive small adipocytes were detected in perivascular regions or at the margins of necrotic zones after week 3. These results suggested that the survived stromal cells began to differentiate into adipocytes; the regenerating phase started.

Recently, Eto et al¹² reported the fate of nVFG in early phase (2wk) and proposed the 3 zones: the surviving area (adipocytes survived), the regenerating area (adipocytes died, ADSCs survived, and dead adipocytes were replaced with new ones), and the necrotic area (both adipocytes and ADSCs died) (Fig. 7). Our long-term study supported their “3 zones” almost completely. Moreover, our approach with combination of in vivo BLI and immunohistochemistry revealed the dynamic process of survival and regeneration of the nVFG.

In our study, although most adipocytes in nVFG died within 1 week, the bioluminescent signal in-

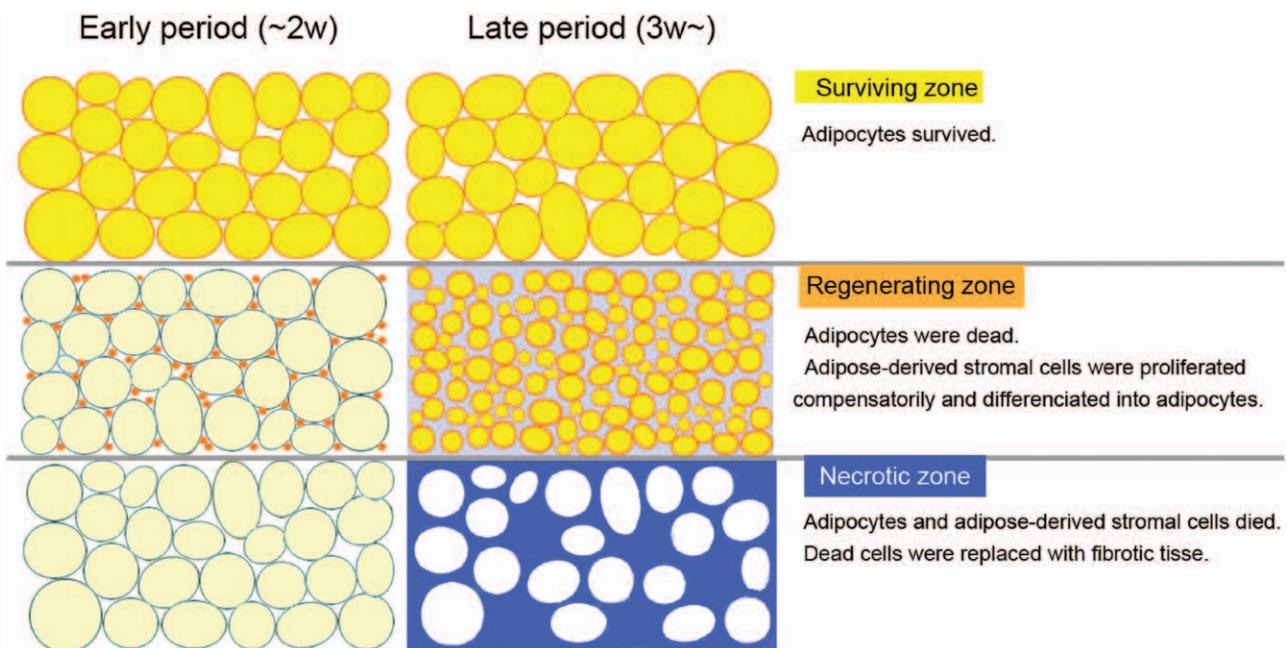


Fig. 7. The schematic diagram of the “3-zone theory.” Surviving zones and necrotic zones located at the periphery and the center in the grafts, respectively. Regenerating zones locate at the intermediate areas between surviving zones and necrotic zones and at the perivascular regions.

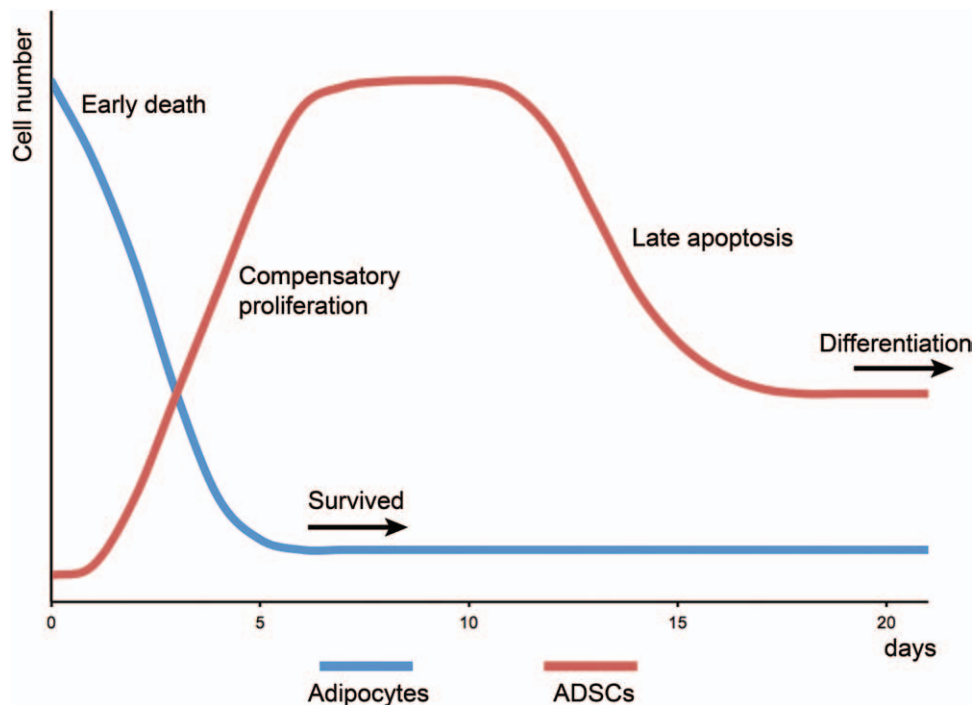


Fig. 8. The change of cell numbers in nVFG. Most adipocytes died within 1 wk after grafting, and only a few adipocytes in the surviving zone survived. ADSCs proliferated within 1 wk compensatorily, but a part of them underwent apoptosis from day 12 through day 17. The survived ADSCs were differentiated into adipocytes.

tensity per transplanted volume was almost equal to VAF. These findings suggested that the ADSCs proliferated just as much as the adipocytes died to restore the adipose tissue to its former state. However, the proliferated ADSCs underwent apoptosis from day 12 through day 17, and the survived ADSCs were differentiated into adipocytes from week 3, resulting in incomplete regeneration of the adipose tissue and replacement with fibrotic tissue (Fig. 8). To improve clinical results of fat grafting, it is essential to clarify the mechanism of the late apoptosis of ADSCs.

In this study, we did not approach the contribution of the recipient-derived cells such as bone marrow-derived cells to regeneration of the grafts. Further study using transgenic rats with the marker genes, such as GFP rats²⁴ or LacZ rats,²⁵ would be valuable.

Recently, several clinical studies reported the high survival rate of nVFG in a large amount using an excellent technique with multiple injective passes of small pieces,²⁶ concurrent transplantation of ADSCs,²⁷ or an external soft-tissue expansion system.²⁸ Their clinical results warrant further studies on the fat graft survival.

CONCLUSION

This study demonstrated the long-term fate of the nVFGs. ADSCs proliferated within 1 week and differentiated into adipocytes after 3 weeks.

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