[Frontiers in Bioscience 19, 768-776, January 1, 2014]

Application of adipose-derived stem cells in critical limb ischemia

Kangkang Zhi[1], Zhiwei Gao[1], Jun Bai[1], Yongfa Wu[1], Sili Zhou[1], Maoquan Li[2], Lefeng Qu[1]

[1]Department of Vascular and Endovascular Surgery, Changzheng Hospital, 415 Fengyang Road, Shanghai, China; [2]Department of Interventional Radiology, Tenth People's Hospital of Tongji University, 301 Yanchang Road, Shanghai,China, 200072

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Characteristics of critical limb ischemia (CLI)
4. ASCs
5. Neovascularization
6. Function and mechanism of action of ASCs in neovascularization and treatment of CLI
6.1. ASCs differentiate into VECs and smooth muscle cells during neovascularisation
6.2. ASCs release angiogenic cytokines to promote neovascularization
6.3. ASC-endothelial crosstalk contributes to neovascularisation
7. Therapeutic potential of ASCs in vascular growth and tissue repair
8. Challenges of functional usage in neovascularization
9. Acknowledgements
10. References

1. ABSTRACT

Peripheral artery disease is growing in global prevalence. Its most severe form, critical limb ischemia (CLI), is associated with high rates of limb loss, morbidity, and mortality. Neovascularization is the cornerstone of limb preservation in CLI. In the field of regenerative medicine, basic research and preclinical studies have been conducted using mesenchymal stem cells (MSCs) from adult tissues, including bone marrow and adipose tissue, to overcome clinical shortcomings. Adipose-derived stem cells (ASCs) display stable growth and proliferation kinetics and can differentiate into osteogenic, chondrogenic, adipogenic, myogenic or neurogenic lineages. ASCs are readily available from autologous adipose tissue, and have significant potential for tissue repair under conditions of myocardial infarction, heart failure, hind limb ischemia, and inflammation. This review highlights some of the key reports underlining the potential of ASCs, particularly in diseases involving neovascularization.

2. INTRODUCTION

Regeneration of circulation is an indispensable step in the treatment of ischemic disease, organ transplantation and tissue injury repair surgery. Blood flow regeneration is dependent on blood vessel formation (revascularization) (1). In the field of regenerative medicine, several basic research and preclinical studies have been conducted using mesenchymal stem cells (MSCs) from adult tissues, including bone marrow (BM) and adipose tissue, to overcome clinical shortcomings (1-4). Although BM-derived MSC (BM-MSC) have remained the primary source of stem cells for tissue engineering applications, recent studies have shown that subcutaneous adipose tissue-derived stem cells (ASCs) have a clear advantage over other sources due to ease of access and isolation from harvested tissues. ASCs display stable growth and proliferation kinetics and can differentiate into osteogenic, chondrogenic, adipogenic, myogenic or neurogenic lineages (4, 5).

Due to the high expectations in terms of utility of these attractive cell populations, recent studies have focused on the safety and efficacy of implanted ASCs in a variety of animal models. Clinical studies using ASCs have been initiated in some medical subspecialties. The differentiation potential of ASCs into vascular endothelial cells (VECs) and vascular smooth muscle cells has been reported. These cells secrete angiogenesis-related cytokines, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), to promote angiogenesis in ischemic tissue (5, 6). Since autologous adult stem ASCs hold several advantages, such as abundant source, stable availability and simple means of collection, they are expected to present a valuable therapeutic option in revascularization and tissue repair in a variety of clinical settings (1, 4). During the past decade, preclinical studies on the safety and efficacy of ASCs have provided considerable data supporting the use of these cells in clinical applications (1, 4, 6). Recent clinical applications of ASCs have demonstrated early safety results and possibility of efficacy in patients with a range of diseases, including acute myocardial infarction, peripheral vascular disease, and skin wounds (6). In this review, the mechanisms of action and therapeutic potential of ASCs in neovascularisation are summarized, along with current preclinical data and ongoing clinical trials and their outcomes in a variety of medical fields.

3. CHARACTERISTICS OF CRITICAL LIMB ISCHEMIA (CLI)

CLI is diagnosed in 12% of the adult population in the U.S. Its clinical presentation varies from no symptoms to intermittent claudication, atypical leg pain, rest pain, ischemic ulcers or gangrene (7-9). CLI is a syndrome combining several peripheral artery diseases with different etiologies and pathogenesis, but with similar prognosis, high morbidity and mortality. Surgical and conservative treatment possibilities of CLI have been almost completely exhausted. These patients suffer severe blockage in arteries of the lower extremities, resulting in markedly reduced blood flow. Limb ischemia is a serious form of peripheral arterial disease (PAD), but less common than claudication. PAD is caused by atherosclerosis, the hardening and narrowing of arteries over time due to the buildup of fatty deposits denoted 'plaque'. CLI results in severe pain in the feet or toes, even while resting (10, 11). Moreover, patients with CLI have a high incidence of cardiovascular comorbidities that reflect a significant systemic atherosclerotic burden. These individuals display increased functional impairment and functional decline. Complications of poor circulation include sores and wounds that do not heal in the legs and feet. If left untreated, these complications often result in amputation of the affected limb (12, 13).

Thus, improved therapeutic strategies against CLI are essential. Cell and gene therapy studies have shown promise in controlling pain and minor ulceration in patients with significant CLI. *In vivo* gene transfer techniques for vascular applications include viral gene transfer, liposomal gene transfer using cationic liposomes, and naked plasmid DNA transfer. Initially, single applications of therapy were normally used, but this has been modified to multiple applications over a 4 to 8-week interval to allow for continued priming of the area targeted for angiogenesis. Numerous angiogenic growth factors, such as VEGF, basic fibroblast growth factor (bFGF) and HGF, continue to be tested in clinical trials. In addition to gene therapy, recent evidence has indicated that BM mononuclear cells (BM-MNC) promote collateral vessel formation in patients with severe PAD (14). BM-MNCs from patients with CLI display impaired phenotype and a lower number of endothelial progenitor cells (EPCs), compared to normal individuals and patients with Buerger's Disease. Multiple strategies have been developed to mobilize and derive cells, with the aim of improving the performance of cell therapy. Cell therapies for severe ischemic diseases, such as CLI, acute myocardial infarction and cerebral ischemia, have been developed through animal and clinical studies (15-17). EPCs are reported to be the active cells for angiogenic cell therapy. EPCs have been extensively investigated to clarify their origin and biology (18, 19). A number of EPC sources have been reported, including a MNC fraction containing $CD34^+$ or $CD133^+$ ($AC133^+$), isolated $CD34^+$ and $AC133^+$ cells, as well as induction and differentiation of EPCs from hematopoietic stem cells (HSCs). However, the *in vivo* mechanisms by which EPCs contribute to neovascularization require clarification. To date, numerous *in vitro*, *in vivo*, and clinical studies have been performed using these cells. Angiogenic cell therapy is expected to become an important regimen for severe ischemic diseases. In addition to BM-MNC and EPCs, ASCs have recently been identified as an ideal candidate in cell therapy, in view of their stability, operability and ease of acquisition.

4. ASCS

Recent studies have consistently shown that mesenchymal cells isolated from subcutaneous adipose tissue display multilineage developmental potential. These cells, termed 'ASCs', can be induced to differentiate into various mesenchymal lineages, including osteogenic, adipogenic, myogenic, and chondrogenic (3, 20).

ASCs express the MSC markers CD10, CD13, CD29, CD34, CD44, CD54, CD71, CD90, CD105, CD106, CD117, and STRO-1. However, the cells are negative for the hematopoietic lineage markers CD45, CD14, CD16, CD56, CD61, CD62E, CD104, and CD106, and the endothelial cell (EC) markers CD31, CD144, and von Willebrand factor. ASCs display many properties in common with MSC, such as BM-MSC (5, 21, 22). The similarities between ASCs and BM-MSC indicate that ASCs are derived from circulating BM-MSCs, which infiltrate the adipose compartment through vessel walls (23). Morphologically, the cells are fibroblast-like and preserve their shape after expansion *in vitro* (23). ASCs and BM-MSCs share broadly similar surface immunophenotypes. Both cell types are positive for CD10, CD13, CD90, and CD106 (3, 23). Stem cell-related surface markers, Sca1(ly-6A/E) and CD44 antigen, are expressed, but not c-kit, Lin, CD11b, CD31, CD34 or CD45, in ASCs

from mice, while mouse MSCs display high levels of Sca1 and CD34, but not c-kit, Lin, CD11b, CD31 or CD459 (23). The CD34 antigen is expressed in freshly isolated human ASCs, but disappears after several days in culture (3, 24, 25). Similarly, bone marrow culture over a two- to three-week time-frame to obtain BM-MSC colonies leads to loss of CD34 antigen, which is expressed in BM-MSCs *in vivo* (26). However, the cells do not express endothelial or hematopoietic cell markers. Similar to BM-MSCs, ASCs are capable of differentiating into multiple cell lineages *in vivo* and *in vitro*. A number of groups have reported the differentiation of human and murine ASCs into osteoblasts, chondroblasts and adipocytes, whereas other studies have shown differentiation into neurons, VECs, smooth muscle cells, cardiomyocytes and skeletal myocytes (5, 27-35). In 2008, a periendothelial pericyte-like subpopulation of ASCs was identified. These cells were $CD34^+$, $CD31^-$, $CD45^-$, and $CD144^-$, and expressed mesenchymal cell markers, smooth muscle antigens, and pericytic markers, including chondroitin sulfate proteoglycan, CD140a, and CD140b (36). Since CD140a and CD140b could not co-localize with CD34 and CD104b, it was concluded that $CD34^+/CD31^-$ cells of adipose vasculature are not pericytes. The differences in surface marker expression in these studies may be explained by the stage of culture growth. Similarly, the variability of CD34 positivity is possibly a consequence of downregulation of this marker within the first few days of *in vitro* expansion.

Adipose tissue participates in endocrine processes by secreting cytokines and growth factors. ASCs secrete high levels of epidermal growth factor (EGF), VEGF, bFGF, keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), HGF, transforming growth factor (TGF)-β, insulin-like growth factor (IGF), and brain-derived neurotrophic factor (BDNF) (36). In addition, these cells secrete cytokines, such as Flt-3 ligand, granulocyte colony stimulating factor, granulocyte/macrophage colony stimulating factor, macrophage colony stimulating factor, interleukin (IL)-6, IL-7, IL-8, IL-11, IL-12, leukemia inhibitory factor (LIF), and tumor necrosis factor. This paracrine secretion of adipose tissue with ASCs may contribute to the elevated cytokine levels in cases of obesity (37). Notably, angiogenic and anti-apoptotic growth factors are secreted by ASCs at a bioactive level, and hypoxic conditions promote their secretion to a significant extent. HGF is possibly the main angiogenic factor secreted by ASCs and plays a central role in paracrine effects on ASCs. Inhibition of HGF receptors has been shown to impair the angiogenic and regenerative effects of ASCs in ischemic tissues. Moreover, knockdown of HGF reduces the ability of ASCs to promote EC proliferation and inhibits the pro-angiogenic effects of HGF *in vitro* (38, 39).

Independent studies have reported data from gene microarray experiments analyzing transcriptomes of undifferentiated human ASCs and BM-MSCs (20, 40). Analysis of a 28-gene panel revealed no significant differences between the two cell types. A more comprehensive comparison using Affymetrix gene chips determined that human ASCs and MSCs share a common transcriptome (41). The transcriptomes of MSCs and ASCs derived from different donors were shown to have a ~50% correlation coefficient, compared to an average correlation coefficient of 71% and 64% between individual donors within the ASC and MSC groups, respectively (41). The data from mRNA transcription analysis suggest that ASCs and MSCs display distinct expression profiles with similar features. However, evaluation of the ASC and adipocyte proteome with mass spectrometry indicated that the identities of >200 proteins in both undifferentiated and adipose differentiated cells (42-44). The human ASC proteome shares similarities with fibroblasts, MSCs and other lineages (42-44). ASCs present a number of features that provide a key advantage for therapeutic applications, such as ease of harvest via minimally invasive liposuction and significantly greater numerical availability for harvest from adipose tissue (where culture expansion is not necessary). To date, autologous ASCs have been used in most clinical cases and human studies, which are simply derived by enzymatic and mechanical processing of adipose tissue to obtain ASC-rich cell pellets without subsequent culture. These would avoid potential harmful transformation during *ex vivo* culture, but the quantity of ASCs available from liposuction depends on donor age, Body Mass Index, and tissue harvest site (45). Limited studies have focused on the qualitative differences among the activities of ASC in relation to the donor source.

5. NEOVASCULARIZATION

The formation of new blood vessels (neovascularization) occurs through two mechanisms, specifically, angiogenesis and asculogenesis. Angiogenesis is the process of neovascularization from preexisting blood vessels, whereas vasculogenesis refers to the generation of blood vessels from angioblast precursor cells (46, 47). Neovascularization is essential in embryonic development, wound repair, ischemia, tumor and inflammation as a result of interactions between cells, cytokines, extracellular matrix and proteolytic enzymes. Under these physiological or pathological conditions, the process is highly regulated, turned on for brief periods and subsequently completely inhibited.

Postnatal neovascularization is thought to result exclusively from proliferation, migration, and remodeling of fully differentiated putative VECs derived from preexisting blood vessels. This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, a term applied to the formation of embryonic blood vessels from EC progenitors or angioblasts (48). In 1997, EC progenitors or angioblasts were isolated from human peripheral blood via magnetic bead selection on the basis of cell surface antigen expression. *In vitro*, these cells differentiated into VECs. These findings suggest that EC progenitors may be useful for augmenting collateral vessel growth to ischemic tissues (therapeutic angiogenesis) and delivering anti- or pro-angiogenic agents, respectively, to sites of pathologic or utilitarian angiogenesis (49).

However, neovascularization is an essential step in the treatment of many disorders, such as cardiovascular diseases, ischemic diseases, tissue repair and

organ

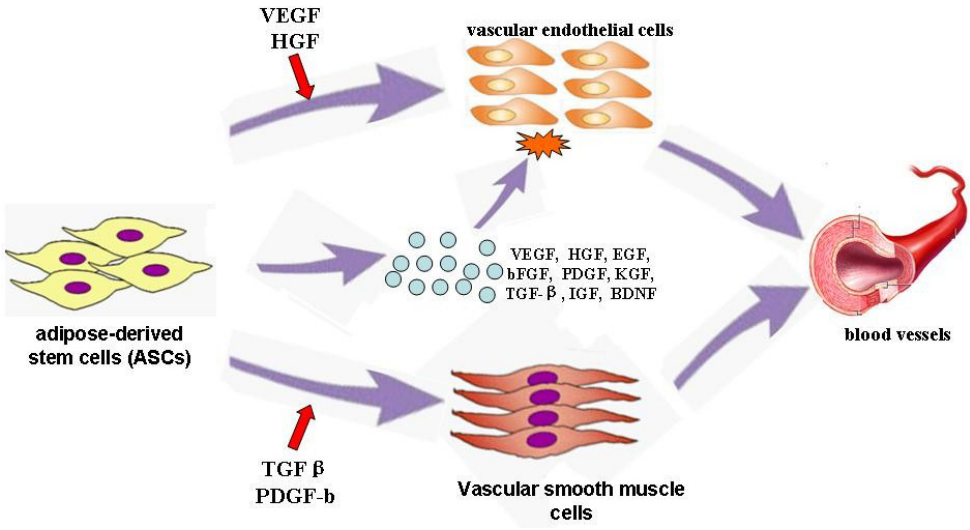

Figure 1. The mechanism of action of ASCs in regeneration of vascular vessels.

transplantation. Neovascularization using angiogenic factors as well as specific gene and cell therapy to promote vascular regeneration and improve the blood supplement may facilitate the cure of several diseases. Cell biology literature suggests that angiogenesis is regulated by angiogenic factors that stimulate vascular cell migration and proliferation, such as VEGFs (VEGF-A□VEGF-B, VEGF-C, VEGF-D) and FGFs. VEGFs are considered the most important growth factor in the process of neovascularization (50). Most VEGF-A isomers effectively stimulate angiogenesis *in vivo* (51). Recently, ASCs were shown to promote new vessels by differentiating into vascular endothelial and vascular smooth muscle cells and secreting the VEGF. In view of the abundant sources and no medical ethics issues, ASCs are expected to play an important role in neovascularization and vascular tissue engineering.

6. FUNCTION AND MECHANISM OF ACTION OF ASCS IN NEOVASCULARIZATION AND TREATMENT OF CLI

Recent studies reported *in vitro* co-culture models using human adipose stem cells and human umbilical vein VECs, where capillary-like networks sprouting from the central lumen wall are formed in perfused tubes. It has developed an *in vitro* 3D model of tissue regeneration whereby human vascularized adipose tissue, human ASCs, and human umbilical vein VECs were co-cultured on 3D aqueous silk scaffolds. After two weeks of co-culture, continuous endothelial lumens formed. Furthermore, it was reported that transplantation of fat tissue with non-cultured ASCs improves long-term graft retention in an *in vivo* murine model. Compared to transplanted fat tissue alone, adipose tissue transplanted with non-cultured ASCs had a higher density of capillaries. These successful results may be attributed to the pro-angiogenic growth factors secreted by ASCs, as described previously. The studies collectively suggest a reciprocal relationship between adipose tissue remodeling and vascular regeneration. Increasing evidence has shown that ASCs can be induced to differentiate into VECs and facilitate neovascularization (24). The molecular mechanisms of ASCs in angiogenesis are summarized below (Figure 1).

6.1. ASCs differentiate into VECs and smooth muscle cells during neovascularisation

As pluripotent stem cells, ASCs have the potential to differentiate into VECs and vascular smooth muscle cells (24). Cells derived from ASCs can be used as building blocks to generate new vascular vessels during neovascularization.

Preadipocytes within adipose tissue depots and VECs exhibit close relationships *in vivo*. Earlier, Williams *et al.* (52) successfully isolated differentiated VECs from human adipose tissue with specific endothelial markers, including CD31, CD144 (VE-cadherin) and von Willebrand factor. Seeding these VECs onto synthetic vascular grafts improved the patency of the grafts after surgery (52). Furthermore, in the presence of VEGF, bFGF and other growth factors, $CD31^-$ and $CD34^-$ cells isolated from adipose tissue showed endothelial cell phenotypes with typical phagocytosis and tube-like structure formation (4, 53). Transplantation of these cells into nude mice ischemic lesions significantly improved perfusion in damaged tissue, in which a proportion of VECs in neoformed blood vessels was obtained from ASC differentiation (4, 53). Accumulating evidence further confirmed potential differentiation into VECs. Cao *et al.* (54) showed that a population of $CD31^-CD34^-CD106^-$, fetal liver kinase-1^+ (flk-1) adipose-derived cells express endothelial markers in response to VEGF. Similarly, Martinez-Estrada and colleagues found that flk-1^+ cells isolated from cultured ASCs display endothelial phenotype in the presence of VEGF (55). In addition, *in vivo* studies extensively support the endothelial differentiation potential

of ASC cells. In a rat ischemic hindlimb model, introduction of the $CD31^-$ population improved angiogenesis and subsequent recovery of vascular supply. This finding has been confirmed by several research groups with reproduction of both intravenous delivery of cells and intramuscular injection in the direct vicinity of ischemic injury (54, 56, 57). Clearly, ASCs can differentiate into VECs and participate in the formation of vascular lumen under the appropriate induction conditions.

In vitro, human ASCs show a smooth muscle phenotype, indicated by the expression of smooth muscle markers, including α-smooth muscle actin, calponin, and SM22. A combination of TGF-β and PDGF-b induces commitment of ASCs into smooth muscle cells, as evident from expression of smooth muscle cell markers, including smooth muscle actin, troponin, and smooth muscle myosin heavy chain, suggesting that differentiation of ASCs into vascular smooth muscle facilitates neovascularization. A critical role of aortic carboxypeptidase-like protein in the mechanism of ASC smooth muscle differentiation was suggested, based on data from a study on preadipocytes from a murine model (58). In addition, the finding that human ASC expression of α-smooth muscle actin and calponin is reduced in a cyclic uniaxial strain indicates that mechanical stimuli are critical in modulating the smooth muscle phenotype (59). After injection of human ASCs into the urinary tract of immunodeficient mice *in vivo*, the cells persisted for up to 12 weeks and displayed smooth muscle cell morphology (60). Moreover, transplantation of BM-MSCs and endothelial precursor cells only formed capillary vessels, whereas transplanted ASCs formed large blood vessel-like structures (61). Technological developments facilitating control of the generation and manipulation of ASC sheets may provide an effective approach to deliver smooth muscle-differentiated ASCs for genitourinary and cardiovascular regenerative procedures.

6.2. ASCs release angiogenic cytokines to promote neovascularization

Although ASCs can integrate as fully functional and differentiated VECs *in vivo*, they may additionally contribute to neovascularization through paracrine pathways. Adipose-derived cells secrete angiogenic cytokines, such as VEGF and HGF, which may contribute to their angiogenic properties. A number of studies have shown that ASCs secrete significant quantities of angiogenic and anti-apoptotic factors, including VEGF and HGF. These findings further encouraged a series of *in vivo* studies that focused on evaluating the therapeutic potential of cells based particularly on their paracrine and angiogenic effects. Intramuscular and intravenous injections of approximately 5×10^5 ASCs have been shown to functionally regenerate the vascular circulation network in ischemic hind limbs in immunocompromised mice.

The processes of neo-vascularization and angiogenesis are regulated by a number of endothelial growth factors, including VEGF and bFGF. ASCs secrete high levels of VEGF, EGF, bFGF, PDGF, KGF, HGF, TGF-β, IGF, and BDNF through autocrine or paracrine mechanisms (62). Furthermore, pathological conditions, such as ischemia, induce the release of bFGF and VEGF from ASCs, leading to the formation of new vascular vessels. VEGF, an important angiogenic factor, promotes the proliferation and migration of VECs maintaining the integrity of vascular vessels (63). VEGF facilitates angiogenesis synergistically with angiogenin. The growth factor affects early blood vessel formation and promotes the generation of a primitive vascular network, while angiopoietin acts on the subsequent alterations of vascular remodeling and promotes the formation of mature vessels and there is spatial structure the vascular network (63). bFGF induces capillary growth and differentiation, leading to an increase in the number of local capillaries. IGF is another strong inducer of ASC differentiation into VECs (64).

6.3. ASC-endothelial crosstalk contributes to neovascularisation

Adipose tissue is not only an energy storage organ, but also an endocrine organ with the capability to secrete a variety of hormone-like factors, including leptin, adiponectin and resistin (38, 65). These factors affect endothelial function and even contribute to vascular disease. VECs also secrete growth factors to regulate the growth and differentiation of adipose precursor stromal cells, affecting adipose tissue growth (38). The collective findings indicate a reciprocal relationship between adipose tissue and VECs (66). Recent studies have shown that adipose tissue microvascular wall ASCs have similar features to pericytes, which wrap around VECs of capillaries and venules throughout the body (36). The synergistic crosstalk between ASCs and VECs promotes neovascularization and angiogenesis. These newly formed functional blood vessels rebuild a network of blood vessels within a few weeks, facilitating improvement of vascular regeneration in organ transplant and ischemic disease (67).

7. THERAPEUTIC POTENTIAL OF ASCS IN VASCULAR GROWTH AND TISSUE REPAIR

In view of accumulating evidence supporting the practical utility of ASCs, their application in ischemic diseases has been highlighted in recent clinical studies, particularly as treatment for ischemic heart disease and peripheral vascular disease (68). The number of patients with ischemic heart disease has gradually increased over the years. However, traditional treatments, such as coronary artery bypass and percutaneous transluminal coronary angioplasty, are less efficient on patients with coronary artery stenosis and insufficient blood diffusion (69, 70). Alternative therapeutic angiogenesis treatment through introduction of ASCs has been shown to be valuable for acute coronary syndrome patients. Earlier reports indicate that intramyocardial injection of ASCs significantly ameliorates left ventricular ejection fraction and fractional shortening in an animal model of acute myocardial infarction (68). According to the described histological effects, ASCs can increase tissue angiogenesis and reduce myocardial apoptosis and inflammatory responses.

Although ASCs have only been applied in animal models, clinical data support their potential in the

treatment of ischemic lesions. In a study on human peripheral vascular disease, the subjects included six cases of Buerger's disease (thromboangiitis obliterans). After administration of autologous ASCs through intramuscular injection, the most painless walking distance, toe-brachial index, ankle-brachial index, continuous laser Doppler blood flow and ulcer healing situation were recorded in these patients (69). Following 24 weeks of treatment, patients completely tolerated autologous ASCs, and resting pain, and other symptoms were significantly improved (70). Recent clinical studies have shown that intramuscular injection of ASCs on the diabetic foot and arteriosclerosis obliterans have a dramatic effect. After six months of injection of ASCs, clinical manifestations included lower resting pain and improved painless walking distance with no complications. Histological assay revealed massive neovascularization and angiogenesis in ischemic tissue, suggesting an important role of ASCs in peripheral vascular revascularization (69).

8. CHALLENGES OF FUNCTIONAL USAGE IN NEOVASCULARIZATION

While ASCs have numerous advantages, many important scientific and medical questions remain in terms of their application, including development of large-scale manufacturing methods with appropriate quality assurance, quality control to generate cells in compliance with current Good Manufacturing Practices, transformation techniques, and oncogenic risk induced by angiogenesis. Purification of ASCs is the principal problem, as there are no uniform appraisal standards. Preferably, these steps should be performed in a closed, sterile container that minimizes the risk of tissue contamination while protecting personnel from exposure to blood-borne pathogens. Cell cultures should perfomed in stirred or rotating flasks, perfused hollow fiber bioreactors or Teflon bags. It is additionally important to determine the optimal methods to store and ship ASCs, and their shelf-life. Second, the differentiation efficiency of ASCs in induction medium is not sufficiently effective for clinical usage. The identification methods for these differentiated cells are too simple to fully detect morphological or surface markers (69). Finally, human ASCs show a capability to form tumors in immunodeficient mice after prolonged culture *in vitro* (71). Therefore, long-term experiments examining the safety of ASC transplantation in appropriate animal models are warranted. Such studies should be designed to ensure that all preclinical questions are addressed before advancing to phase I studies in patients.

9. ACKNOWLEDGEMENTS

Lefeng Qu and Maoquan Li are the co-corresponding authors. This work was supported by the Program of Science and Technology Commission of Shanghai Municipality 13ZR1414500.

10. REFERENCE

1. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T and Imaizumi T: Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet* 360, 427-35 (2002)

2. Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO and Gimble JM: Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. *Tissue Eng* 7, 729-41 (2001)

3. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P and Hedrick MH: Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 13, 4279-95 (2002)

4. Nakagami H, Morishita R, Maeda K, Kikuchi Y, Ogihara T and Kaneda Y: Adipose tissue-derived stromal cells as a novel option for regenerative cell therapy. *J Atheroscler Thromb* 13, 77-81 (2006)

5. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP and Hedrick MH: Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 7, 211-28 (2001)

6. Tobita M, Orbay H and Mizuno H: Adipose-derived stem cells: current findings and future perspectives. *Discov Med* 11, 160-70.(2011)

7. Kaneko E, Izumimoto N, Toyoshima K, Abe Y and Shimokado K: Therapeutic angiogenesis for critical limb ischemia. *Nihon Ronen Igakkai Zasshi* 50, 366-8.(2013)

8. Attanasio S and Snell J: Therapeutic angiogenesis in the management of critical limb ischemia: current concepts and review. *Cardiol Rev* 17, 115-20 (2009)

9. Motukuru V, Suresh KR, Vivekanand V, Raj S and Girija KR: Therapeutic angiogenesis in Buerger's disease (thromboangiitis obliterans).patients with critical limb ischemia by autologous transplantation of bone marrow mononuclear cells. *J Vasc Surg* 48, 53S-60S; discussion 60S (2008)

10. Ouma GO, Zafrir B, Mohler ER, 3rd and Flugelman MY: Therapeutic angiogenesis in critical limb ischemia. *Angiology* 64, 466-80.(2013)

11. Tara S, Miyamoto M, Takagi G, Fukushima Y, Kirinoki-Ichikawa S, Takano H, Takagi I, Mizuno H, Yasutake M, Kumita S and Mizuno K: Prediction of limb salvage after therapeutic angiogenesis by autologous bone marrow cell implantation in patients with critical limb ischemia. *Ann Vasc Dis* 4, 24-31.(2011)

12. Tongers J, Roncalli JG and Losordo DW: Therapeutic angiogenesis for critical limb ischemia: microvascular therapies coming of age. *Circulation* 118, 9-16 (2008)

13. Marui A, Tabata Y, Kojima S, Yamamoto M, Tambara K, Nishina T, Saji Y, Inui K, Hashida T, Yokoyama S,

Onodera R, Ikeda T, Fukushima M and Komeda M: A novel approach to therapeutic angiogenesis for patients with critical limb ischemia by sustained release of basic fibroblast growth factor using biodegradable gelatin hydrogel: an initial report of the phase I-IIa study. *Circ J* 71, 1181-6 (2007)

14. Subrammaniyan R, Amalorpavanathan J, Shankar R, Rajkumar M: Application of autologous bone marrow mononuclear cells in six patients with advanced chronic critical limb ischemia as a result of diabetes: our experience. *Cytotherapy* 13, 993-9.(2011)

15. Balaz P, Rokosny S, Chlupac J, Janousek L and Adamec M: Intraarterial peroperative thrombolysis in therapy of the acute limb ischemia. *Rozhl Chir* 90, 55-8.(2011)

16. Taleb M, Burket MW and Cooper CJ: A role for embolic protection in the management of acute limb ischemia. *Tech Vasc Interv Radiol* 14, 75-9.(2011)

17. Murphy MP, Lawson JH, Rapp BM, Dalsing MC, Klein J, Wilson MG, Hutchins GD and March KL: Autologous bone marrow mononuclear cell therapy is safe and promotes amputation-free survival in patients with critical limb ischemia. *J Vasc Surg* 53, 1565-74 e1.(2011)

18. Hu Z, Zhang F, Yang Z, Yang N, Zhang D, Zhang J and Cao K: Combination of simvastatin administration and EPC transplantation enhances angiogenesis and protects against apoptosis for hindlimb ischemia. *J Biomed Sci* 15, 509-17 (2008)

19. Yagi T, Sadamori H, Nakagawa H, Ishikawa T, Sasaki H, Ohta K, Ishine N, Inagaki M and Tanaka N: Hepatropic soluble vitamin E derivative EPC-K1 prevents warm ischemia/reperfusion injury of non-heart-beating donor liver transplantation in pigs. *Transplant Proc* 29, 1390-3 (1997)

20. Katz AJ, Tholpady A, Tholpady SS, Shang H and Ogle RC: Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS).cells. *Stem Cells* 23, 412-23 (2005)

21. Gimble J and Guilak F: Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 5, 362-9 (2003)

22. Miyazaki M, Zuk PA, Zou J, Yoon SH, Wei F, Morishita Y, Sintuu C and Wang JC: Comparison of human mesenchymal stem cells derived from adipose tissue and bone marrow for *ex vivo* gene therapy in rat spinal fusion model. *Spine* (Phila Pa 1976).33, 863-9 (2008)

23. Arrigoni E, Lopa S, de Girolamo L, Stanco D and Brini AT: Isolation, characterization and osteogenic differentiation of adipose-derived stem cells: from small to large animal models. *Cell Tissue Res* 338, 401-11 (2009)

24. Elabd C, Chiellini C, Massoudi A, Cochet O, Zaragosi LE, Trojani C, Michiels JF, Weiss P, Carle G, Rochet N, Dechesne CA, Ailhaud G, Dani C and Amri EZ: Human adipose tissue-derived multipotent stem cells differentiate *in vitro* and *in vivo* into osteocyte-like cells. *Biochem Biophys Res Commun* 361, 342-8 (2007)

25. Rodriguez AM, Elabd C, Amri EZ, Ailhaud G and Dani C: The human adipose tissue is a source of multipotent stem cells. *Biochimie* 87, 125-8 (2005)

26. Simmons PJ and Torok-Storb B: CD34 expression by stromal precursors in normal human adult bone marrow. *Blood* 78, 2848-53 (1991)

27. Song YH, Gehmert S, Sadat S, Pinkernell K, Bai X, Matthias N and Alt E: VEGF is critical for spontaneous differentiation of stem cells into cardiomyocytes. *Biochem Biophys Res Commun* 354, 999-1003 (2007)

28. Planat-Benard V, Menard C, Andre M, Puceat M, Perez A, Garcia-Verdugo JM, Penicaud L and Casteilla L: Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. *Circ Res* 94, 223-9 (2004)

29. Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H and Guilak F: Chondrogenic potential of adipose tissue-derived stromal cells *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 290, 763-9 (2002)

30. Estes BT, Wu AW and Guilak F: Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. *Arthritis Rheum* 54, 1222-32 (2006)

31. Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P and Hedrick MH: Myogenic differentiation by human processed lipoaspirate cells. *Plast Reconstr Surg* 109, 199-209; discussion 210-1 (2002)

32. Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM and Rice HE: Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun* 294, 371-9 (2002)

33. Safford KM, Safford SD, Gimble JM, Shetty AK and Rice HE: Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells. *Exp Neurol* 187, 319-28 (2004)

34. Ashjian PH, Elbarbary AS, Edmonds B, DeUgarte D, Zhu M, Zuk PA, Lorenz HP, Benhaim P and Hedrick MH: *In vitro* differentiation of human processed lipoaspirate cells into early neural progenitors. *Plast Reconstr Surg* 111, 1922-31 (2003)

35. Hicok KC, Du Laney TV, Zhou YS, Halvorsen YD, Hitt DC, Cooper LF and Gimble JM: Human adipose-derived adult stem cells produce osteoid *in vivo*. *Tissue Eng* 10, 371-80 (2004)

36. Traktuev DO, Merfeld-Clauss S, Li J, Kolonin M, Arap W, Pasqualini R, Johnstone BH and March KL: A

population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. *Circ Res* 102, 77-85 (2008)

37. Folkman J: Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285, 1182-6 (1971)

38. Kougias P, Chai H, Lin PH, Yao Q, Lumsden AB and Chen C: Effects of adipocyte-derived cytokines on endothelial functions: implication of vascular disease. *J Surg Res* 126, 121-9 (2005)

39. Kang S, Bajnok L, Longo KA, Petersen RK, Hansen JB, Kristiansen K and MacDougald OA: Effects of Wnt signaling on brown adipocyte differentiation and metabolism mediated by PGC-1alpha. *Mol Cell Biol* 25, 1272-82 (2005)

40. Winter A, Breit S, Parsch D, Benz K, Steck E, Hauner H, Weber RM, Ewerbeck V and Richter W: Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells. *Arthritis Rheum* 48, 418-29 (2003)

41. Fraser JK, Wulur I, Alfonso Z and Hedrick MH: Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol* 24, 150-4 (2006)

42. Gronborg M, Kristiansen TZ, Stensballe A, Andersen JS, Ohara O, Mann M, Jensen ON and Pandey A: A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phospho-specific antibodies: identification of a novel protein, Frigg, as a protein kinase A substrate. *Mol Cell Proteomics* 1, 517-27 (2002)

43. Guo L, Eisenman JR, Mahimkar RM, Peschon JJ, Paxton RJ, Black RA and Johnson RS: A proteomic approach for the identification of cell-surface proteins shed by metalloproteases. *Mol Cell Proteomics* 1, 30-6 (2002)

44. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M and Pandey A: A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics* 1, 213-22 (2002)

45. Strem BM, Hicok KC, Zhu M, Wulur I, Alfonso Z, Schreiber RE, Fraser JK and Hedrick MH: Multipotential differentiation of adipose tissue-derived stem cells. *Keio J Med* 54, 132-41 (2005)

46. Tepper OM, Galiano RD, Kalka C and Gurtner GC: Endothelial progenitor cells: the promise of vascular stem cells for plastic surgery. *Plast Reconstr Surg* 111, 846-54 (2003)

47. Bdolah Y, Sukhatme VP and Karumanchi SA: Angiogenic imbalance in the pathophysiology of preeclampsia: newer insights. *Semin Nephrol* 24, 548-56 (2004)

48. Plate KH, Breier G, Weich HA and Risau W: Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature* 359, 845-8 (1992)

49. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G and Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964-7 (1997)

50. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M and Isner JM: VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 18, 3964-72 (1999)

51. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z and Ferrara N: VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 5, 623-8 (1999)

52. Williams SK, Wang TF, Castrillo R and Jarrell BE: Liposuction-derived human fat used for vascular graft sodding contains endothelial cells and not mesothelial cells as the major cell type. *J Vasc Surg* 19, 916-23 (1994)

53. Schaffler A and Buchler C: Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. *Stem Cells* 25, 818-27 (2007)

54. Cao Y, Sun Z, Liao L, Meng Y, Han Q and Zhao RC: Human adipose tissue-derived stem cells differentiate into endothelial cells *in vitro* and improve postnatal neovascularization *in vivo*. *Biochem Biophys Res Commun* 332, 370-9 (2005)

55. Martinez-Estrada OM, Munoz-Santos Y, Julve J, Reina M and Vilaro S: Human adipose tissue as a source of Flk-1+ cells: new method of differentiation and expansion. *Cardiovasc Res* 65, 328-33 (2005)

56. Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R and Bouloumie A: Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* 110, 349-55 (2004)

57. Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L and Casteilla L: Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation* 109, 656-63 (2004)

58. Abderrahim-Ferkoune A, Bezy O, Astri-Roques S, Elabd C, Ailhaud G and Amri EZ: Transdifferentiation of preadipose cells into smooth muscle-like cells: role of

aortic carboxypeptidase-like protein. *Exp Cell Res* 293, 219-28 (2004)

59. Lee WC, Maul TM, Vorp DA, Rubin JP and Marra KG: Effects of uniaxial cyclic strain on adipose-derived stem cell morphology, proliferation, and differentiation. *Biomech Model Mechanobiol* 6, 265-73 (2007)

60. Jack GS, Almeida FG, Zhang R, Alfonso ZC, Zuk PA and Rodriguez LV: Processed lipoaspirate cells for tissue engineering of the lower urinary tract: implications for the treatment of stress urinary incontinence and bladder reconstruction. *J Urol* 174, 2041-5 (2005)

61. Zhao J, Qu XF, Zhao CY, Cao FL, Zhou T, Li WM and Huang YL: Effects of metoprolol treatment on a disintegrin metalloproteinase expression and extracellular matrix remodeling after myocardial infarction in rats. *Chin Med J (Engl)*.120, 1549-52 (2007)

62. Cousin B, Caspar-Bauguil S, Planat-Benard V, Laharrague P, Penicaud L and Casteilla L: Adipose tissue: a subtle and complex cell system. *J Soc Biol* 200, 51-7 (2006)

63. Tolosa L, Mir M, Olmos G and Llado J: Vascular endothelial growth factor protects motoneurons from serum deprivation-induced cell death through phosphatidylinositol 3-kinase-mediated p38 mitogen-activated protein kinase inhibition. *Neuroscience* 158, 1348-55 (2009)

64. Amos PJ, Shang H, Bailey AM, Taylor A, Katz AJ and Peirce SM: IFATS collection: The role of human adipose-derived stromal cells in inflammatory microvascular remodeling and evidence of a perivascular phenotype. *Stem Cells* 26, 2682-90 (2008)

65. Sattar N and Nelson SM: Adiponectin, diabetes, and coronary heart disease in older persons: unraveling the paradox. *J Clin Endocrinol Metab* 93, 3299-301 (2008)

66. Frye CA, Wu X and Patrick CW: Microvascular endothelial cells sustain preadipocyte viability under hypoxic conditions. *In vitro Cell Dev Biol Anim* 41, 160-4 (2005)

67. Traktuev DO, Prater DN, Merfeld-Clauss S, Sanjeevaiah AR, Saadatzadeh MR, Murphy M, Johnstone BH, Ingram DA and March KL: Robust functional vascular network formation *in vivo* by cooperation of adipose progenitor and endothelial cells. *Circ Res* 104, 1410-20 (2009)

68. Cai L, Johnstone BH, Cook TG, Tan J, Fishbein MC, Chen PS and March KL: IFATS collection: Human adipose tissue-derived stem cells induce angiogenesis and nerve sprouting following myocardial infarction, in conjunction with potent preservation of cardiac function. *Stem Cells* 27, 230-7 (2009)

69. Hong SJ, Traktuev DO and March KL: Therapeutic potential of adipose-derived stem cells in vascular growth and tissue repair. *Curr Opin Organ Transplant* 15, 86-91.(2010)

70. Moon MH, Kim SY, Kim YJ, Kim SJ, Lee JB, Bae YC, Sung SM and Jung JS: Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. *Cell Physiol Biochem* 17, 279-90 (2006)

71. Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC and Bernad A: Spontaneous human adult stem cell transformation. *Cancer Res* 65, 3035-9 (2005)

Abbreviations: ASCs, adipose-derived stem cells; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BM, bone marrow; BM-MNC, bone marrow mononuclear cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; CLI, critical limb ischemia; ECs, endothelial cells; EGF, epidermal growth factor; EPCs, endothelial progenitor cells; flk-1, fetal liver kinase-1; HGF, hepatocyte growth factor; HSCs, hematopoietic stem cells; IGF, insulin-like growth factor; IL, interleukin; KGF, keratinocyte growth factor; LIF, leukemia inhibitory factor; MNC, mononuclear cells; MSCs, mesenchymal stem cells; PAD, peripheral arterial disease; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VECs, vascular endothelial cells; VEGF, vascular endothelial growth factor

Key Words: Adipose-Derived Stem Cells, Neovascularization, Therapeutic Vasculogenesis, Therapeutic Angiogenesis, Critical Limb Ischemia, Review

Send correspondence to: Lefeng Qu, Department of Vascular and Endovascular Surgery Changzheng Hospital 415 Fengyang Road, Shanghai, China, Tel: 86-13816263943, Fax: 86-21-81886531, E-mail: qulefeng@yeah.net