How Does Fat Survive and Remodel After Grafting?

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KEYWORDS

• Fat grafting • Adipose-derived stem/stromal cell • Tissue regeneration • Macrophages
• Vascular endothelial cells

KEY POINTS

- Under severe ischemia, adipocytes die within 24 hours; adipose-derived stem/stromal cells (ASCs) survive up to 3 days and are activated, contributing to the repairing process through adipogenesis, angiogenesis, and paracrine effects.
- Adipocyte fate after fat grafting is categorized into three zones depending on the distance from the surface: survival, regeneration, and necrosis.
- ASCs do not die and give rise to new adipocytes in the regenerating zone; they die in the necrotizing zone. The balance between regeneration and degeneration determines the final volume retention after fat grafting.
- Dead adipocytes under better conditions (regenerating zone) are phagocytized by macrophages and are successfully replaced by new adipocytes.
- Dead adipocytes under worse conditions (necrotizing zone) are replaced with cicatrization or oil cyst formation depending on the size of oil drops.

INTRODUCTION

Adipose tissue and adipose-derived stem/stromal cells (ASCs) obtained from liposuction were shown to have potential for regenerative therapeutic use. However, clinical outcomes of fat grafting remain unpredictable and, to improve the outcomes, it is crucial to elucidate the detailed mechanism of engraftment of fat tissue. The "cell survival theory," which maintains that transplanted adipocytes partly survive once they receive adequate nutrients and remain alive in the recipient site, had been accepted for a long time.[1–3] In contrast, our recent studies showed how ASCs work in response to microenvironmental changes, such as ischemia and applied mechanical force,[4,5] and revealed the "cell replacement theory," which holds that most adipocytes undergo ischemic death and subsequent replacement with next generation during the first 3 months after fat grafting.[6,7] Further details, such as the cellular origin of adipose regeneration and the mechanism of cicatrization and oil cyst formation, were also demonstrated.[7]

BASIC SCIENCE: FUNCTIONAL ROLES OF ADIPOSE-DERIVED STEM/STROMAL CELLS IN TISSUE REMODELING

Adipose Tissue Biology

Adipose tissue is not only an organ of energy storage, but also an endocrine organ (releasing multiple adipose-derived hormones, such as leptin and adiponectin) that regulates metabolic

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homeostasis. Adipose tissue consists predominantly of adipocytes, ASCs, vascular endothelial cells (VECs), pericytes, fibroblasts, and connective tissue as well as adipose tissue-resident macrophages and lymphocytes (**Fig. 1**).[8] Our rough estimation of cellular component numbers are as follows; 1 cm^3 intact adipose tissue contains several millions cells; 1 million adipocytes, 1 million ASCs, 1 million VECs, and 1 million other cells (adipose-resident macrophages and lymphocytes, pericytes, fibroblasts, etc).[8] Adipose tissue is rich in capillary and every single adipocyte is attached to the capillary network. The size of adipocyte is 50 to 150 μm in diameter (if it becomes larger, it dies from ischemia) and its life span is several to 10 years in humans. ASCs are located perivascularly along the capillaries between adipocytes like pericytes. ASCs have been shown to release angiogenic factors responding to ischemia[4] and to differentiate physiologically into adipocytes and VECs.[9] A small subpopulation of ASCs (1%–2%) may have greater multipotency, corresponding with stem cells called multilineage differentiating stress enduring (Muse) cells.[10] The enlarged adipocytes in obese individuals occasionally die from relative ischemia and are subsequently surrounded by infiltrated M1 inflammatory macrophages (crownlike structure). The crownlike structure is seen after any types of adipocyte death (**Fig. 2**).

Adipose-Derived Stem/Progenitor Cells in Adipose Tissue Remodeling

ASCs are the main cell population contributing to adipocyte (re)generation in any types of adipose tissue remodeling/expansion, such as developmental growth, hyperplasia in obesity, repair processes after injury/ischemia,[4] or tissue expansion induced by internal/external mechanical forces.[5] These remodeling processes are in balance between adipocyte apoptosis/necrosis and adipogenesis managed by ASCs. In ASC-deficient tissues, such as irradiated or chronically inflamed tissues, any type of adipose tissue remodeling or expansion is impaired and thus fat grafting to fertilize such stem cell–depleted condition would be theoretically the right solution.[11] Adipose-tissue atrophy over aging is likely owing to a decrease in number of ASCs and consequent impaired physiologic turnover, as is commonly seen in other tissues and organs.

Ischemia to Adipose Tissue

Subcutaneous adipose tissue has the highest tissue partial oxygen tension (ptO_2; 40–60 mm Hg) among organs. The high ptO_2 of adipose tissue probably reflects high density of capillaries and low oxygen consumption rate of the tissue. Diabetic adipose tissue is relatively ischemic with low-grade chronic inflammation, which causes

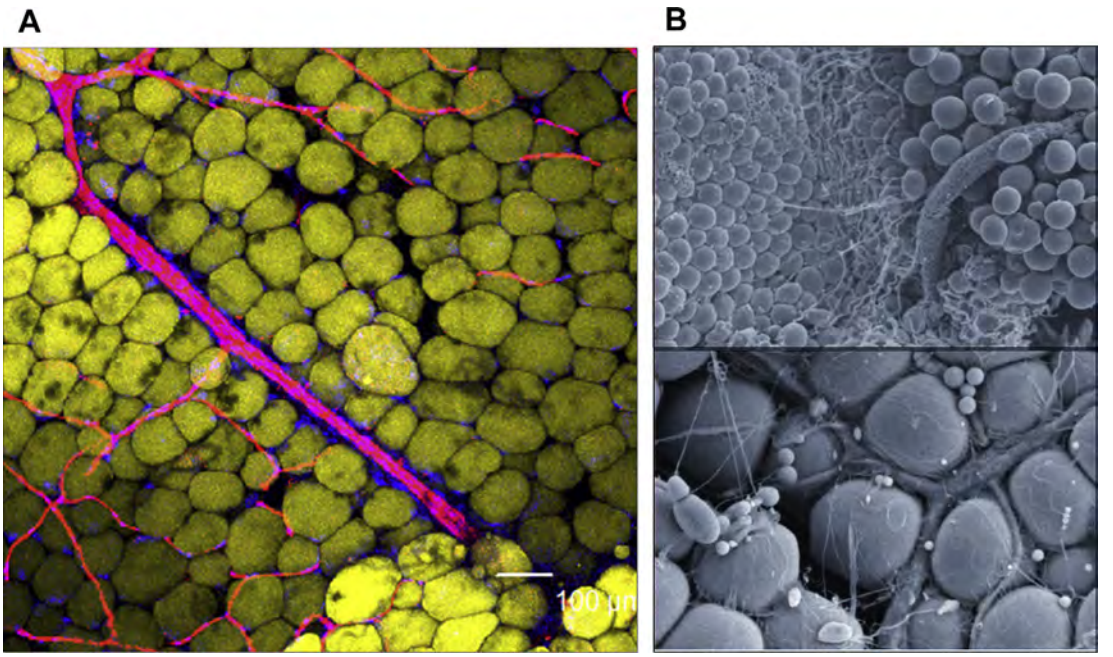

Fig. 1. Structure of human adipose tissue. (*A*) Adipose tissues are triple stained with BODIPY (adipocytes; *yellow*), lectin (endothelial cells; *red*), and Hoechst 33,342 (nuclei; *blue*). Adipose tissue is packed with adipocytes with scarce connective tissue and is rich in capillary network, though each adipocyte is exceptionally large in size. Scale bars = 100 μm. (*B*) Scanning electromicroscopic images.

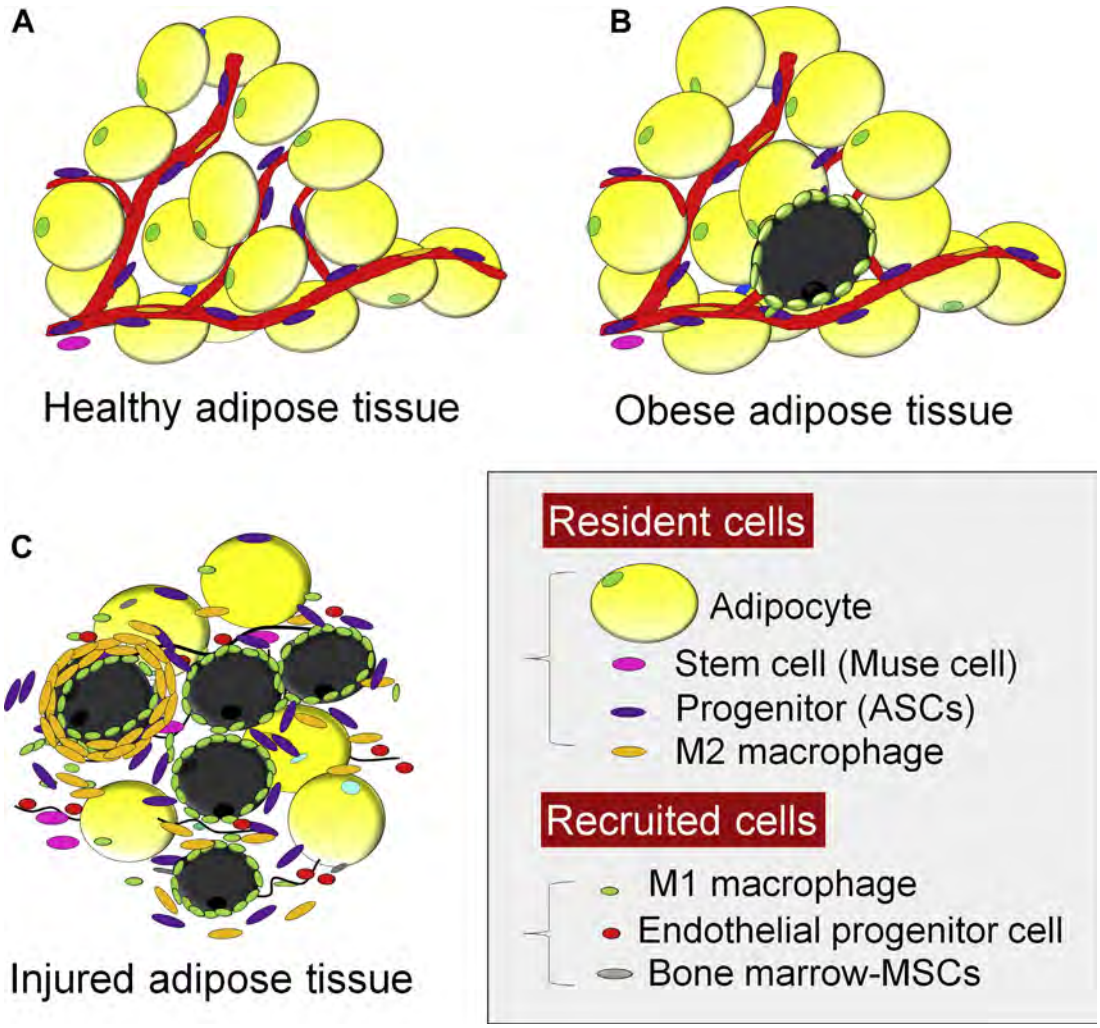

Fig. 2. Schema for structure of intact, obese, and injured adipose tissues. (*A*) Intact adipose tissue has not only adipocytes but also many other types of cells such as ASCs and vascular endothelial cells. (*B*) Obese adipose tissue has some dead adipocytes surrounded by infiltrated M1 macrophages (crownlike structure) and shows low-grade chronic inflammatory condition. (*C*) In injured adipose tissue, ASCs are activated and many types of progenitor/stem cells are recruited from bone marrow to repair the tissue damage. MSC, mesenchymal stem cell.

adipose endocrine dysfunction, insulin resistance, and the metabolic syndrome, whereas lipoma tissue is not ischemic, probably owing to upregulated angiogenesis.[12]

Among cellular components of adipose tissue, adipocytes are most susceptible to death under ischemic conditions such as 15 mm Hg of ptO_2.[6] When severe ischemia prolongs, VECs and blood-derived cells start to die next. In contrast, ASCs can remain alive up to 3 days, even under severely ischemic conditions.[6] Over the 3 days, they can be activated by signals from dying cells and contribute to the adaptive repairing process, such as by adipogenesis and angiogenesis.[6,7]

Injury to Adipose Tissue

Tissue injury also causes adipose tissue degeneration with inflammatory cell recruitment and release of inflammatory cytokines. After injury, degenerative changes such as adipocyte death occur, and primary injury factors such as basic fibroblast growth factor and other factors from aggregated platelets such as platelet-derived growth factor, epidermal growth factor, and transforming growth factor-β are first released into the injured site and trigger a cascade of wound healing processes.[4,13] Basic fibroblast growth factor is released from damaged connective tissue and

acts through a c-Jun N-terminal kinase signaling pathway to stimulate ASCs not only to proliferate, but also to secrete secondary factors such as hepatocyte growth factor and vascular endothelial growth factor, and contributes to the regeneration of adipose tissue and suppression of fibrogenesis during the first week after injury.[13] In parallel, a variety of stem/progenitor cells such as endothelial progenitor cells are recruited from bone marrow and collaborate with activated ASCs in an orchestrated repair of the damaged adipose tissue (see **Fig. 2**).

Mechanical Force to Adipose Tissue

Mechanical forces, whether external (shear, stretch, tension, distraction and compression) or endogenous (forces that are generated within the

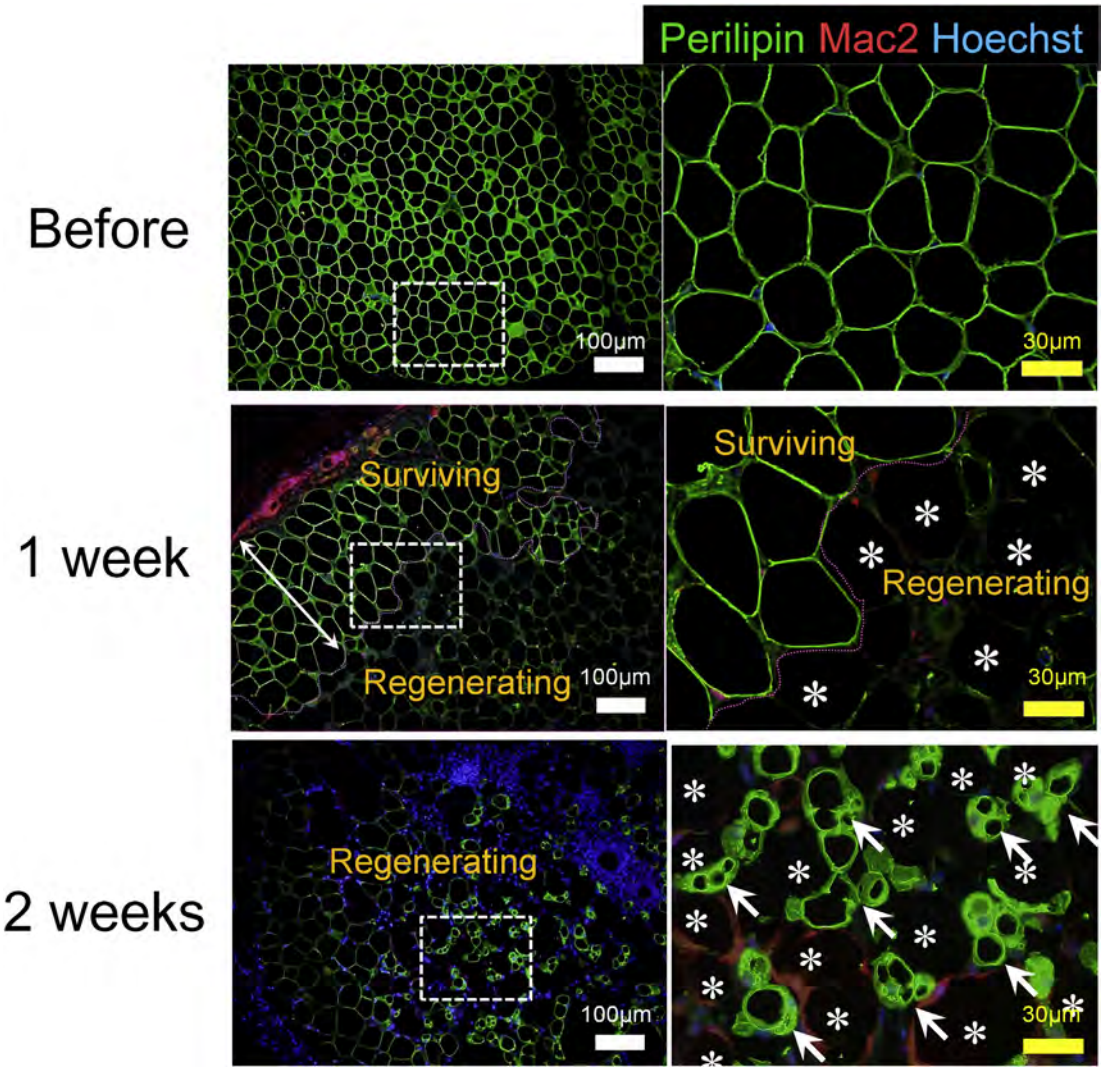

Fig. 3. Immunohistology of grafted fat tissue in mice (Before, 1 week and 2 weeks). Harvested tissue samples [before (*top*), 1 week (*middle*), and 2 weeks (*bottom*) after grafting] were immunostained for perilipin (cytoplasm of viable adipocytes; *green*), MAC2 (monocytes/macrophages; *red*) and Hoechst 33,342 (nuclei; *blue*). Rectangles in the low magnification images (*left column*; *yellow* scale bars = 100 μm) were further magnified in the right column (*white scale bars* = 30 μm). Demarcation between the surviving and regenerating zone became clear at 1 week (*interrupted line*); dead adipocytes (*asterisk*) were perilipin negative and surviving adipocytes were strongly positive for perilipin. Small-sized preadipocytes with multiple intracellular lipid droplets (*arrows*) appeared between dead adipocytes at 2 weeks; the dead adipocytes were surrounded by a single layer of macrophages (*red*). (*Adapted from* Kato H, Mineda K, Eto H, et al. Degeneration, regeneration, and cicatrization after fat grafting: dynamic total tissue remodeling during the first 3 months. Plast Reconstr Surg 2014;133:306e; with permission.)

active cytoskeleton), affect tissue growth, cellular function, and even survival. Moreover, physical interactions with the extracellular matrix can significantly influence stem cell behavior.[14] Continuous external tissue expansion (Brava®) is attempted for expansion of the breast tissue.[15] Experimentally, 4 weeks of external suspension caused enlargement of the subcutaneous tissue, particularly adipose tissue, although the enlargement was reversible.[5] The regenerating potential has been attributed to the number (density) and potential of ASCs; thus, irradiated tissue has a limited potential for expansion.

RELEVANCE TO CLINICIANS: WHAT HAPPENS AFTER FAT GRAFTING?

Acute Events Immediately After Fat Grafting

The grafted nonvascularized adipose tissue is placed under ischemia (hypoxia) and is nourished only by plasmatic diffusion from the surrounding host tissue for a few days until revascularization occurs. This results in the death of many adipocytes within 24 hours and release of multiple cell death and injury-associated factors from the dying donor tissue and injured host tissue (**Fig. 3**).[6,13] Inflammatory cells, such as macrophages and lymphocytes, are infiltrated and inflammatory cytokines, such as interleukins, are secreted. Despite the death of adipocytes, ASCs, which can be functional for up to 72 hours even under severe ischemia, are activated and try to repair the damaged tissue in collaboration with infiltrated stem and progenitor cells from the bone marrow.[6,7]

Regeneration After Ischemic Tissue Damage: Three Zones with Differential Cell Fates

Based on our recent studies, the first 3 months after fat grafting is a period of tissue remodeling; adipogenesis does not occur after this period.[11] The grafted fat is categorized into three zones from the periphery to the center: (1) survival (superficial), (2) regeneration (intermediate), and (3) necrosis (central; **Fig. 4**).[7] The demarcation of the surviving zone (100–300 μm thick) from the regenerating

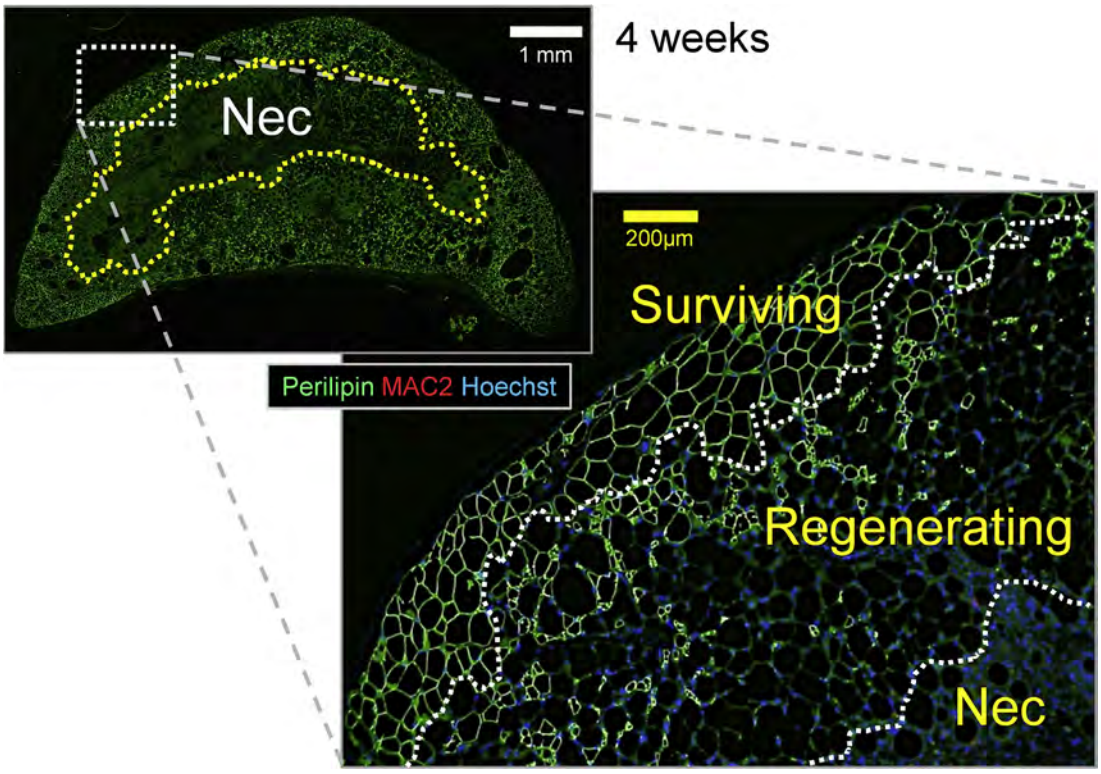

Fig. 4. Immunohistology of grafted fat tissue in mice (4 weeks). Immunohistology of a graft sample at 4 weeks showed demarcated surviving, regenerating, and necrotizing zones. (*Left*, *above*) A low-magnification image of perilipin staining showed the necrotizing zone (*yellow interrupted line*) with no adipogenesis. White scale bar = 1 mm. (*Right*, *below*) A high-magnification image showed demarcated (with *white interrupted lines*) surviving (perilipin-positive adipocytes), regenerating (perilipin-positive small adipocytes), and necrotizing zones (no viable adipocytes). Yellow scale bar = 100 μm.

zone became clear at 1 week, whereas the demarcation between the regenerating and necrotizing zones was obvious between 2 and 4 weeks (see **Fig. 3**).

Adipocytes superficially located 100 to 300 μm from the tissue edge remain alive (surviving zone), and all the rest of adipocytes (regenerating and necrotizing zones) die within 24 hours after grafting. The dead adipocytes are surrounded by M1 macrophages for phagocytosis (see **Fig. 3**), but the absorption process takes weeks or months, depending on the size and therefore the grafted fat maintains its original size for the first 4 weeks despite adipocyte death. ASCs in the regenerating and necrotizing zones are activated and start to repair the tissue. New, small preadipocytes appeared around the dead adipocytes (surrounded by a single layer of macrophages) at 1 to 2 weeks in the regenerating zone (600–1200 μm thick), whereas no adipogenesis was observed in the necrotizing zone (see **Fig. 4**). In the regenerating zone, the hypoxic condition is improved by revascularization within 3 days and ASCs give rise to new adipocytes, which finally replace the dead adipocytes by 3 months. On the other hand, in the necrotizing zone, the microenvironment is not improved within 3 days and ASCs also die, leading to central necrosis of the graft tissue.

The ratio between the necrotizing and surviving/regenerating zones, which determines the final volume retention after fat grafting, varies depending on the recipient microenvironment, based on factors such as vascularity, as well as the size of the grafted fat, grafting technique, and postoperative care. Our experimental study using a mouse model revealed that oxygenation of the recipient bed with normobaric 60% oxygen for 3 days postoperatively promotes survival, regeneration, and

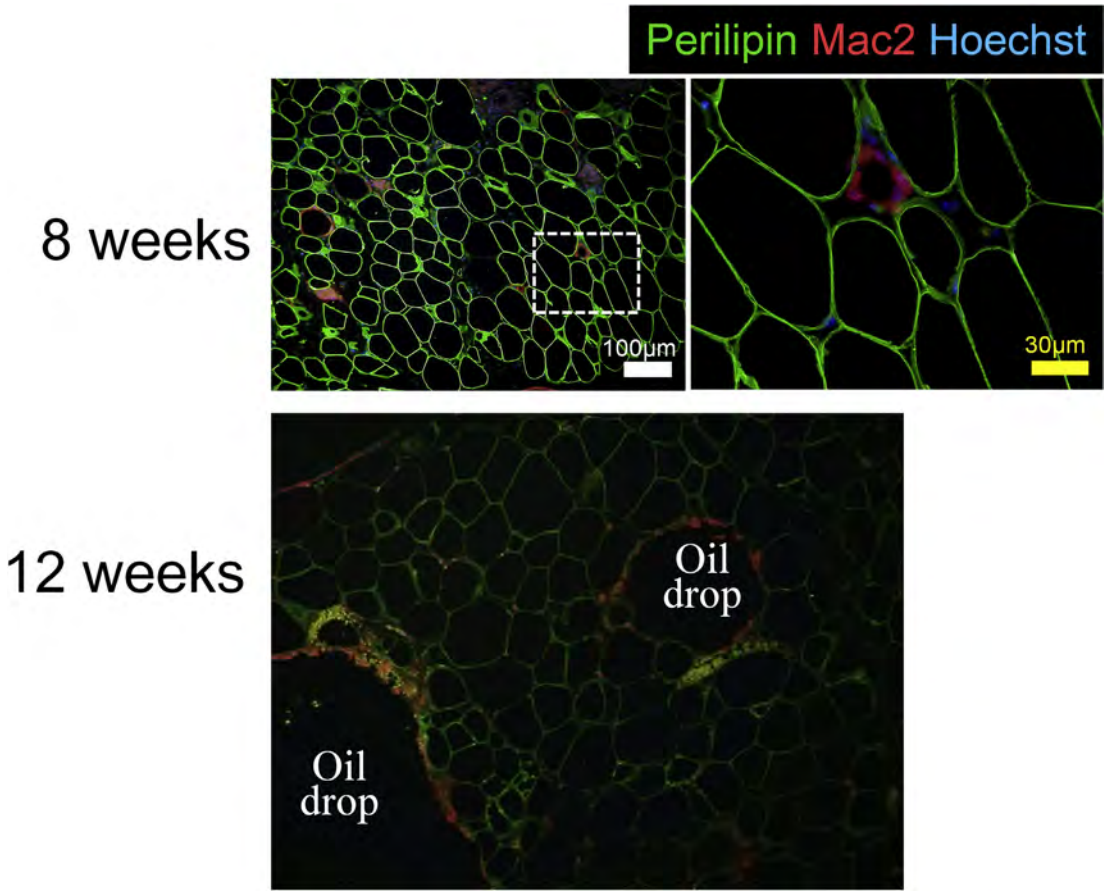

Fig. 5. Immunohistology of grafted fat tissue in mice (8 and 12 weeks). Harvested tissue samples (8 weeks [*top*] and 12 weeks [*bottom*] after grafting) were immunostained for perilipin (cytoplasm of viable adipocytes; *green*), MAC2 (monocytes/macrophages; *red*) and Hoechst 33,342 (nuclei; *blue*). There are few small new adipocytes, which means that adipose regeneration seemed to be finished by 12 weeks. Large-sized lipid drops surrounded by M1 macrophages are left in the tissue. (*Modified from* Kato H, Mineda K, Eto H, et al. Degeneration, regeneration, and cicatrization after fat grafting: dynamic total tissue remodeling during the first 3 months. Plast Reconstr Surg 2014;133:306e.)

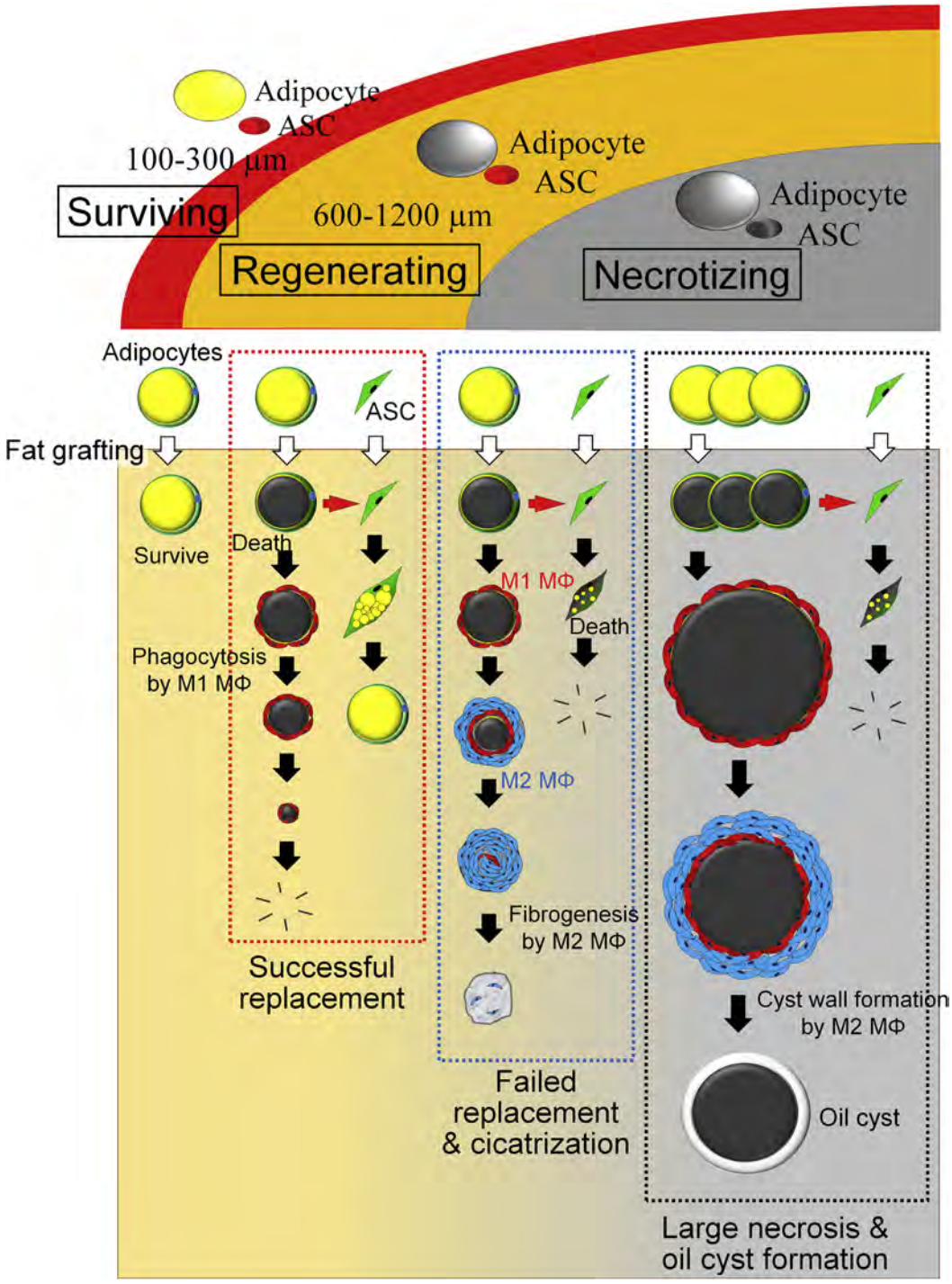

Fig. 6. Conclusive schema for the fate of adipocytes in grafted fat. During the first 3 months of adipose tissue remodeling, transplanted adipocytes have differential fates depending on their microenvironments. In this schema, complex cellular events are simplified and the adipocyte fate is categorized into 4 patterns: survival, successful regeneration, failed regeneration (cicatrization), and oil cyst formation. Cicatrization and oil cyst formation are often not complete at 3 months. The most superficial zone is the "surviving zone," which is less than 300 μm thick. In the surviving zone, both adipocytes and adipose-derived stem cells (ASCs) survive. The second zone is the "regenerating zone," the thickness of which varies (600–1200 μm) depending on the microenvironmental conditions. In this zone, adipocytes die, but ASCs survive and provide new adipocytes to replace the dead ones. The most central zone is the "necrosis zone," where both adipocytes and ASCs die, no regeneration is expected, and the dead space will be absorbed, be filled with fibrosis, or develop into an oil cyst. (*Modified from* Kato H, Mineda K, Eto H, et al. Degeneration, regeneration, and cicatrization after fat grafting: dynamic total tissue remodeling during the first 3 months. Plast Reconstr Surg 2014;133:312e.)

final retention of transplanted fat.[16] The thickness of surviving and regenerating zones were increased, suggesting superior survival of adipocyte and resident ASCs, respectively.

Long-Term Stabilization Process (Lipid Absorption and Cicatrization)

In parallel with the regenerating events, stabilizing events, such as lipid absorption (phagocytosis) and lipid replacement with scar tissue (fibrosis), occur in the regenerating and necrotizing zones.[7] Although the adipogenesis/regeneration process in the regenerating zone peaks at 4 weeks and is completed by 3 months (**Fig. 5**), the stabilizing process persists for at least several more months, as suggested by clinical observations that volume reduction after fat grafting continues until the end of the first year. Small-sized oil droplets were absorbed or temporarily filled with multilayered M2 macrophages, inducing the dead space replacement with fibrogenesis in parallel with lipid absorption. On the contrary, substantially larger oil drops (>8 mm) form oil cysts in several months and remain permanently, which are considered the worst outcome of fat grafting accompanied by chronic inflammation and calcification.[7,11,17] We summarize differential fates of adipocytes depending on the microenvironment in **Fig. 6** and the postoperative time course of fat grafting in **Fig. 7**.

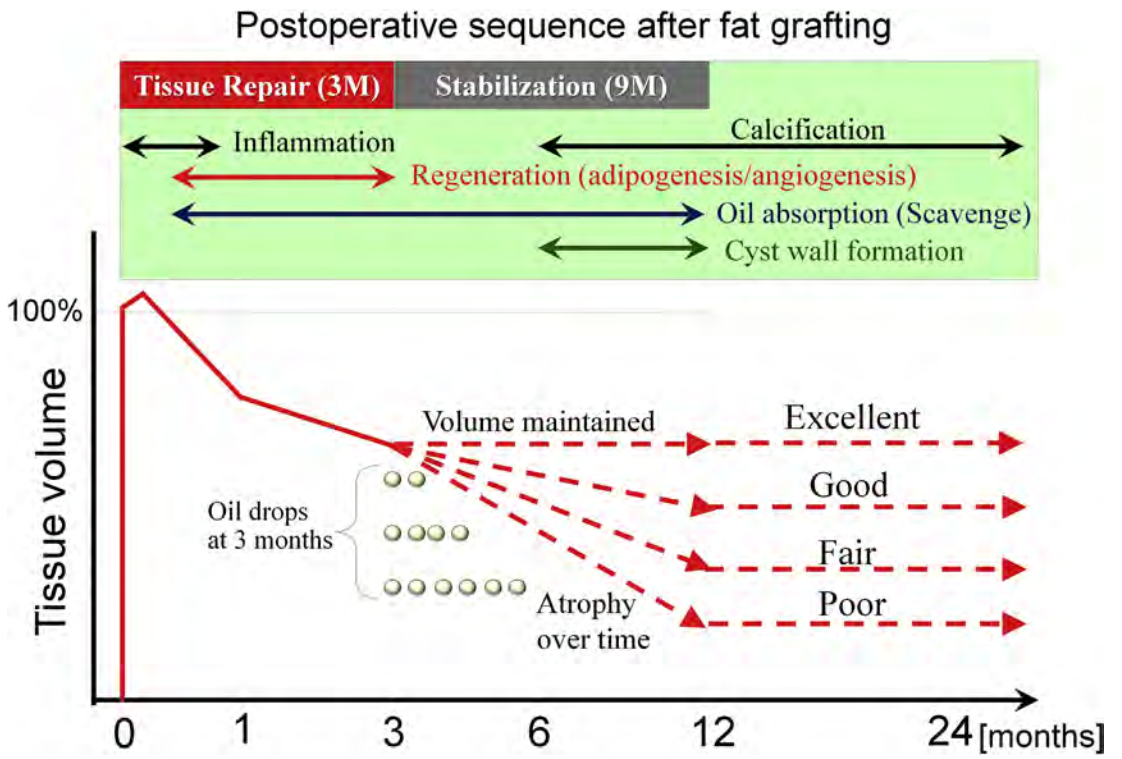

Fig. 7. Long-term postoperative sequence after fat grafting. Adipogenesis after adipocyte death is exerted by activated adipose-derived stem cells (ASCs). Some of the dead adipocytes are replaced with new adipocytes of next generation and the adipogenesis is finish by 3 months (tissue "repair" phase). Dead adipocytes remaining at 3 months are absorbed during the next 9 months (tissue "stabilization" phase). Lipid droplets (dead adipocytes) are absorbed by macrophage phagocytosis, but the absorption is very slow and the absorption period depends on the diameter of the lipid droplets; when the lipid droplet diameter was large, such as 10 mm, the cyst wall is formed before completing absorption and the cyst wall start to calcify over time. The final volume retention after fat grafting is determined by the rate of successful replacement of adipocytes. If grafted adipose has only small lipid droplets and absorption is finished by 3 months, the volume will not substantially change after 3 months (shown as "excellent"). On the other hand, many large lipid droplets remain at 3 months, tissue will atrophy between 3 and 12 months (shown as "poor"). (*Adapted from* Yoshimura K, Eto H, Kato H, et al. In vivo manipulation of stem cells for adipose tissue repair/reconstruction. Regen Med 2011;6(6 Suppl):38; with permission.)

What Are the Origins of Next-Generation Cells After Fat Grafting?

Our recent study using green fluorescent protein mice revealed the origin of cell components in grafted fat.[18] Mature adipocytes are mostly derived from ASCs in the graft. Although vascular wall constituents (smooth muscle cells) are chiefly graft derived; capillaries (VECs) originated equally from the graft and the host bone marrow. ASCs of the regenerated fat are an admixture of grafted, host non–bone marrow, and host bone marrow cells. These findings highlight the importance of ASCs contained in the grafted fat for regeneration of adipocytes. Also, host bone marrow and local tissues contribute substantially to capillary networks and the provision of new ASCs, which can contribute to future remodeling. Thus, although ASCs can be provided by bone marrow or other tissues, they have to get ready by staying adjacent to adipocytes in of contributing to adipocyte regeneration after adipocyte death.

Clinical Implications: How We Can Improve the Engraftment of Grafted Fat?

Recent advancements in the understanding of the underlying mechanisms provide a number of clinical implications. It is considered that the size and thickness of surviving zone are influenced by the surrounding recipient tissue. Better vascularity and greater oxygen tension of recipient tissue increase the surviving zone. Preconditioning of recipient tissue, negative pressure, and/or hyperoxygenation may help for this purpose. Excessively high internal pressure keeps the recipient tissue ischemic and reduces the surviving zone. As with skin grafts, immobilization should help the capillary to grow into the graft during the first week, which improves the oxygen tension of the regenerating zone and rescues ASCs from ischemic death. The size and surface area of grafted fat is a critical factor to minimize the central necrotizing zone; the diameter of grafted fat particles or noodles would be recommended to be as small as 2 mm. For adipogenesis after fat grafting, it is very important to have a good number of both viable adipocytes and ASCs in the graft (not helped from the outside). Adipocytes can release crucial factors to activate adjacent ASCs and lead them to differentiation into adipocytes. This finding strongly suggests that it is worth considering preparing a better number and ratio of adipocytes and ASCs during the tissue processing before grafting (discussed in the article by Kuno and Yoshimura elsewhere in this issue).

SUMMARY

ASCs act as main players in any types of adipose tissue regeneration, including after fat grafting, by differentiating into adipocytes or VECs and releasing angiogenic growth factors. The fate of grafted fat depends on its size and the microenvironment of cellular components, such as adipocytes. Adipocytes remain alive in the surviving zone, whereas they die shortly after grafting in the regenerating and necrotizing zones. Adjacent perivascular ASCs are activated by adipocyte death and begin to proliferate and differentiate to repair the damaged tissue in collaboration with infiltrated stem/progenitor cells in the regenerating zone. Dead adipocytes are phagocytized by M1 macrophages and are replaced successfully by new adipocytes without residual fibrosis in a better condition of the regenerating zone. In contrast, dead adipocytes under worse conditions in the regenerating or necrotizing zones are replaced partly with fibrosis or oil cysts; M2 macrophages act in the fibrogenesis process. Interestingly, dead adipocytes work as spacers and keep the space for new adipocytes during the regeneration process. The final volume retention after fat grafting is determined by the balance between degeneration and regeneration of adipose tissue and affected by many surgeons' factors including the microenvironments of graft regenerating zone and surrounding recipient tissue. Adipogenesis after fat grafting depends greatly on ASCs resident in the graft tissue, suggesting the importance of tissue processing before transplantation.

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