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Significance of cellular cross-talk in stromal vascular fraction of adipose tissue in neovascularization

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Abstract

Adult stem cell-based therapy has been regarded as a promising treatment for tissue ischemia because of its ability to promote new blood vessel formation. Bone marrow-derived mesenchymal stem cells are the most used angiogenic cells for therapeutic neovascularization, yet the side effects and low efficacy have limited their clinical application. Adipose stromal vascular fraction (SVF) is an easily accessible, heterogeneous cell system comprised of endothelial, stromal, and hematopoietic cell lineages, which has been shown to spontaneously form robust, patent, and functional vasculatures *in vivo*. However, the characteristics of each cell population and their specific roles in neovascularization remain an area of ongoing investigation. In this review, we summarize the functional capabilities of various SVF constituents during the process of neovascularization and attempt to analyze whether the cross-talk between these constituents generates a synergetic effect, thus contributing to the development of new potential therapeutic strategies to promote neovascularization.

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Introduction

Adult stem cell-based therapy has become one of the most promising methods to promote collateral vessel formation for ischemia diseases[1, 2]. It is generally recognized that there are two processes during the formation of a vascular network: angiogenesis and vasculogenesis[3]. Angiogenesis refers to the development of new vessels by budding from an existing vascular network in an adult[4]. On the other hand, vasculogenesis is defined as the *in situ* blood vessel formation by hemangioblasts or vascular stem/progenitor cells and is mostly used to describe vasculature formation in the embryonic period[5]. However, the formation of stable vessels is a complex process involving both angiogenesis and vasculogenesis and requires orchestrating interactions between endothelial cells (ECs), mural cells, and the surrounding environment[5]. Thus, the term “neovascularization” is used to indicate these two processes, which represent different aspects of the complex process of vascular network formation[6, 7].

Postnatal neovascularization is supposed to be initiated by recruitment and differentiation of vascular precursors, which may be derived from the bone marrow, vascular endothelium, or other mature tissues[8–12]. For a long time, bone marrow-derived mesenchymal stem cells (BM-MSCs) have been the most extensively used stem cells for therapeutic neovascularization. They are multipotent progenitors regarded as having the capability to differentiate into vascular cells and promote neovascularization[2, 13, 14]. However, the side effects and possible morbidity of the bone marrow harvest procedure and the relatively low content of BM-MSCs in the bone marrow aspirate have limited their clinical application[15].

Adult adipose tissue provides an alternative source of accessible autologous adult stem cells with a high content of endothelial progenitor cells (EPCs) and multipotent mesenchymal stem cells (MSCs)[16]. Adipose tissue is a highly vascularized tissue in the body, and the remodeling of existing vessels may also play an important role in the physiological functions of adipose tissue[17]. In 2001, Zuk et al. first described a population of fibroblast-like cells in the stromal vascular fraction (SVF) of adipose tissue, which could differentiate into adipogenic, myogenic, chondrogenic, and osteogenic cells *in vitro*[18]. Since then, numerous studies have shown that SVF contains a biologically and clinically interesting heterogeneous cellular population, which can self-assemble into complex vascular networks by supplying ECs or secreting cytokines[19, 20]. SVF is known to contain ECs, smooth muscle cells, mural cells, fibroblasts, macrophages, and MSCs/other stem cell phenotypes[21]. Among these cells, most of the attention has been focused on the characteristics and function of adipose-derived mesenchymal/stem cells, which were usually called adipose-derived stem cells (ADSCs) or

adipose tissue-derived MSCs (AT-MSCs), in the process of neovascularization. Nevertheless, due to the heterogeneity of SVF and the lack of consensus surface markers to distinguish the definitive cell population, some studies achieved different and even contradictory conclusions in research on SVF induced neovascularization. For instance, whether ADSCs can differentiate into ECs and be directly involved in neovascularization or ADSC transplantation is just “cell-based cytokine therapy” for ischemic disease is still controversial[20, 22–25]. In addition, although ADSCs have shown efficacy in regenerative and reconstructive medicine, SVF was considered to have superiority compared to ADSC treatment alone[26]. In a study of a rat cavernous nerve injury model, the SVF treatment group showed statistically better results, with a higher smooth muscle/collagen ratio and more endothelial cell content compared to the ADSC group[27]. The better therapeutic outcomes observed with SVF treatment are considered to be related to the synergistic promoting effect of heterogeneous cellular composition[27–29]. How to make the most of SVF in therapeutic neovascularization needs additional investigation.

To clarify the specific mechanisms of neovascularization induced by SVF, the diversity of the cell population isolated by different laboratories and alterations to cell components during *in vitro* culture must be taken into consideration. This paper will focus on the role of each subpopulation during the SVF neovascularization process and attempt to analyze whether cross-talk between constituents generates a synergetic effect for neovascularization.

Cell subpopulations of SVF and their dynamic changes

It is important to recognize that SVF is a heterogeneous, versatile cellular system, and the degree of heterogeneity is dependent on a variety of factors, such as the adipose tissue isolation site, the digestion protocol, and the patient’s own pathological status. To date, there is no consensus definition to distinguish the specific proportions of these constituents to one another[30, 31]. In fact, because the previously used markers overlap some cell populations, it is theorized that the composition of the SVF is somewhat like a mosaic. What is clear is that the SVF is a dynamic population of cells with a potentially significant clinical utility; a hallmark characteristic of SVF cells is their ability to self-assemble into a hierarchical, branched, perfused vasculature *in vivo*[21].

Many researchers have proposed different classifications according to different surface markers. Among these markers, the most commonly used in previous studies are CD31 and CD34. CD31 is a classic surface marker for ECs and their progenitors, and CD34 is expressed by both ECs and hematopoietic stem/progenitor cells[32]. Klar et al. divided the SVF cells into a CD34+/CD31+ cell population and a CD34+/CD31− cell population by flow cytometry sorting[33]. They observed that the CD31+ population comprised 25% of all cells in the SVF; this subset showed a specific endothelial phenotype, could express CD31 and vascular endothelial growth factor receptor 2 (VEGFR2), and could uptake fluorescent labeled acetylated low-density lipoprotein. On the contrary, the CD31− cell population from human adipose tissue showed stromal stem cell properties, expressed surface markers typical of MSCs, and was positive for CD90 and vimentin[33]. In addition to endothelial and stromal cell lineages, hematopoietic cells are also a major component of SVF. Morris et al. reported that myeloid cells accounted for about 22% of SVF cells of mice[34].

Notably, culturing SVF cells for even one passage would profoundly alter their cellular composition. Nunes et al. examined the differences in the proportions of these cells in fresh versus cultured human SVF isolates[35]. They found that about 33% of freshly isolated SVF cells were comprised of CD31+ ECs, and that culturing SVF cells significantly reduced this number to about 10% of the total population. Similar trends were also observed in CD14+ monocytes/macrophages and c-Kit+ progenitors[35]. Harada et al. revealed that, when the freshly isolated SVF cells were cultured in Dulbecco's modified eagle medium, these cells expressed significantly lower levels of CD31, CD34, and CD45 but a higher level of CD90 even in early passages[36]. There are two possible reasons for these alterations: 1) most of the hematopoietic cells cannot adhere to the flask during *in vitro* culture and were removed during the course of passaging; 2) an unsuitable culture medium for an endothelial lineage resulted in the decrease of CD31+ ECs. Szoke et al. reported that cell surface markers, such as CD34 and CD90, quickly disappeared in the adipose tissue derived CD31+ cell population while CD34 and THY1 (CD90) mRNA remained for a period of time even after cell surface expression disappeared, suggesting that the culture conditions might induce a phenotype change[37].

The International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) issued a joint statement that hematopoietic, endothelial, and stromal cells are the main subpopulations of the nucleated cells in SVF[32]. In this paper, we will review the characteristics of these cell lineages and discuss the cross-talk between them in the process of neovascularization. According to previous studies, the cell lineages isolated from SVF and other tissues, including peripheral blood, vessel wall, and bone marrow, share almost identical characteristics[38–42]. Thus, we also refer to the research results of these cell populations isolated from different tissues in addition to SVF. The commonly used cell positive and negative surface markers and characteristics are listed in Table 1.

Endothelial lineage

ECs play a vital role in many physiological activities, including neovascularization, hemostasis, and immune responses[43]. Many studies have indicated that ECs are capable of forming vascular networks spontaneously *in vitro* or within a previously avascular tissue[3]. Koh et al. reported that ECs from freshly isolated SVF implantation can rapidly rebuild a vascular network at the site of implantation[19]. They also found that depleting ECs from freshly isolated SVF and the cultured SVF cells failed to effectively create a vascular network[19].

Patel et al. indicated that endovascular progenitors had self-renewal and colony-forming capacity but differentiated ECs did not[11]. These progenitor cells exist in many tissues and can form new vascular networks when transferred to a new environment. Some other investigators also believed that neovascularization required vascular progenitor cell homing or recruitment, proliferation, and differentiation into ECs to form a primitive vascular network[5, 44, 45]. In 1997, Asahara et al. reported the identification of EPCs from peripheral blood, which contributes to the formation of a new vascular network[45]. However, because there is no specific definition to date, a wide variety of cell populations are named EPCs. It

has been demonstrated that the circulating putative EPCs reported in the literature contained two distinct subtypes: 1) myeloid angiogenic cells (MACs) or early outgrowth cells, which stem from the monocyte/macrophage lineage with no/low proliferative activity and 2) endothelial colony-forming cells (ECFCs) or late outgrowth cells, which have a high proliferative capacity[41, 46–49]. ECFCs represent an EC type with potent intrinsic angiogenic capacity, which are capable of forming a vascular network *de novo* and contributing to the repair of injured vascular endothelium[46, 50]. However, the frequency of peripheral blood ECFC colonies was estimated as about only 0.017 per million blood mononuclear cells in healthy adults[46]. This low content of ECFCs in adult peripheral blood has limited its clinical application, thus an alternative sustainable source of ECs needs to be found. Besides the adult circulating blood, progenitor cells with EC potential were also found in the vascular wall of several organs[10, 51, 52]. Ingram et al. isolated a hierarchy of ECFC population from the aorta and umbilical vein, which had a similar colony-forming ability to peripheral blood-derived ECFCs[51]. These results suggest that the vasculature might be a possible source of resident ECFCs[53].

Considering the cell surface marker expression pattern of adipose tissue, CD31+ ECs are almost identical to the ECFCs isolated from peripheral blood[19]; it is postulated that the vasculature of adipose tissue could serve as a dependable source of ECFCs. Lin et al. obtained 10^9 homogeneous ECFCs from 1 g of white adipose tissue after 30 days of *in vitro* culture; these cells were consistent with adult blood-derived ECFCs in surface markers and colony-forming ability[38]. There are several advantages of adipose tissue derived ECFCs (AT-ECFCs). First, adipose tissue is a steady source of ECFCs with easy availability. Second, AT-ECFCs have remarkable expansion potential *in vitro* and highly pure ECFCs can be obtained after several passages. Third, AT-ECFCs have a stable phenotype similar to blood-derived ECFCs. Fourth, culture expanded ECFCs have a robust vasculogenic potential *in vivo* and *in vitro*[38]. These characteristics make adipose tissue a more practical source of ECFCs than peripheral blood.

Although cell surface markers of various ECFC populations (high proliferation versus no proliferation) were not distinguishable from one another or from ECs, Ingram et al. suggested that the high proliferative potential-endothelial colony-forming cells (HPP-ECFCs), low proliferative potential-endothelial colony-forming cells (LPP-ECFCs), and mature ECs may represent different stages of ECFC differentiation[49, 51]. They found that HPP-ECFCs could generate all other subsequent stages of endothelial progenitors, while LPP-ECFCs do not form secondary colonies but rather mature EC clusters[49, 51]. Patel et al. also reported an entirely novel endothelial hierarchy including endovascular progenitor (EVP), transit amplifying cell, and a definitive differentiated cell population in normal endothelium; only EVP cells had self-renewal and colony-forming capacity[11]. Thus it is concluded that ECs isolated from SVF might be composed of a hierarchical distribution of ECFCs and mature ECs, which jointly provide “building blocks” during the process of SVF induced neovascularization. The degree of neovascularization may be dependent, in part, on the amount of resident ECFCs with proliferative potential or circulating ECFCs that can be recruited into the tissue[54].

Stromal/mesenchymal cell lineage

ADSCs are defined as the adherent cell population of SVF; they remain their phenotype and plasticity toward the stromal lineage even after being cultured for several passages[32]. ADSCs are currently accepted to be positive for stromal markers (such as CD13, CD73, and CD90) and retain these markers in culture, but negative for hematopoietic markers (such as CD11b and CD45)[32]. CD34 is generally considered to be expressed by hematopoietic and endothelial progenitors; nevertheless, it is transiently expressed on ADSCs up to 8–12 passages in culture[32]. In many ways, ADSCs are similar to BM-MSCs, such as morphology, immune phenotype, colony frequency, and differentiation capacity, which has led some investigators to regard these two populations as identical[39, 40, 55]. However, numerous features have distinguished ADSCs and BM-MSCs, and the difference between them may be related to the different microenvironment where these cells reside[32]. ADSCs were reported to exhibit higher proliferative capacity and better efficacy in angio-inductive ability than BM-MSCs[56]. Besides, the frequency of MSCs within SVF is reported to be at least 500-fold higher than in bone marrow mononuclear cells[57]. These results imply that ADSCs from SVF may be a more suitable candidate than BM-MSCs for cell therapy of ischemic disease.

It is generally accepted that ADSCs may be obtained in high numbers from SVF by removing CD31+ cells from adherent cells[58]. ADSCs have been reported to differentiate into several cell types *in vitro*, including adipocytes, chondrocytes, and osteoblasts[18]. In 2004, Planat-Benard et al. first suggested that ADSCs might differentiate into ECs and promote neovascularization[20]. They reported that the SVF cells cultured *in vitro* represent a homogeneous CD34+/CD13+ cell population, which could differentiate into ECs and express endothelial surface markers, such as CD31 and von Willebrand factor (vWF). After that, many studies reported the capacity of ADSCs to express endothelial surface markers and their ability to self-assemble into vascular networks[24, 59–62].

However, some other studies were unable to prove the differentiation capacity of ADSCs toward an endothelial lineage[22, 25]. Antonyshyn et al. reported that ADSCs without CD45+ leukocytes and CD31+ ECs have limited endothelial differentiation by well-established biochemical stimuli[25]. Although ADSCs are isolated from SVF, they are definitely not a homogeneous population[32]. It is a significant challenge to completely sort out the endothelial lineage from ADSCs due to the absence of definitive surface markers with specificity to both of these cell lineages[25]. Most importantly, the difference of cell components of SVF between different studies due to different passages, protocols, and adipose tissue sites, can result in various outcomes. One potential reason for the different conclusions is possible contamination of ECs or ECFCs. Some experiments did not deplete the CD31+ cell population completely from the cultured cells; if a small amount of HPP-ECFCs are contained in the cultured cells, they can expand rapidly in suitable environments and become the dominant cell population, which will give a false impression that ADSCs differentiate into ECs. Thus it remains controversial whether ADSCs can actually differentiate into mature ECs.

Nakagami et al. reported that ADSCs could significantly improve neovascularization in the mouse ischemic hind limb model mainly through secretion of angiogenic growth factors rather than differentiating into ECs directly[22]. Rehman et al. also proved that ADSCs could

enhance perfusion in the ischemic hind limb through secretion of multiple angiogenic and antiapoptotic growth factors when delivered to ischemic tissue. In an *in vitro* experiment, they found that vascular endothelial growth factor (VEGF) secretion increased five-fold when ADSCs were cultured in hypoxic conditions, and the conditioned media obtained from hypoxic ADSCs could significantly promote EC proliferation[63]. In addition to VEGF, ADSCs also secrete significant amounts of angiogenesis-related mediators, such as hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β)[63, 64].

There is another opinion suggesting that ADSC populations may be identical to pericytes[65, 66]. Traktuev et al. demonstrated that CD34+ ADSCs are indeed resident pericytes that interacted with ECs mutually and stabilized the vascular structure[67]. Crisan et al. identified a population of CD146+/NG2+/PDGFRβ+/ALP+/CD34−/CD45−/vWF−/CD144− cells as pericytes in several tissues and described links between MSCs and pericytes. These perivascular cells express the classic MSC markers but not endothelial markers, and also exhibit adipogenic, osteogenic, and chondrogenic differentiation potentials[65].

Hematopoietic lineage

It has been demonstrated that adipose SVF contains a significant proportion of hematopoietic cells, which also play critical roles in SVF's ability to self-assemble into mature vasculature[19, 33, 34]. Eto et al. reported that the resident hematopoietic cells make up about 10% of the total cells in human adipose tissue[69]. Adipose tissue-resident hematopoietic cells were composed predominantly of macrophages (about 60%) and lymphocytes (about 40%), mixing with a small amount of helper T cells and NK cells, but no B cells[42]. Currently, the cells from the myeloid lineage which are able to offer angiogenic support for neovascularization in adults are called "MACs"[7]. According to the genotypic, proteomic, and immunophenotypic perspectives, MACs are considered to be monocytes in nature[70].

Urbich et al. reported that both CD14+ and CD1−mononuclear cells could express endothelial surface markers and delivery of these MAC-like cells improved the neovascularization in a hind limb ischemia mouse model[71]. Thus, it may be postulated that MACs can differentiate into ECs. However, Medina et al. reported that MACs did not directly incorporate into the vascular network and differentiate into ECs but remained remote from the vasculature and retained their original myeloid phenotype[72]. It was also found that MAC conditioned media could enhance angiogenesis in an *in vitro* tubulogenesis assay, which demonstrated that MACs exhibit pro-angiogenic ability through the release of cytokines and chemokines, such as interleukin 8 (IL-8), monocyte chemotactic protein-1 (MCP1), and matrix metallopeptidase 9 (MMP9)[72]. Endothelial markers on monocytes, such as CD31, VEGFR2, and Tie2, possibly originate from platelet microparticles which are uptaken by the mononuclear cells[73].

Adipose tissue macrophages, which are derived from blood monocytes and remain resident as tissue macrophages, are regarded to be involved in angiogenesis regulation[74–76]. Macrophage subsets are divided into two major groups: M1 (classically activated) and M2 (alternatively activated) macrophages. M1 macrophages can promote inflammation and

secrete several kinds of pro-inflammatory cytokines, while M2 macrophages regulate inflammation and contribute to angiogenesis, arteriogenesis, and wound healing[76]. It has been demonstrated that resident macrophages expressing M2 markers were the predominant adipose tissue macrophage subtype, and there are few M1 macrophages detected in adipose tissue[42, 77]. Coincidentally, MACs isolated from peripheral blood also display typical M2 markers, such as CD204, CD206, IL-10, and CD163, suggesting that MACs exhibit an M2 macrophage phenotype[41, 78]. Thus it can be deduced that adipose tissue-resident macrophages might play the role of MACs in the process of SVF induced neovascularization.

Lymphocytes of SVF cells may also be involved in the process of neovascularization. It was reported that Th-1 cells could promote the tumor-associated blood vessel formation directly and Th-2 cells could secrete IL-4 to stimulate M2-like activation of macrophages[79]. Weirather et al. found that therapeutic T_{reg} cell activation was also related with an M2-like monocyte differentiation after myocardial infarction[80]. In addition, ADSC transplantation was reported to create a suitable microenvironment in local ischemia for tissue regeneration by stimulating M2 macrophage polarization in the mouse ischemic model[81]. The cross-talk between T cells, ADSCs, and resident M2 macrophages of SVF may provide a favorable environment for neovascularization in the ischemic area.

Synergistic effects of SVF subpopulations

Although both fresh and cultured SVF cells can promote neovascularization, the vessel density of vasculature formed by cultured SVF is lower than fresh SVF. Additionally, the fresh SVF can generate more small capillary-like vessels than the cultured SVF[35]. Harada et al. also reported that transplantation of freshly isolated SVF cells in mice could significantly improve the capillary density in transplanted ischemic limbs, whereas these effects were not observed in the cultured SVF cell group[36]. The cells of cultured SVF were found mainly in the perivascular position, while the cells of fresh SVF were detected in both endothelial and perivascular regions. Further investigation found that the number of CD31+ cells contained in the cultured SVF cell population was much less than in fresh SVF cells[35]. A different proportion of cell components, especially ECs, may be the reason for this discrepancy. Nevertheless, ECs alone are also considered to be insufficient to form a mature vasculature without supporting cells[82, 83]. The support cells within the SVF, such as perivascular cells, MSCs, and macrophages, may be important[42]. It was also reported that the freshly isolated cells transcribed a host of genes related to angiogenesis with the production of extracellular matrix (ECM); therefore, they also secreted significantly higher levels of angiogenic factors than cultured cells in the *in vitro* study[36, 84]. Based on the above results, the interrelations between each subpopulation of SVF contribute to the process of neovascularization (the possible mechanism is represented in Figure 1).

Synergistic effects of ECs/ECFCs and ADSCs

Molecular interactions between ADSCs and ECs, including secreted factors and cell-cell interactions, are appealing because ADSCs co-cultured with ECs can promote vascular formation[67, 85–89]. To determine the molecular interactions between them *in vitro*, there are

two major methods: direct co-culturing or culturing ECs and ADSCs in each other's conditioned medium, respectively. Rehman et al. reported that conditioned medium obtained from ADSCs in a hypoxic environment significantly promoted EC proliferation and reduced apoptosis[63]. Traktuev et al. reported that ADSC conditioned medium did not stimulate the proliferation of ECs but supported endothelial survival, while EC conditioned medium exhibited a significant chemotaxis and mitogenic effect on ADSCs[67]. Other investigations revealed that ADSC conditioned medium had a positive effect on the proliferation of the EC lineage but the ADSC conditioned medium alone did not result in fully branched networks, which indicated that direct cell-cell contact was also necessary for vascular formation[85–87]. Interestingly, the phenotype of the ADSCs was also found to be changed in the co-culture system. It was revealed that, in a co-culture system, the ADSCs around the endothelial cord-like structures expressed a high level of α-smooth muscle actin (α-SMA) and eventually stabilized the structures to represent physiological microvasculature[85, 87]. Lin et al. indicated that, when ADSCs were directly co-cultured with AT-ECFCs or circulating ECFCs, the expression of smooth muscle specific marker, smooth muscle myosin heavy chain (SM-MHC), was upregulated significantly[38].

In vivo experiments also proved that ADSCs combined with EC lineage implantation could result in more dense and robust vessel formation[37]. Traktuev et al. reported that the combination of ECFC and ADSC implantation yielded a higher neovascularization capacity than either ECFC or ADSC implantation alone[90]. They demonstrated that the majority of CD34+ ADSCs from freshly isolated SVF adopted a supportive role similar to pericytes, which associate with the EC lineage and stabilize the vascular network[67]. Lin et al. proved that human AT-ECFC co-implantation with ADSCs into immune-deficient mice could quickly form an extensive vascular network. The endothelium of the newly formed vessel was lined with AT-ECFCs, while the perivascular region was occupied by ADSCs[38]. If ADSCs and ECFCs are appropriately combined, the reciprocal interaction between them might promote neovascularization in a synergistic manner *in vivo*.

To investigate EC and ADSC interactions in the co-culture system, the angiogenic proteins were determined. The protein expression of supernatants from ECFCs, ADSCs mono-culture, ECFCs cultured in ADSC conditioned medium, and ADSC-ECFC co-culture system were determined after four days of incubation. Compared with ECFC mono-culture, ADSCs expressed much higher levels of different angiogenesis-related proteins, including both pro-angiogenic factors and angiogenesis inhibitors, which further indicates that ECs alone are not sufficient to form a tube-like structure. ADSCs might regulate neovascularization by the interplay of pro- and anti-angiogenic/regulatory proteins. Comparing the results of ECFCs cultured with supernatant from ADSCs and the ECFC-ADSC co-culture system, there was a were more than two-fold increase of several pro-angiogenic factors in the co-culture system, including platelet-derived endothelial cell growth factor (PD-ECGF), fibroblast growth factor (FGF-2), MMP-9, angiopoietin-2 (Ang-2), and pentraxin-3. These angiogenic proteins seemed to be important for neovascularization because ADSC conditioned medium alone failed to induce ECFCs to form a vessel structure[86].

The different gene expression levels were also evaluated *via* quantitative reverse transcription polymerase chain reaction in a co-culture system to investigate molecular

interactions of ECs and ADSCs. When ECs were cultured alone, there was no significant change in angiogenesis-related gene expression except vWF, which increased two-fold after one week. The expression levels of genes encoding for CD31, VE-cadherin, VEGFR2, and vWF were upregulated in the co-culture system; however, platelet-derived growth factor (PDGF) and Tie-2 expression remained stable. Both CD31 and VE-cadherin are cellular adhesion molecules which are needed for cells to attach to each other to form networks[91, 92]. It is considered that the upregulation of CD31, VE-cadherin, VEGFR2, and vWF will contribute to prevent uncontrolled vessel growth. The expression of PDGF and Tie-2 might increase after long-term co-culture because they are mainly involved in vessel stabilization[86].

In the ADSC and EC co-culture system, it was reported that ECM production increased, which was also considered to be related to vessel formation[87]. Collagen IV was not detected in ADSC mono-culture, and a low level was present in EC mono-culture. However, ECs expressed a significantly higher level of collagen IV in co-culture. In addition, perlecan I and fibronectin expression also significantly increased in a co-culture system compared to ADSC and EC mono-culture. The accumulation of this protein was distributed in proximity to the EC-cord structures. Interestingly, laminin was also found to gather around EC vascular structures despite no significant change of protein expression[87]. Taking together, Collagen IV, fibronectin, perlecan I, and laminin are regarded to be associated with vessel formation, because they are components of the basement membrane and promote the proliferation of SVF ECs into monolayer to form a tube-like structure[93]. According to these findings, the close interaction of ADSC-EC plays an important role in ECM production and therefore on vessel formation.

Synergistic effects of EC/ECFCs and MACs

Although ECFCs are widely regarded as “building blocks” and are directly involved by forming the endothelial lining of vasculature, these cells alone have yet to be used clinically[41, 53]. Koh et al. described the role of SVF macrophages on vascular assembly, noting that macrophages were required for proper vascular structural organization. To support this supposition, the depletion of CD11b+ and F4/80+ macrophages from SVF yielded vessels that were blunted and disconnected[19]. Medina et al. found that MACs significantly increased EC tubulogenesis approximately two-fold when MACs were co-cultured with ECs; however, MACs did not incorporate into the vessel lumen and often remained completely isolated from the vasculature[41]. Fantin et al. reported that the cytokine or growth factor concentration gradient released by MACs could stimulate tip cell protrusion and vessel sprouting, while the resident macrophages also promoted the fusion of tip cells to add to new circuits within the existing vascular[94].

It has been proven that a mixed implantation of MACs and ECFCs could result in superior neovascularization *in vivo* compared to either cell alone[95]. MACs cannot differentiate into ECs but indirectly augmented proliferation, migration, and the tube forming capability of ECFCs in a paracrine manner by secretion of various cytokines[71, 96–98]. MACs secreted large amounts of VEGF and IL-8, and correspondingly, ECFCs showed significantly higher expressions of a receptor of VEGF (KDR) and IL-8 (CXCR-1)[95]. Both of these cytokines

are known to promote endothelial proliferation, tube formation, and migration. Furthermore, when the two kinds of cells were mixed and incubated together, the migrating distance of both types of cells was significantly increased beyond that of any single cell alone; however, the migration could also be significantly inhibited by the neutralizing antibodies of VEGF and IL-8[95]. It was reported that MAC-derived IL-8 could transactivate VEGFR2 of mature ECs independently of extracellular VEGF[41]. In addition, MACs also contribute to neovascularization by secretion of cytokines and MMP-9, whereas ECFCs secrete MMP-2[99]. The co-culture increased the active form of MMP-9 and the invasion depth of MACs, which was not observed when MACs were cultured alone. MMP-2 can mediate the activation of MMP-9 and enhance matrix degradation[100].

The prospect of SVF in stem cell therapy for ischemic disease

The majority of preclinical and clinical trials of human cell therapies for ischemic disease using autologous BM-MSCs have displayed modest regenerative or reparative capability[101]. Compared to bone marrow cells, SVF cells seem to be a more attractive cell population because human adipose tissue can provide many more cells suitable for autologous implantation and it is an easily accessible and dispensable tissue[102]. Moreover, SVF comprises abundant endothelial and mesenchymal progenitors in addition to hematopoietic cells; the mixed SVF cell population by a certain percentage is considered highly angiogenic compared to other stem cells alone[103].

At present, it is not clear how to mix these heterogeneous cells to get the best result in stem cell therapy. To maximize the regenerative potential of SVF cells, investigators have made some attempts to transplant stem cells within their physiological niche. Hamdi et al. implanted an SVF cell sheet into cardiac infarct mice and got greater post-infarct survival than intra-myocardial injections of the same cells[104]. It was also reported that implantation of a three-dimensional (3D) graft with SVF cells or intact microvascular segments from adipose tissue could promote the development of coronary microvasculature after acute myocardial infarction[105, 106]. In summary, the relative ease of isolation and the synergic effects of different cell subpopulations makes SVF a clinically relevant, promising cell source for stem cell therapy of ischemic disease. However, the variety of cell components between different studies and passages makes it hard to elucidate the mechanism underlying neovascularization. How to mix different cell types in an ideal ratio to result in the optimal vascular network and how to organize these cells still needs to be investigated. To solve this problem, there might be three methods: 1) using freshly isolated SVF cells; 2) providing an appropriate growth environment for many cell subpopulations to avoid the loss of EC and the hematopoietic cell lineage; and 3) mixing these cells in specific proportions or arranging them in a 3D location according to different ischemic environments.

Besides differentiating into vascular cells, the other possible fates of the SVF cells when in the ischemic environment also should be noted. For one thing, it is postulated that some of the SVF cells, such as ADSCs, will differentiate into adipocytes because they are generally regarded as adipocyte precursors[107]. However, adipogenesis by ADSC differentiation is regulated by the specific microenvironment, such as ECM[107, 108]. Whether the ischemic microenvironment can provide a suitable adipogenic niche for ADSCs still needs further

investigation. Also, does ADSC therapy promote oncogenesis? To date, no tumor formation was reported in human recipients of ADSCs[15]. Nevertheless, long-term follow-up and more clinical experience are needed to confirm the safety of ADSC therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ADSC	adipose-derived stem cell
AT-MSC	adipose tissue-derived mesenchymal stem cell
BM-MSC	bone marrow-derived mesenchymal stem cell
ECM	extracellular matrix
EC	endothelial cell
ECFC	endothelial colony-forming cell
EVP	endovascular progenitor
EPC	endothelial progenitor cell
HGF	hepatocyte growth factor
HPP-ECFC	high proliferative potential-endothelial colony-forming cell
IL-8	interleukin 8
LPP-ECFC	low proliferative potential-endothelial colony forming cell
MCP1	monocyte chemotactic protein-1
MMP	matrix metallopeptidase
SVF	stromal vascular fraction
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2

vWF	von Willebrand factor

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Highlights

1. Adipose stromal vascular fraction (SVF) is a heterogeneous, versatile cellular system; cellular composition profoundly alters after being cultured. Fresh SVF was considered to have superiority compared to cultured SVF during the process of neovascularization.
2. Endothelial colony-forming cells (ECFCs) isolated from SVF can proliferate and differentiate into endothelial cells and supply “building blocks” for tube network formation.
3. Adipose-derived stem cells (ADSCs) from SVF promote ECFC proliferation and differentiation by secreting proangiogenic factors; ADSCs can differentiate into pericytes to stabilize the newly formed vessel structure.
4. The hematopoietic cells of SVF may provide a favorable environment for neovascularization in ischemic areas through the release of IL-8, MCP1, MMP9, and VEGF. Correspondingly, ECFCs showed significantly higher expressions of the receptors for VEGF (KDR) and IL-8 (CXCR-1).

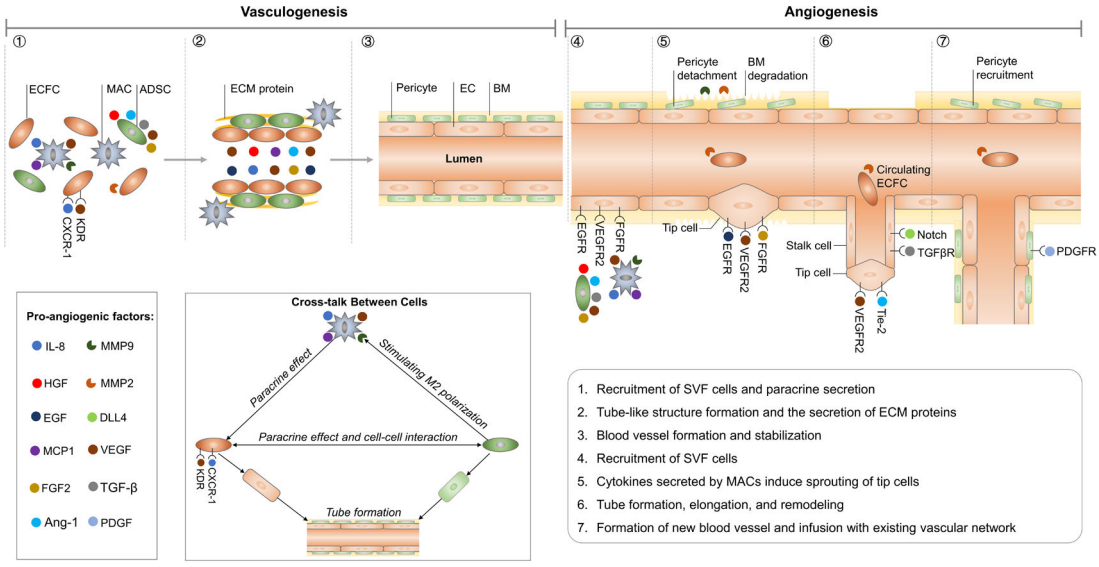

Figure 1:

The possible mechanisms underlying neovascularization induced by SVF implantation:

1. ECFCs can proliferate and differentiate into ECs and supply “building blocks” for tube network formation.
2. ADSCs promote ECFC proliferation differentiation by secreting VEGF, HGF, placental growth factor (PGF), FGF-2, TGF-β, and angiopoietin-1 and through cell-cell contact.
3. ADSCs and ECs co-produce ECM proteins; ADSCs can differentiate into pericytes, both of which stabilize the newly formed vessel structure.
4. MACs enhance angiogenesis tubulogenesis assay through the release of IL-8, MCP1, MMP9, and VEGF2; correspondingly, ECFCs showed significantly higher expressions of the receptors for VEGF (KDR) and IL-8 (CXCR-1).
5. Cytokines may induce the resident or circulating ECFCs to aggregate in the ischemic area and promote neovascularization; the angiogenic factor gradient could induce EC tip cell proliferation and migration.

Table 1.

The main subpopulations of SVF and their characteristics

Classification	Function in neovascularization	Nomenclature	Origin/species	Cell surface marker	Ref
Endothelial lineage	1) Serving as "building blocks" for vessel formation 2) Producing ECM protein to separate the ECs from the surrounding environment	EC	Mouse, aorta, wound, tumor	CD31+/VEGFR2+/CD34+/VE-Cadherin+/CD45−/Ter119−	11, 19
		ECFC	Human, adipose tissue, and peripheral blood	CD31+/vWF+/VE-cadherin+/CD146+/CD45−/CD14−/CD90−	38, 47
		HPP-ECFC LPP-ECFC	Human, umbilical cord blood, umbilical vein, and aortic ECs	CD31+/CD141+/CD105+/ CD146+/CD144+/vWF+/Flk-1+/CD45−/CD14−	49, 51
		EC-SP	Mouse, large and peripheral vessels	VE-cadherin+/ Flk-1+/ Sca-1+/CD133+/CD31+/ CD157+CD200+/CD45−/c-kit-/PDGFR-β-/Hoechst$_{lo}$	10, 109
		EVP	Mouse, aorta, wound, tumor	CD34+/VE-Cadherin+/CD45-/CD31$_{lo}$/VEGFR2$_{lo}$	11
		OEC	Human, blood	CD31+/VEGFR2+/Tie-2+/Ve-cadherin+/VEGFR2+/vWF+/CD36+/CD146+/CD105+	70, 110, 111
Stromal lineage	1) Cooperating with ECs to stabilize newly forming endothelial networks 2) Improving neovascularization through secretion of angiogenic growth factors	ADSC	Human and rat, adipose tissue	CD34+/CD10+/CD13+ /CD90+/NG2+CD140a+/CD140b+/calponin+/α-SMA+/CD31−/CD45−/CD144-	32, 67, 112
		ADSC	Human, adipose tissue	CD13+/CD29+/CD44+/CD73+/CD90+/CD105+/CD166+/CD10−/CD14−/CD24−/CD31−/ CD34−/ CD36−/CD38−/ CD45−/CD49d−/CD117−/CD133−	113
		ADSC	Mouse, adipose tissue	Sca-1+/CD44+/c-kit−/Lin−/CD34−/CD45−/CD11b−/CD31−	22
		Pericyte	Human, adipose tissue, muscle, pancreas, placenta	CD44+/CD73+/CD90+/ CD105+/CD146+/CD34−/CD45−/CD56−	65
Hematopoietic lineage	Inducing endothelial tube formation *in vitro* and vascular repair *in vivo* by secreting pro-angiogenic factors	MAC	Human, peripheral blood	CD45+/CD14+/CD68+/MSR1+/MRC1+/CD163+/CD146−/ CD133−/Tie2-	41, 47
		Early EPC	Human, blood	CD45+/CD14+	70
		Macrophage	Human, adipose tissue	CD45+, CD14 +, CD34+, CD206+	42
			Mouse, adipose tissue	CD11b+ and F4/80+	19

Abbreviation: ADSC, adipose-derived stem cell; α-SMA, α-smooth muscle actin; EC, endothelial cell; ECFC, endothelial colony-forming cell; ECM, extracellular matrix; EC-SP, EC-side population; EPC, endothelial progenitor cell; EVP, endovascular progenitor; HPP-ECFC, high proliferative potential-endothelial colony-forming cell; LPP-ECFC, low proliferative potential-endothelial colony-forming cell; MAC, myeloid angiogenic cell; MSR1, macrophage scavenger receptor 1; MRC1, mannose receptor C type 1; OEC, outgrowth endothelial cell; PDGFR-β, platelet-derived growth factor receptor-β; Sca-1, stem cell antigen-1; VEGFR2, vascular endothelial growth factor receptor 2; vWF, von Willebrand factor.