Stromal Vascular Fraction From Adipose Tissue Forms Profound Vascular Network Through the Dynamic Reassembly of Blood Endothelial Cells

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Objective—Tremendous efforts have been made to establish effective therapeutic neovascularization using adipose tissue-derived stromal vascular fraction (SVF), but the efficiency is low, and underlying mechanisms and their interaction with the host in a new microenvironment are poorly understood.

Methods and Results—Here we demonstrate that direct implantation of SVF derived from donor adipose tissue can create a profound vascular network through the disassembly and reassembly of blood endothelial cells at the site of implantation. This neovascular structure successfully established connection with recipient blood vessels to form a functionally perfused circuit. Addition of vascular growth factors to the SVF implant improved the efficiency of functional neovascularization formation. In contrast, spheroid culture of SVF before implantation reduced the capacity of vasculature formation, possibly because of cellular alteration. Implanting SVF into the mouse ischemic hindlimb induced the robust formation of a local neovascular network and salvaged the limb. Moreover, the coimplantation of SVF prevented fat absorption in the subcutaneous adipose tissue graft model.

Conclusion—Freshly isolated SVF can effectively induce new vessel formation through the dynamic reassembly of blood endothelial cells and could be applied to achieve therapeutic neovascularization for relieving ischemia and preventing fat absorption in an autologous manner. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: angiogenesis ■ ischemia ■ vascular biology

SVF is a mixture of blood endothelial cells (BECs), adipocyte progenitors, immune cells, fibroblasts, pericytes, and stromal cells, as well as yet undefined stem/progenitor cells.1–4 Previous studies have shown that mesenchymal stem/progenitor cells in stromal vascular fraction (SVF) can be cultured to successfully differentiate into adipocytes, pericytes, osteoblasts, chondrocytes, and myocytes under optimized conditions.1,2,5,6 These observations of in vitro culture prompted the application of cells isolated from SVF in fields of regenerative medicine, such as the use of adipose-derived stem/progenitor cells (ADSCs) or adipose-derived regenerative cells, which are known to be multipotent mesenchymal stem cells.7,8 In most cases, the main strategy is to isolate ADSCs from small quantities of subcutaneous adipose tissue (SAT) retrieved by minimally invasive surgery, further expand them in vitro, and implant the cells into damaged tissues.7,8 However, several drawbacks of such cell therapy do exist. First, the intrinsic stemness of freshly isolated SVF is rarely maintained during culture. Second, the outcome of implanted ADSCs has not been measurable in terms of cell survival and function. Third, the in vitro ADSC differentiation is not readily reproducible in the in vivo microenvironment, and therefore, after implantation, ADSCs would often fail to establish the intended cell population. Moreover, our understanding in the nature of ADSCs and their ultimate fate after implantation remains far from satisfactory, raising safety concerns.

Reasonable means to achieve neovascularization that would ensure circulation should be pursued to relieve chronic complications and minimize the acute/subacute staged ischemic penumbra of occlusive vascular disorders. Tremendous efforts have been made to establish protocols using angiogenic cytokines, adult stem cells, umbilical vein endothelial cells, and endothelial progenitor cells (EPCs) supplemented with the extracellular matrix to attain effective therapeutic neovascularization.9–13 Several attempts14–18 have been made

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to attain therapeutic neovascularization, adopting either BECs derived from adipose tissue or ADSCs. Early studies showed that freshly enriched BECs from adipose tissue could be beneficial for therapeutic reendothelialization. Interestingly, implantation of freshly isolated microvessel fragments from adipose tissue underwent angiogenic sprouting and interfaced with the recipient vessels within a day. Some researchers have claimed that CD34+/CD31− cells derived from cultured human ADSCs are capable of differentiating into BECs, and subsequent augmentation of blood flow through increased vasculature of ischemic hindlimbs takes place. However, whether ADSCs are actually capable of differentiating into BECs remain controversial. Recently, Traktuev et al demonstrated that the majority of CD34+ cells of freshly isolated SVF are actually resident pericytes, which associate with BECs and stabilize the vascular network. Moreover, they extended their study and showed that implants adopting the combination of EPCs and ADSCs yield a much higher neovascularization capacity than when either EPCs or ADSCs were implanted alone and that the implanted EPCs and ADSCs contribute to form the vascular mural layer. This reciprocal interaction between ADSCs and EPCs indicates that if they are appropriately combined, neovascularization can be induced in a synergistic manner.

Nowadays, millions of people receive autologous fat tissue augmentation for cosmetic and reconstructive purposes, but one of the major concerns for this procedure is the shrinkage of adipose tissue over time, mainly due to the loss of adipocytes (fat absorption) and replacement of dead space with fibrotic tissue. Although the primary cause for fat absorption is believed to be insufficient blood supply or supporting matrix to nurture and maintain the implanted adipose tissues, the actual mechanism of this process is poorly understood. In this study, we determined the cellular events occurring in the process of SVF-derived vascular network formation and explored the possibilities of freshly isolated SVF as a source candidate for cell therapy. Here, we suggest their potential therapeutic applications. We showed that freshly isolated donor SVF can readily provide BECs to create a vascular network through a disassembly and reassembly mechanism, which can establish functional communication with the circulation of the recipient. We investigated the clinical implications of this phenomena and demonstrated that SVF delivery can be applied to rescue a mouse hindlimb ischemia or prevent fat absorption in SAT implants.

Methods

Animals
Specific pathogen-free C57BL/6J mice (B6), Tie2-GFP mice, Tie2-GFP mice, and Tie2-GFP mice to avoid contamination from nonadipose tissues as previously described. Implantation of SVF
A total of 3¢10^5 SVF cells were mixed with Matrigel (100 µL, growth factor reduced, BD Biosciences) or collagen type I gel (100 µL, BD Biosciences) containing phosphate-buffered saline (PBS), bovine serum albumin (BSA) (100 ng), vascular endothelial growth factor (VEGF) (50 ng), or COMP-angiopoietin I (hereafter Ang I) (100 ng) and implanted into the flank region subcutaneously of 8-week-old B6, GFP, or FVB/NJ mice.

Supplemental Methods are available online at http://atvb.ahajournals.org.

Results

Direct Implantation of SVF With Matrigel Forms a Profound Vascular Network
First, we were curious what kind of structure would be formed if we implanted SVF directly into a subcutaneous compartment. To avoid contamination from nonadipose tissues, we isolated the SVF from EAT, which is free from other tissues as we previously described. To distinguish implanted donor SVF from that of recipients, we harvested SVF from GFP, dsRed+, or Tie2-GFP+ mice and implanted them into their syngeneic normal or GFP+ mice. Two weeks after implantation, the implanted SVF formed a group of disorganized cells (Figure 1A). In contrast, SVF implanted as a mixture with Matrigel (growth factor reduced) formed a network suggestive of vascular architecture (Figure 1A). To identify this network, we harvested SVF from dsRed+ mice, mixed them with Matrigel, and implanted the mixture into GFP+ mice (Figure 1B). The entire PECAM-1+ vascular structure were shown to be derived from the donor dsRed+ SVF, without identifiable contribution from the GFP+ recipient (Figure 1B). In parallel, we isolated the SVF from SAT of GFP+ mice, mixed them with Matrigel, and implanted the mixture into syngeneic normal mice (Supplemental Figure IA and IB). Formation of the SVF-derived vascular network between the SAT and EAT was almost identical (Supplemental Figure IA and IB). In addition, to distinguish the effect of supporting matrix from growth factors in the SVF-derived vascular network formation, we mixed SVF from GFP+ mice with Matrigel or collagen type I gel and compared the effects at 2 weeks after implantation. Activities of the SVF-derived vascular network formation between the Matrigel and collagen type I gel were almost identical (Supplemental Figure IC and ID). These data indicate that direct implantation of SVF gives rise to vascular structures in a syngeneic recipient but requires a supporting matrix. We observed the SVF-derived vascular network to persist in the recipient throughout the entire experimental period of up to 6 months (Supplemental Figure IE). The other donor cells in the Matrigel that failed to participate in the established vascular network gradually disappeared over time, and corresponding fluorescent signal was detected mainly in the spleen (data not shown), presumably reflecting a degradation process.

The process of SVF-derived vascular formation seems to be substantially influenced by alterations of populations and cellular senescence. Freshly isolated SVF derived from EAT successfully managed to form a de novo vascular network. However, the implants of spheroids produced by

Isolation of SVF From Adipose Tissue
Because epididymal adipose tissue (EAT) has no adjacent tissues, we isolated the SVF from the EAT of B6, dsRed+, GFP+, and

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hanging drop culture of SVF after 2 passages often, but not always, produced vascular networks in the matrix (Supplemental Figure IIA and IIB). In contrast, implants of single-cell suspension of SVF or cultured SVF spheroids after 6 passages failed to form a vascular network in the matrix (data not shown and Supplemental Figure IIA and IIB). In fact, our flow cytometric analyses revealed that matrix (data not shown and Supplemental Figure IIA and IIB). In contrast, implants of SVF of Tie2-GFP+ blood vessels in establishing connections with the recipient are rarely detected. Scale bars=200 μm. C, Diagram for subcutaneous implantation of Tie2-GFP+ SVF mixed with Matrigel into the flank region of FVB/NJ mice. Images show Tie2-GFP+/PECAM-1+ blood vessels derived from Tie2-GFP+ SVF 2 weeks after implantation. Higher magnification of regions 1 and 2 (dotted-line squares) in the peripheral region is presented. White arrows indicate the junction between donor and recipient blood vessels. Scale bars=50 μm. D to G, 3×10^6 SVF cells were harvested from dsRed− mice; mixed with 100 μL of Matrigel containing 50 ng of VEGF-A (VEGF), 100 ng of Ang I (Ang1), VEGF+Ang I (V+A), or 100 ng of BSA; and subcutaneously implanted into the flank region of B6 mice. Two weeks after implantation, FITC-lectin was injected through the tail vein. After 15 minutes, the tissues containing the SVF were whole-mounted and immunostained for PECAM-1. D, Diagram for the FITC-lectin perfusion test. E, Images of FITC-lectin perfusion study showing dsRed−/PECAM-1− (donor) and dsRed+/PECAM-1+ (recipient) blood vessels in BSA (SVF only) or SVF and V+A supplement-implanted mice. Dotted lines indicate margin between donor and recipient blood vessels. Note that FITC-lectin perfusion was detected only in the recipient blood vessels (white arrow) of BSA-supplemented implants, whereas it was detected in both donor and recipient blood vessels in V+A-supplemented implants. White arrows indicate FITC-lectin-containing dsRed+/PECAM-1− blood vessels from the donor. Scale bars=100 μm. F and G, Comparisons of relative SVF-derived blood vessel (SVF-BV) area density per mm² and relative FITC-lectin-perfused SVF-BV area density per mm² in the Matrigel among the treatment groups. Each group, n=5. *P<0.05 vs BSA. #P<0.05 vs VEGF.

Figure 1. Implanted SVF forms a profound vascular network. A to C, 3×10^6 SVF cells were harvested from GFP+ mice, dsRed− mice, or Tie2-GFP+ mice, mixed with 100 μL of PBS or Matrigel, and subcutaneously implanted into the flank region of B6 mice, GFP+ mice, or FVB/NJ mice. Two weeks after implantation, the regions containing the SVF were whole-mounted and immunostained for PECAM-1. A, Diagram for subcutaneous implantation of GFP+ SVF mixed with PBS or Matrigel into B6 mice. Images show vessel-like structure derived from GFP+ SVF (green) 2 weeks after implantation. SVF was delivered in a mixture with either PBS or Matrigel. Scale bars=200 μm. B, Diagram for subcutaneous implantation of dsRed− SVF cells mixed with Matrigel into the flank region of GFP+ mice. Images show dsRed+/PECAM-1+ blood vessels derived from donor dsRed− SVF. Note that GFP−/PECAM-1− blood vessels derived from recipient are rarely detected. Scale bar=200 μm. C, Diagram for subcutaneous implantation of Tie2-GFP+ SVF mixed with Matrigel into the flank region of FVB/NJ mice. Images show Tie2-GFP+/PECAM-1+ blood vessels derived from Tie2-GFP+ SVF 2 weeks after implantation. Higher magnification of regions 1 and 2 (dotted-line squares) in the peripheral region is presented. White arrows indicate the junction between donor and recipient blood vessels. Scale bars=50 μm.

SVF Forms Connecting Blood Vessel Networks Sparsely With Recipient Blood Vessels

The Tie2-GFP+ mouse is an useful model with which to label BECs and a subset of hematopoietic cells.29 To trace the fate of SVF-derived BECs in the process of vascular network formation, we implanted SVF of Tie2-GFP+ mice into syngeneic normal mice (Figure 1C). A vascular network consisting of Tie2-GFP+/PECAM-1− blood vessels was observed 2 weeks after implantation, indicating the foundation of the structure to be the implanted Tie2-GFP+ SVF (Figure 1C). Therefore, we interpret BECs to be the main cellular components responsible for SVF-derived vascular network formation. Higher magnification of the peripheral regions of the implanted Matrigel revealed sparse connections between the organized SVF-derived vasculatures and GFP+/PECAM-1− recipient vasculatures, indicating the low efficiency of the newly formed vessels in establishing connections with the recipient vessels (Figure 1C).

SVF-Derived Vasculatures Require Vascular Growth Factors to Achieve Functional Circulation

The above findings led us to perform a perfusion assay to determine whether the SVF-derived vascular network was functional. SVF from dsRed− mice in Matrigel was implanted and maintained for 2 weeks. Then fluorescein isothiocyanate (FITC)–lectin was injected into the tail vein 15 minutes before the SVF containing region was sampled (Figure 1D). Both the donor (dsRed+/PECAM-1+) and recipient (dsRed−/PECAM-1−) origin vessels were detected, but FITC-lectin was not readily detectable in the donor-contributed vasculatures (Figure 1E). In contrast, FITC-lectin was well perfused in the recipient-originated dsRed−/
To test whether adding vascular growth factors would improve the perfusion of SVF-derived vasculatures, 2 major vascular growth factors—VEGF-A and Ang I—were added into Matrigel before implantation. Compared with the control, vascular perfusion in vessels formed by SVF was promoted 4.3-fold by the supplementation of VEGF-A (50 ng), 3.6-fold by supplementation of Ang I (100 ng), and 9.7-fold by supplementation of VEGF-A (50 ng) plus Ang I (100 ng) (Figure 1E and 1G). In addition, supplementation of these vascular growth factors slightly but not significantly increased the vascular density (Figure 1F). In fact, the effect of supplemented growth factors, in terms of proliferative activity, on SVF-derived vasculatures and surrounding recipient vasculatures was limited or even negligible. However, the sprouting activity of SVF-derived vasculatures was promoted by growth factor supplementation (Supplemental Figure III). Conversely, we inhibited the action of VEGF-A and angiopoietins by adding copious amounts of VEGF-A blocker (VEGF-Trap) and angiopoietin blocker (sTie2-Fc). Compared with the control mice treated with Fc, the SVF-derived vascular density decreased by 27.6% by treating with VEGF-Trap, 48.9% by treating with sTie2-Fc, and 72.4% by treating with both VEGF-Trap and sTie2-Fc (Supplemental Figure IV). Collectively, vascular growth factors are essential for SVF-derived vasculatures to achieve functional perfusion and maintain vessel density.

Vascular Networks Formed By SVF Require Macrophages for Proper Structural Organization

SVF also contains macrophages, which secrete a battery of vascular growth factors and cytokines.30 To investigate the role of macrophages in SVF-derived vascular network formation, we depleted macrophages (>95%, measured by flow cytometric analysis for CD11b+ and F4/80+ cells and immunohistochemistry for F4/80, Supplemental Figure VA and VB) in the donor and recipient mice pretreated with either CL or CDL before harvesting or implanting SVF (Figure 2A). As a control, control liposome (CL) was treated to donor mice. Consequently, macrophage-depleted SVF or control SVF were implanted into either the recipient macrophage-depleted mice or control mice, respectively (Figure 2A). Marked reduction of vascular networks in the central and peripheral regions of the implants was observed in the macrophage-depleted mice bearing the macrophage-depleted donor SVF (group 4, Figure 2). Interestingly, these mice frequently exhibited large but blunt-ended GFP + PECAM-1+ disconnected vessels in the central region of the implant (Figure 2C). Although the macrophage-depleted mice that received control SVF (group 2) displayed relatively abundant vasculature in the central region of the implant, still a marked reduction of vascular networks in the peripheral region was detected (Figure 2). In comparison, the control mice that received the macrophage-depleted donor SVF (group 3) displayed relatively abundant vasculature (Figure 2). These observations indicate that the recipient macrophages actively...
participate in the process of establishing a vascular network between the implanted donor SVF and recipient mice vasculature. Furthermore, these abnormalities of vascular network of implanted donor SVF by macrophage depletion were partially (77.2% and 59.8% in the central and peripheral regions, respectively) recovered by supplementation of VEGF-A (50 ng) (Supplemental Figure VC and VD), indicating that VEGF-A and other angiogenic growth factors secreted from the macrophage contribute to forming the vascular network.

**SVF Forms Hybrid Vascular Networks Through Dynamic Reassembly of BEC Network in the Adipose Tissue**

We questioned how SVF forms an organized vascular network in such a quick and efficient manner. We hypothesized that the vascular network formed by SVF in the Matrigel could be built simply by rearranging the preexisting blood vessel components, because adipose tissues possess relatively abundant blood vasculartures (Supplemental Figure VIA). Consistent with previous finding, our flow cytometric analyses revealed that approximately 7% of SVF consist of BECs (Supplemental Figure VIB). To validate our hypothesis, we isolated the CD31⁺/CD45⁻/Ter119⁻ BECs or non-BECs, including CD31⁺/CD45⁻/Ter119⁻, CD31⁺/CD45⁺/Ter119⁻, and CD31⁺/CD45⁺/Ter119⁺, and CD31⁺/CD45⁻/Ter119⁻, from SVF using fluorescence-activated cell sorting, and each was plated onto Matrigel-coated plate to examine vascular network formation in vitro (Figure 3). In comparison, the non-BECs-enriched implants failed to achieve vascular network formation in vitro, whereas isolated CD31⁺/CD45⁻/Ter119⁻ cells were able to form a vascular network in vitro (Supplemental Figure VID). Moreover, the CD31⁺/CD45⁻/Ter119⁻ BECs or non-BECs, including the combination of CD31⁺/CD45⁻/Ter119⁻, CD31⁺/CD45⁻/Ter119⁻, and CD31⁺/CD45⁻/Ter119⁺, from SVF were mixed with Matrigel that contained either vascular growth factors (50 ng of VEGF-A and 100 ng of Ang I) or BSA (100 ng), before implantation (Figure 3A). Only the BEC-enriched implants formed a vascular network, and the addition of vascular growth factors increased the vessel density by 1.34-fold compared with BSA-supplemented SVF (Figure 3). In comparison, the non-BECs-enriched implants failed to form a vascular network in spite of the presence of vascular growth factor supplements (Figure 3). To trace the origin of the BEC components in the SVF, we harvested SVF from GFP⁺ mice and dsRed⁺ mice, mixed the cells together at a 1:1 ratio in Matrigel, and implanted the mixture into a normal B6 recipient (Figure 4A). Two weeks later, CD31⁺ vascular networks were found that were composed of intermingled GFP⁺ BECs and dsRed⁺ BECs (Figure 4B), suggesting that the new vessels were built by rearranging the BECs contained in the donor SVF. Collectively, SVF-derived BECs establish a hybrid vascular network in the recipient through the disassembly and reassembly of the preexisting blood vessel components in the donor adipose tissue (Figure 4C).

**SVF-Derived Vascular Networks Rescue Hindlimb Ischemia**

To explore whether the SVF-derived vascular network formation can provide potential benefits to relieve ischemia, we implanted SVF directly into the muscles of an ischemic mouse hindlimb (Supplemental Figure VIIA). Implanted SVF effectively formed new vascular networks (Figure 5). In the case of SVF implantation into the ischemic hindlimb, unlike normal B6 recipients, the presence or absence of Matrigel did not cast a significant difference in the blood vessel density (Figure 5A and 5B). These SVF-derived vessels frequently exhibited connections with the adjacent
recipient blood vessels, which were found to be well perfused by FITC-lectin (Figure 5C and Supplemental Figure VIIB). Noticeably, SVF did not contribute to form pericytes in the ischemic tissue (Supplemental Figure VIIC). Compared with the implantation of an equal number of 3T3 cells, the SVF implantation even without Matrigel induced a 5.8-fold increase of blood perfusion in the ischemic limb on day 7, achieving an 8.2-fold increase of hindlimb salvage (Figure 5D to 5G). Moreover, the SVF implantation induced more pronounced proliferative activity, antiapoptosis, and antiinflammation in the injection areas for salvage of the ischemic limb compared with the implantation of 3T3 cells (Supplemental Figure VIID to VIIG).

**SVF-Derived Blood Vessels Supplemented With Vascular Growth Factors Can Prevent Fat Absorption of the Coimplanted SAT**

Fat absorption of the SAT implant is a major drawback for autologous fat tissue transplantation in the field of plastic surgery.25,34 We extended our study to see whether the SVF-derived vascular network could be applied to inhibit fat absorption of SAT implants. To address this matter, we attempted coimplantation of dsRed+ SAT with GFP+ SVF into syngeneic recipients (Supplemental Figure VIII A). The dsRed+ SAT implanted recipients were categorized into 4 study groups according to whether the coinjected Matrigel contained BSA only (group 1), vascular growth factors only (50 ng of VEGF-A and 100 ng of Ang I) (group 2), or GFP+ SVF either with or without vascular growth factor supplements (groups 3 and 4) (Figure 6A, Supplemental Figure VIII A). Overall, group 4 showed profound dsRed+ SAT-derived and GFP+ SVF-derived vascular networks in both the central and peripheral regions of SVF implant (Figure 6B to 6E). To clarify the presence or absence of adipocytes in the implants, we incubated the tissues with BODIPY, a specific marker for neutral lipid. The SAT implanted only with BSA (group 1) had almost no remaining adipocytes (Figure 6B to 6E). In comparison, the adipocyte content increased by 17.8-fold when vascular growth factors were supplemented, 33.6-fold when SVF was coimplanted, and 40.8-fold when both vascular growth factors and SVF were coimplanted (Figure 6B and 6E). Our confocal microscopic analysis revealed that all BODIPY+ cells in the central portion of implants were dsRed+ adipocytes derived from implanted...
SAT (data not shown). In group 4, hybrid vasculatures composed of BECs originating from both the GFP+/SAT and dsRed+/SAT were frequently observed at the implant periphery (Supplemental Figure VIII B). However, hybrid vessels were rarely detectable in the implant center (Figure 6B).

**SVF Forms Abnormal Blood Vessel Structures and BEC Junctions in the Allograft**

Allografting with BECs directly activates CD8+ T cells and triggers immune rejection.35 To investigate the degree of immune rejection, we harvested SVF from GFP+/mice (B6 background, H-2b haplotype), mixed the cells with Matrigel, and implanted them into FVB/NJ mice (H-2q haplotype) (Supplemental Figure IX A). Two weeks later, we found abnormal vascular networks, which were characterized by disconnected and blunt-ended vessels (Supplemental Figure IX B). Moreover, disarrangements of PECAM-1, an endothelial cell junction marker, were detected at the blunt-ended vessels (Supplemental Figure IX C).

**Discussion**

In this study, we show that direct implantation of freshly isolated SVF derived from adipose tissue can induce a rapid and robust process of vascular network formation through the dynamic reassembly of BECs at the site of implantation in syngeneic mice. This neovascular formation is quite different from the traditional concept in which new vessels are formed by either vasculogenesis or angiogenesis.36,37 Therefore, we addressed the following question: How could SVF form such a speedy and profound vascular network? We identify this phenomenon to occur mainly through a process of recycling the factorized preexisting cellular components in SVF instead of building them de novo, namely the disassembly-reassembly of BECs mechanism, which differs from either vasculogenesis or angiogenesis.

Adipose tissue is abundant in a fine, delicate, and well-organized blood vessel network, of which BECs have been measured as making up ~7% of the cellular components.32 We found that most of the vasculatures in the adipose tissue are disassembled during the preparation procedures of SVF by collagenase digestion, resulting in a BEC suspension. Our data based on the implantation of CD31+/CD45-/Ter119− BECs enriched from SVF and the implantation of an SVF mixture obtained from both GFP+ mice and dsRed+ mice provide compelling evidence that the BECs in freshly isolated SVF do form hybrid vascular networks. These observations support our hypothesis regarding the disassembly-reassembly of BECs. However, we assumed that angiogenesis would also be required for connecting the SVF-derived vessels with the recipient vessels. In fact, we found that the implanted SVF-derived BECs require appropriate supporting matrix
(Matrigel), endogenous or supplemental vascular growth factors, and macrophages of the recipient to successfully complete the construction of a new vascular network. In particular, vascular growth factor supplements were critical to provoke the implanted SVF-derived BECs to complete a functional perfusion circuit with the recipient vessels. Moreover, consistent with a recent report, the host myeloid cells including macrophages showed profound contribution in the process of establishing engineered vascular network. During this process, sprouting activity was quite active, whereas proliferative activity was infrequently detectable in the BECs of implanted SVF. Therefore, the sprouting process could be responsible for the connection and establishment of the SVF-derived vessels with the recipient vessels.

In addition, our results suggest that population alteration or cellular senescence has a substantial influence over the capacity of SVF in vessel formation. Spheroids formed with >5 passages of SVF failed to effectively create a vascular network. Previous studies using ADSCs describe limited vascular network formation, which could be due to the loss of BECs and senescence from excessive SVF culture passages. Therefore, it would be advisable to use freshly isolated SVF instead of the products of in vitro culture. For therapeutic purposes, using freshly isolated SVF is also beneficial in terms of convenience and safety. However, it remains to be determined whether the non-BECs could induce adverse side effects, although we observed the non-BEC components of SVF to be deposited and presumably degraded in the spleen. In fact, the use of pure BECs from SVF could be considered to avoid possible side effects. However, the neovascular efficacy using pure BECs is less than that using the total SVF, and an additional step for purification is required, which is quite laborious and difficult. Moreover, we have not found any difference between the ability of SAT and EAT in terms of convenience and safety. However, it remains to be determined whether the non-BECs could induce adverse side effects, although we observed the non-BEC components of SVF to be deposited and presumably degraded in the spleen.

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pericytes, were described to improve the degree of neovascularization and recovery of blood flow in the ischemic limbs through paracrine mechanism.\textsuperscript{41} Therefore, it would be intriguing to see whether the combination of freshly isolated SVF and cultured pericytes could achieve a much higher neovascularization capacity than freshly isolated SVF alone in the future. Within the scope of clinical application, SAT would serve as a feasible and practical source of SVF. However, we found a limitation using SVF as allograft for therapeutic neovascularization. A possible limitation of the clinical applications using this approach is the risk of immune rejection caused by major histocompatibility complex I mismatching between the implanted donor SVF and the recipient. Thus, the implantation of freshly isolated SVF is restricted to autograft and isograft approaches.

Taking the advantages of SVF, we next attempted to explore the potential therapeutic applications of SVF in preclinical studies. We focused on 2 aspects: protective effect against ischemia and supportive role in fat autografts. To test whether SVF implants can treat ischemia, we adopted the mouse ischemic hindlimb model, which we assume to represent human peripheral arterial occlusive disease. Compared with the control, the SVF-implanted mice showed improved perfusion, as well as an encouragingly higher rate of hindlimb preservation. This suggests that SVF does possess protective characteristics against ischemia and may be able to minimize the ischemic penumbra during the acute/subacute stage of an ischemic crisis. Autologous fat tissue augmentation is a widely adopted method for a variety of cosmetic and reconstructive surgeries.\textsuperscript{23,27,42} However, the major drawback of fat autograft is the inevitable fat absorption that occurs up to 70%,\textsuperscript{25} in which the graft is interrupted. In such cases, the compromised circulation of the graft and a major shift in the microenvironment would jeopardize the integrity of the transplanted adipocytes. Therefore, we postulated that enriching the vascular supply with coimplantation of SVF might improve the microenvironment and survival of the graft. Our results suggest that the coimplantation of SVF might prevent or delay fat absorption in autologous fat augments and could be exploited as an adjuvant therapy of fat transplantation.

In conclusion, SVF found in adipose tissue can generate a rapid and robust construction of a vascular network through a mechanism that we attribute to the dynamic reassembly of BECs. Although the possible clinical applications and protocols for SVF-based cell therapy must be further optimized, we have demonstrated the possibility of therapeutic applications of SVF to be promising for treatment of diseases that could benefit from therapeutic neovascularization and prevention of fat absorption in autologous fat augments.

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Disclosures
None.

References


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Supplemental Figure Legends

**Supplemental Figure I.** The SVF-derived vascular network formations are almost identical between SAT and EAT, and Matrigel and collagen gel, and persists up to 6 months

(A and B) 3x10^5 SVF cells were harvested from EAT or inguinal SAT of GFP^+ mice, mixed with 100 μl of Matrigel (Mat) or collagen type I gel (collagen gel or Col), and subcutaneously implanted into the flank region of B6 mice. 2 weeks or 6 months after implantation, the regions containing the SVFs were whole-mounted and immunostained for PECAM-1. (A) Images showing GFP^+/PECAM-1^+ blood vessels derived from SVF of EAT and SAT at 2 weeks after implantation. Scale bars, 200 μm. (B) Comparison of relative area density of SVF-derived blood vessel (SVF-BV) per mm^2 in the Matrigel between two groups. Each group, n=3. (C) Images showing GFP^+/PECAM-1^+ blood vessels derived from SVF of EAT at 2 weeks after implantation. Scale bars, 200 μm. (D) Comparison of relative area density of SVF-derived blood vessel (SVF-BV) per mm^2 in the Matrigel and collagen gel. Each group, n=3. (E) Images showing GFP^+/PECAM-1^+ blood vessels derived from SVF of EAT at 6 months after implantation. Scale bars, 100 μm.

**Supplemental Figure II.** SVF spheroids fail to form blood vessel networks

(A) SVF of GFP^+ mice were cultured on dish (adherent culture) that was used to produce spheroids by a hanging drop culture of primary cultured SVF at passage 2 and 6. 1x10^3 SVF spheroids were mixed with 100 μl Matrigel, and subcutaneously implanted into the flank region of B6 mice. (B) 2 weeks after implantation, the tissues containing SVF spheroids were whole-mounted, immunostained for PECAM-1 and NG-2. Images showing GFP^+/PECAM-1^+ blood vessels, GFP^-NG-2^+ cells and GFP^-NG-2^+ cells. Higher magnifications of regions 1 and 2 (dotted-line insets) in the Matrigel are shown. Scale bars, 50 μm. Note that GFP^+/PECAM-1^+ blood vessel-like structure is rarely detectable. (C) At passage 2 and 6, cultured SVF cells were analyzed by a FACS analyzer after staining for CD31 and CD45. Three independent experiments showed similar results. (D) At passage 2 and 6, cultured SVF cells were stained for senescence-associated β-galactosidase (SA-β-Gal, arrows). Scale bars, 50 μm. Three independent experiments showed similar results.

**Supplemental Figure III.** Vascular growth factors promote sprouting of blood vessel
formed by SVF

3×10^5 SVF cells were harvested from GFP^+ mice, mixed with 100 μl Matrigel containing 50 ng of VEGF-A (VEGF), 100 ng of Ang1 (Ang1), VEGF+Ang1 (V+A), or 100 ng of BSA (BSA), and subcutaneously implanted into the flank region of B6 mice. 3 days after implantation, the tissues containing the SVF were whole-mounted, immunostained for PECAM-1. (A) Images showing GFP^+/PECAM-1^+ sprouting blood vessels. Scale bars, 50 μm. (B and C) Comparisons of number and length of SVF-derived blood vessel (SVF-BV) sprouts in the Matrigel among the treatment groups. Each group, n=5. *p<0.05 vs. BSA; #p<0.05 vs. VEGF.

Supplemental Figure IV. SVF requires endogenous vascular growth factors for proper formation of blood vessel networks

3×10^5 SVF cells were harvested from GFP^+ mice, mixed with 100 μl Matrigel containing either 10 μg of Fc, VEGF-Trap (VT), sTie2-Fc (T2) or VT+T2, and subcutaneously implanted into the flank region of B6 mice. 1 week after implantation, the tissues containing the SVF were whole-mounted and immunostained for PECAM-1. (A) Images showing GFP^+/PECAM-1^+ blood vessels and GFP^+/PECAM-1^- cells, of which the identity cannot be determined. Scale bars, 200 μm. (B) Comparison of the SVF-derived blood vessel (SVF-BV) density per mm^2 in the Matrigel among the treatment groups. Each group, n=5. *p<0.05 vs. Fc; #p<0.05 vs. Fc+T2.

Supplemental Figure V. Supplementation of VEGF-A partially rescues the abnormality of SVF-derived vessel network formation by depletion of macrophages

(A and B) B6 mice were treated with CL or CDL twice by a 3-day interval. One week after treatment, SVF was analyzed by a flow cytometer after staining for F4/80 and CD11b, and EAT was immunostained for F4/80. (C and D) 3×10^5 SVF cells were harvested from GFP^+ mice that were treated with CDL twice by a 3-day interval. The SVF was then mixed with 100 μl Matrigel containing 50 ng of BSA or 50 ng of VEGF, and subcutaneously implanted into the flank region of B6 mice that were treated with CDL twice by a 3-day interval. 1 week after implantation, the implanted tissues were whole-mounted. (C) Images showing GFP^+ blood vessel networks formed by SVF in the central and peripheral regions of the Matrigel implants. Red arrows indicate disconnected, blunt-ended and enlarged vessel structure. Higher magnification of insets (dotted-line square)
shows that VEGF-A partially rescues the networks in the absence of macrophages. Scale bars, 100 μm. (D) Comparison of the relative SVF-derived blood vessel (SVF-BV) area density per mm² in the Matrigel among the treatment groups. Each group, n=3. *p<0.05 vs. CL+BSA; #p<0.05 vs. CDL+BSA.

**Supplemental Figure VI. Adipose tissue vasculatures and tube formation by the purified BEC in SVF**

(A) 50 μg of FITC-lectin was injected intravenously into 8-week-old B6 mice through the tail vein. 15 min after the injection, EAT was harvested and whole mounted. Image showing FITC-lectin⁺ blood vessels in the EAT. (B) 1.5×10⁵ SVF cells were harvested from B6 mice, and analyzed by a FACS analyzer after staining for CD31, CD45 and Ter119. 5 different fractions were identified; ~7.0% of CD31⁺/CD45⁻/Ter119⁻ BEC, ~27% of CD31⁻/CD45⁻/Ter119⁻ cells, of which the identity cannot be determined, ~25% of CD31⁻/CD45⁺/Ter119⁻ erythrocytes, ~30% of CD31⁻/CD45⁻/Ter119⁻ hematopoietic cells, or ~0.4% of CD31⁻/CD45⁺/Ter119⁻ cells, of which the identity cannot be determined. Three independent experiments showed similar results. (C) Purity of BEC and non-BEC by a a FACS analyzer. (D) 3×10⁴ of sorted CD31⁺/CD45⁻/Ter119⁻, CD31⁻/CD45⁻/Ter119⁻, CD31⁻/CD45⁺/Ter119⁻ or CD31⁻/CD45⁻/Ter119⁺ cells from SVF were plated onto the Matrigel-coated plate, incubated for 24 hr, and images of the cells were captured. Three independent experiments showed similar results.

**Supplemental Figure VII. Blood perfusion, proliferation, anti-apoptosis and anti-inflammation are detected in the SVF implanted region of ischemic hindlimb.**

(A-C) 1×10⁶ SVF cells were harvested from dsRed⁺ mice, mixed with 200 μl of PBS or Matrigel, and intramuscularly implanted into the ischemic region of hindlimb of athymic nude mice. One week later, FITC-lectin was injected through the tail vein, and the implanted tissues were sampled 15 min after FITC-lectin injection. The tissue was H&E stained or immunostained for PECAM-1, NG-2, ki-67, caspase-3 or F4/80. (A) H&E stained images showing the needle tracks (# and ##) for injection of SVF and Matrigel+SVF into the muscles of ischemic hindlimbs. Scale bars, 200 μm. (B) Images showing dsRed⁺/PECAM-1⁺/FITC-lectin⁺ perfused vessels derived from dsRed⁺ SVF cells and dsRed⁺/PECAM-1⁺/FITC-lectin⁻ non-perfused vessels derived from recipient in the ischemic hindlimb. Higher magnifications (dotted-line square) of the upper panels are presented in the lower panels. Note the presence of profoundly perfused dsRed⁺/PECAM-1⁺/FITC-lectin⁺ vessels (white arrows). Scale bars,
50 μm. (C) Images showing dsRed+/FITC-lectin+ blood perfused vessels derived from donor and dsRed-/NG-2+ pericyte-like structures (white arrows) derived from recipient. Note that SVF does not contribute to the pericytes in the ischemic tissue. Scale bar, 50 μm. (D-F) Images showing Ki-67, caspase-3 and F4/80 immuno-positive cells in the ischemic hindlimb regions. Scale bars, 100 μm. (G) Comparison of Ki-67, caspase-3 and F4/80 positive cell numbers per mm² in the ischemic hindlimb regions between two groups. Each group, n=5. *p<0.05 vs. 3T3.

**Supplemental Figure VIII. Hybrid blood vessels formed by implanted SVF, implanted SAT and host blood vessels in the SVF-SAT co-implantation**

150 μl of dsRed⁺ SAT was implanted into the flank region subcutaneously of B6 mice. Then, 3×10⁵ SVF cells harvested from GFP⁺ mice were mixed with 100 μl of Matrigel containing 50 ng of VEGF-A and 100 ng of Ang1 (V+A), and implanted into the SAT implantation site. At 4 weeks after implantation, the tissues containing SAT and SVF were sampled, H&E stained or immunostained for PECAM-1. (A) H&E stained images showing implanted SAT and the Matrigel (M). Scale bars, 20 μm. (B) Upper and middle images showing hybrid blood vessels networks formed by implanted SVF (green arrowheads), implanted SAT (red arrowheads), and host blood vessels (blue arrowheads) at the peripheral regions of the Matrigel. Scale bars, 20 μm. Middle images are drawn in color (lower pictures) for distinguishable visualization of each cell contribution to form a hybrid vessel networks.

**Supplemental Figure IX. SVF forms abnormal blood vessels structures and endothelial cell junctions in the allograft.**

3×10⁵ SVF cells were harvested from GFP⁺ mice (B6, H-2b haplotype), mixed with 100 μl of Matrigel, and subcutaneously implanted into the flank region of FVB/NJ mice (H-2q haplotype). At 2 weeks after implantation, the tissues containing the SVF were whole-mounted and immunostained for PECAM-1. (A) Diagram for subcutaneous implantation of GFP⁺ SVF cells mixed with Matrigel implantation into the flank region of FVB/NJ mice. (B) Images showing GFP⁺/PECAM-1⁺ blood vessels derived from GFP⁺ SVF. Higher magnifications of regions 1 and 2 (dotted-line squares) are presented. Note that blunt-ended blood vessel structures are frequently detected in the allograft. Scale bars, 50 μm. (C) Dissected images showing normal (white arrows) and abnormal (white arrowheads) PECAM-1⁺ blood endothelial cell junctions of image B2. Note that abnormal blood endothelial cell
junctions are detected at blunt-ended blood vessel structures in the allograft. Scale bars, 50 μm.
A

Adherent culture → Spheroid culture → Implantation

B

GFP-SVF | PECAM-1 | NG-2 | Merged

1

2

C

2 passages | 6 passages

CD45-APC | CD31-PE

5.3% 0.0% | 0.7% 94.0%

2.5% 0.0% | 0.1% 97.4%

D

2 passages | 6 passages

SA-β-Gal

Supplemental Fig. II
A

GFP-SVF  PECAM-1  Merged

BSA  VEGF  Ang1  A+V

B

Number of SVF-BV sprouts

C

Length of SVF-BV sprouts (µm)
Supplemental Fig. IV

A

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B

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* * *
A

CL

CDL

21.3%

1.0%

CD11b - FITC

CD11b - FITC

F4/80 - PE

B

F4/80

CL

CDL

C

Central region

CDL+BSA

CDL+VEGF

Peripheral region

D

Supplemental Fig. V

Relative SVF-BV area density per mm² (%)

0

10

20

30

40

50

60

CL  BSA  CDL  BSA  VEGF

CDL  BSA  CDL  BSA  VEGF

*  #  *  #  *

CDL  BSA  CDL  BSA  VEGF

*  #  *  #  *
Supplemental Fig. VI

A

B

C

D

CD45 Ter119 - APC

CD31- PE

98.1%

99.8%

99.8%

Non-BEC

BEC

CD31- PE

29.9%

0.4%

25.3%

7.0%

27.4%

27.4%

CD31- PE

CD31+/CD45+/Ter119+

CD31-/CD45+/Ter119+

CD31+/CD45+/Ter119+

CD31-/CD45+/Ter119+
Supplemental Fig. VII-Continued

**D**

3T3

Ki-67 DAPI

**E**

Caspase-3 DAPI

**F**

F4/80 DAPI

**G**

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Cell number per mm$^2$

- Ki-67
- Caspase-3
- F4/80

* indicates statistical significance.
A

Matrigel  |  GF  |  SVF  |  SVF + GF

B

GFP-SVF  |  dsRed-SAT  |  PECAM-1  |  Merged

Supplemental Fig. VIII
A

GFP-SVF → Matrigel → FVB/NJ mouse (H-2q haplotype)

B

Supplemental Fig. IX

GFP-SVF (B6)  PECAM-1  Merged

C

PECAM-1

a  b  c

d  e  f

PECAM-1

GFP+ mouse (B6 - H-2b haplotype)
Supplemental Data

Stromal Vascular Fraction from Adipose Tissue Forms Profound Vascular Network through the Dynamic Reassembly of Blood Endothelial Cells

Young Jun Koh, Bong Ihn Koh, Honsoul Kim, Hyung Joon Joo, Ho Kyoung Jin, Jongwook Jeon, Chulhee Choi, Dong Hun Lee, Jin Ho Chung, Chung Hyun Cho, Won Seok Park, Ji-Kan Ryu, Jun Kyu Suh, and Gou Young Koh
Supplemental Methods

Animals
The mice were bred in our pathogen-free animal facility, and 8- to 10-week-old male mice were used for this study. Animal care and experimental procedures were performed under approval from the Animal Care Committee of KAIST. All mice were provided water and a standard diet (PMI LabDiet, Purina Mills Inc). All mice were anesthetized by an intramuscular injection of a combination of anesthetics (80 mg/kg ketamine and 12 mg/kg xylazine) before sacrifice.

Isolation of SVF from adipose tissue or BECs from SVF
For a comparison experiment, we isolated the SVF from the SAT of GFP+ mice. To enrich the EC from the GFP+ SVF suspension, suspended SVF were incubated with the following antibodies: PE-conjugated anti-mouse CD31 antibody, rat clone MEC13.3, APC-conjugated anti-mouse CD45 antibody, rat clone 30-F11, and APC-conjugated anti-mouse Ter119 antibody, rat clone Ter119 (all from eBioscience) and sorted using FACS Aria II (Becton Dickinson). CD31+/CD45−/Ter119− cells were regarded as BEC and CD31−/CD45−/Ter119−, CD31−/CD45+/Ter119− and CD31−/CD45+/Ter119+ cells regard as non-BEC, and were confirmed the purities of putative BEC and non-BEC by FACS Aria II (Becton Dickinson) after incubation with CD31 antibody. The purity of EC was >98%, whereas the purity of each population of non-EC was >99% (Supplemental Figure VI C). To exclude blood contamination in the adipose tissue, systemic perfusion with heparinized PBS was performed before harvesting or washing isolated adipose tissues with PBS. Then, the adipose tissues were incubated in Hank’s balanced salts solution (HBSS; Sigma) containing 0.2% collagenase type 2 (Worthington) for 60 min at 37°C with constant shaking. After inactivating collagenase activity with 10% fetal bovine serum (FBS; Hyclone) containing Dulbecco’s Modified Eagle’s Medium (DMEM; Hyclone), the cell suspension was filtered through a 40-μm nylon mesh (BD Biosciences), followed by centrifugation at 420xg for 5 min. Floating adipocytes and supernatant were removed from the SVF pellet. The SVF pellet was washed and resuspended in sterilized PBS. To count the cell number, we used a hemocytometer (Marienfeld, Germany). From the cell suspension, 10 μl was taken and mixed with 90 μl trypan blue solution (Sigma-Aldrich). Ten μl of the mixture was added to the hemocytometer, and the number of viable cells was counted under inverted microscope (Carl Zeiss).
**Cellular senescence assay**
At passage 2 and 6, cultured SVF cells were fixed using 0.25% glutaraldehyde for 5 min at room temperature (RT). After several washes using PBS, the SVF cells were incubated with senescence-associated β-galactosidase (SA-β-Gal) solution constituted of 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 40 mM citrate-Na₂HPO₄ buffer, 150 mM NaCl₂ and 1 mg/ml X-gal for 24 hr while being protected from light at 37°C. After several washes using PBS, blue stained senescence cells were detected with Axiovert25 (Carl Zeiss) equipped with Infinity X (DeltaPix).

**In vitro tube formation assay**
3×10⁴ of sorted CD31⁺/CD45⁻/Ter119⁻, CD31⁻/CD45⁻/Ter119⁻, CD31⁻/CD45⁺/Ter119⁻ or CD31⁻/CD45⁻/Ter119⁺ cells from SVF by FACS were plated onto the Matrigel (50 μl per well)-coated 96-well plate, incubated with EGM-2 media (Lonza) for 24 hr in a 5% CO₂ incubator at 37°C, and then each tube formation was captured by inverted microscope (Axiovert 25, Carl Zeiss) equipped with Infinity X (DeltaPix).

**Implantation of SVF or BECs**
A total of 3 × 10⁵ SVF cells were mixed with Matrigel (100 μl, growth factor reduced; BD Biosciences) or collagen type I gel (100 μl, BD Biosciences) containing PBS, BSA (100 ng), VEGF (50 ng) or COMP-Ang1 (hereafter Ang1) (100 ng) ¹, and implanted into the flank region subcutaneously of 8-week-old B6 mice, GFP⁺ mice or FVB/NJ mice. To block VEGF or/and angiopoietins in Matrigel containing SVF, mice were treated with VEGF-Trap (10 ug/100 ul of Matrigel) ²,³ and/or soluble Tie2-Fc protein (10 ug/100 ul of Matrigel). As a control, dimeric-Fc protein (20 ug/100 ul of Matrigel) was applied in the same manner. For systemic depletion of macrophages, control liposome as control or clodronate liposome (25 mg/kg) was started to inject into intraperitonium of 8-week-old B6 and GFP⁺ mice every 3 days until end of the experiment. To examine blood vessel abnormalities by macrophage depletion, 3×10⁵ SVF cells were harvested from GFP⁺ mice that were treated with CDL twice by a 3-day interval. The macrophage-depleted SVF was then mixed with 100 μl Matrigel containing 50 ng of BSA or VEGF-A, and subcutaneously implanted into the flank region of B6 mice that were treated with either CDL twice by a 3-day interval. 1 week after implantation, the implanted tissues were whole-mounted for histological analyses. To implant...
EC or non-EC, 2×10^4 sorted EC or 3×10^5 were mixed with 100 μl of Matrigel containing BSA (100 ng) or VEGF-A (50 ng) plus Ang1 (100 ng), and implanted subcutaneously into the flank region of 8-week-old B6 mice. After the indicated number of days, implants and adjacent tissues were harvested for histologic analyses.

**Implantation of SVF into ischemic hindlimb of mouse**
Hindlimb ischemia was induced by ligation and excision of the right femoral artery and vein in the 8-week-old BALB/cByJ athymic nude male mice under ketamine/ non-EC were mixed with 100 μl of Matrigel containing BSA (100 ng) or VEGF-A (50 ng) plus Ang1 (100 ng), and implanted subcutaneously into the flank region of 8-week-old B6 mice. After the indicated number of days, implants and adjacent tissues were harvested for histologic analyses.

**SVF spheroid culture and implantation**
The isolated SVF cells from GFP^+ mice were cultured in a complete medium consisting of DMEM-high glucose (Hyclone), 10% FBS (Hyclone), and penicillin/streptomycin (5 U/ml; Invitrogen) on a cell culture dish at a density of 1×10^6 cells/cm^2. At 90% of confluence, the cells were sub-cultured at 1:3 ratio. At passage 2 and 6, spheroids were made with ~300 SVF cells in 20 μl of complete media by a hanging drop culture for 24 hr. 1×10^3 SVF spheroids (~ 3×10^5 SVF cells) were washed three times with PBS, mixed with 100 μl of Matrigel, and implanted subcutaneously into the flank region of B6 mice.

**Implantation of SVF into ischemic hindlimb of mouse**
Hindlimb ischemia was induced by ligation and excision of the right femoral artery and vein in the 8-week-old BALB/cByJ athymic nude male mice under ketamine/xylazine anesthesia. For an implantation study, the mice were divided into four groups; Matrigel (200 μl), 1X10^6 dsRed^+ SVF, 1X10^6 dsRed^+ SVF in Matrigel (200 μl), and 1X10^6 3T3 cells (American Type Culture Collection). The implants were intramuscularly implanted into the ischemic region of hindlimb as multiple (6 times) micro-injections. Serial ICG perfusion imaging was performed according to previously described immediately after surgery and on postoperative day 3 and 7 for measurements of blood perfusion both in the ischemic and contralateral hindlimb regions, and the blood perfusion rate was calculated as percentage by ICG intensity of the ischemic region over the corresponding contralateral hindlimb region. Two independent,
blinded investigators estimated the hindlimb salvage by intactness of foot in the ischemic hindlimbs, and inter-investigator variation was <5%.

**Co-implantation of SAT and SVF**

SAT implantation was performed according to previously described. Briefly, SAT was harvested from dsRed+ mice, then immediately loaded in a 1 ml syringe through the plunger end, so that warm ischemia time was <10 min. 150 μl of dsRed+ SAT was injected subcutaneously into the flank region of B6 mice using 16.5-gauge needle to minimize physical damage on adipocytes during injection. Immediately after SAT injection, 3×10^5 SVF cells harvested from GFP+ mice were mixed with 100 μl of Matrigel containing BSA (100 ng) or VEGF-A (50 ng) plus Ang1 (100 ng) were implanted into the SAT injection site as multiple (6 times) micro-injections. 4 weeks later, implants and adjacent tissues were harvested for histologic analyses.

**Histological and morphometric analysis**

At the indicated days, the mice were anesthetized. Indicated implants and tissues were fixed by systemic vascular perfusion of 1% paraformaldehyde in PBS, removed, and whole-mounted or embedded with tissue freezing medium (Leica, Germany). For histologic analysis, the cryosectioned tissues were hematoxylin and eosin (H&E) stained. To detect blood perfusion, FITC-lectin (Vector Laboratories) was injected 15 min before perfusion-fixation. After blocking with 5% donkey serum in PBST (0.3% Triton X-100 in PBS) for 1 hr at RT, the whole-mounted or sectioned tissues were incubated with one or more of the following primary antibodies: anti-mouse PECAM-1 antibody, hamster clone 2H8 (Chemicon International); anti-mouse NG2 antibody, rabbit polyclonal (Chemicon International); anti-mouse Ki-67 antibody, rabbit polyclonal (Abcam); anti-mouse caspase-3 antibody, rabbit polyclonal (R&D); anti-mouse F4/80 antibody, rat monoclonal (eBioscience). After several washes in PBST, the samples were incubated for 2 hrs at RT with the following secondary antibodies: Cy3-, Cy5- or FITC conjugated anti-hamster IgG antibody (Jackson ImmunoResearch), Cy3- or Cy5- conjugated anti-rabbit antibody (Jackson ImmunoResearch), FITC conjugated anti-rat IgG antibody (Jackson ImmunoResearch). To visualize adipocytes in the implants, neutral lipid staining agent, 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503, 1.0 μg/ml in PBS; Invitrogen) was applied for 15 min at RT. For control experiments, the primary antibody was omitted or substituted with
preimmune serum. Photos were taken by a digital camera (Canon EOS 500D). Fluorescent signals were visualized, and digital images were obtained using a Zeiss LSM 510 confocal microscope equipped with argon and helium-neon lasers (Carl Zeiss) and a Zeiss Stemi SV6 stereoscope equipped with ProgRes C14 (Carl Zeiss). Z-stacked images were obtained using a confocal microscope which was post-processed to achieve 3D construction. For analyses of these images, the z-stacked single-color images were converted into 8-bit gray scale mode to quantify the occupied pixels either by using ImageJ (http://rsb.info.nih.gov/ij) or image analysis (AxioVision, Carl Zeiss) software. Each relative area density per field of SVF-derived blood vessels, functional blood vessels or SAT-derived adipocytes was measured from the pixels in the region of interest; only the pixels with a signal intensity exceeding an empirically predefined threshold level (> 50 intensity value) were counted as valid signals while the remaining was considered as background fluorescence. Density measurements of GFP+/PECAM-1+ or dsRed+/PECAM-1+ or dsRed+/PECAM-1+/FITC-lectin+ blood vessels in the implants were made on each five field in the central and peripheral regions at a screen magnification of 100×, each 5.25 mm² in area, and 5 mice were used per group. Values were obtained per mm² and expressed as relative densities. To avoid non-specific fluorescence of FITC-lectin, the signal was corrected with the values of SVF containing Matrigel that was not injected with FITC-lectin. The central region in the implant was defined as a 1 mm diameter region from the center and the peripheral region in the implant was defined as a 1 mm diameter region from the margin. To measure implant volume in the SAT-SVF implantation, the width and length of the implant were measured by an electronic caliber, and 5 mice were used per group and the measurement of implanted dsRed+ fluorescent SAT was made on one field at a screen magnification of 8×, and 5 mice were used per group. The measurement of BODIPY+ adipocyte content in the implant region was made on 5 fields in the central regions at a screen magnification of 100×, each 1 mm² in area, and 5 mice were used per group. Values were obtained as mm³ or expressed as relative densities.

Statistics
Values are presented as mean ± standard deviation (SD). Significant differences between means were determined by an analysis of variance followed by the Student-Newman-Keuls test. Statistical significance was set at p<0.05.
Supplemental References


지방흡입 후 바로 분리한 기질-혈관 분획은 배양한 지방유래 줄기세포보다 새로운 혈관을 만드는데 효과적이다

조형재 교수
서울대학교병원 순환내과

Summary

목적
지방조직에서 분리한 기질-혈관 분획과 이를 배양한 지방유래 줄기세포를 이용하여 지방적 혈관신생을 도모하고자 하는 많은 연구가 이루어지고 있다. 하지만 직접 효율이 낮고, 주입한 세포와 숙주(host)의 현재 조직 간의 상호관계에 대한 과학적 기전이 잘 알려져 있지 않아, 세포 주입 후 미세환경의 변화를 연구하고자 하였다.

방법 및 결과
본 연구자들은 지방조직에서 분리한 기질-혈관 분획을 추가적인 배양 없이 바로 생체 내에 주입하였을 때 혈관 형성이 매우 높다고 관찰하였고, 이러한 결과는 세포를 주입한 부위에서 기질-혈관 분획 유래 내피세포의 허체/조직 과정이 통제된 해체를 통해 혈관 네트워크가 형성되는 것임을 증명하였다.
지방조직에서 분리한 기질-혈관 분획 주입에 의해 새로운 형성된 혈관들은 숙주에 원래 존재하던 기존 혈관 조직들과 효과적으로 연결됨을 증명하였으며, 기질-혈관 분획 주입에 대해 혈관신생인자(VEGF 또는 angiopoietin)를 추가로 주입한 경우 새로운 혈관 형성이 더욱 속전되었다. 하지만, 기질-혈관 분획을 구체(spheroid)로 만들어 주입한 경우 혈관 형성의 효과가 감소하여, 효능 환상을 위한 사전 세포조작이 오히려 효과적이지 못함을 증명하였다.
마우스의 하지혈관 혈관 부위에 지방조직 유래 기질-혈관 분획을 주입하였을 때 새로운 혈관 형성을 통한 치료적 효과가 관찰되었다. 또한, 지방이식 시 기질-혈관 분획을 동시에 주입하게 되면 이러한 지방이 유수되는 정도를 감소시켜 지방이식의 효과를 배가할 수 있었다.

결론
지방조직에서 분리한 기질-혈관 분획을 추가적인 조직배양 없이 바로 주입하는 것이 혈관 부위의 혈관 생성 지료에 지방이식의 효과 증진에 효과적일 수 있음을 보여준 연구라 생각된다.
지난 10년간 줄기세포를 이용한 재생 의학분야가 급격히 발전하고 있다. 실제 임상에 적용하기 위해서는 재생 세포가 가장 적절한 기준에 대한 논란과 연구는 계속 진행 중이다.

임상적응을 위한 적절한 줄기세포가 갖추어야 할 조건은 1) 입과 안전한 과정을 가진 2) 증분한 양을 확보할 수 있어야 하고, 3) 원하는 세포로 분화가 용이해야 하며, 4) 세포의 안전성과 안정성이 확보되어야 하고, 5) 투약 후 호흡 및 조직의 활력을 유지해야 한다. 안전한 분리가 아닌 세포 배양과정을 거친 경우를 제외하고는 GMP 준수에 맞도록 배양과정과 시설이 다이어트되어 있어야 한다.

이러한 의미에서 지방조직에서 분리하는 기질-혈관 분화를 위한 연구가 진행되고 있으며, 이러한 과정에서 증상의 억제에 대한 논의가 나누어져 있어야 한다.

지방조직에서 지방조직에서 분리한 기질-혈관 분화를 위한 연구가 진행되고 있으며, 이러한 과정에서 증상의 억제에 대한 논의가 나누어져 있어야 한다.

| Table 1 | 지방 줄기세포와 관련된 용어 |
|-----------------|
| 1. Subcutaneous adipose tissue (SAT): 피하지방조직, 지방 조직에서 양을 얻을 수 있는 용어 |
| 2. Adipose tissue-derived stromal vascular fraction (SVF): 지방조직에서 분리한 기질-혈관 분화를 위한 용어 |
| 3. Adipose-derived stem/progenitor cells (ADSCs): 지방조직에서 분리한 줄기세포와 관련된 용어 |
식시 주입한 지방이 시간이 지남에 따라 흩어져서 나타나는 결과가 반감되는 경우를 흔히 경험하게 되는데, 기질-혈관 분화을 동시에 주입하게 되면 이식한 지방이 혈관생성을 도와, 흩어지지 않고 형태를 잘 유지할 수 있음을 증명하였다. 실제로 이러한 세포분화를 임상적 적용하기 위해서는 여러 가지 기술적 문제를 극복하고 복잡한 임상시험과정을 거쳐야 하겠지만, 이번 연구는 추가적인 조직 없이 지방조직 유래 기질-혈관 분화을 바로 주입하는 방법이 임상적으로 훨씬히 가능성이 있는 세포치료 전략임을 보여 주는 결과로 다자란 연구라 하였다.

REFERENCES

Figure 1. 지방조직으로부터 기질-혈관 분화 획득하여 지방유래 줄기세포를 배양하는 과정.