Published in final edited form as:

Ann Plast Surg. 2014 February ; 72(2): 234–241. doi:10.1097/SAP.0b013e318264fd6a.

Paracrine Mechanism of Angiogenesis in Adipose-derived Stem Cell Transplantation

Hirota Suga, M.D., Jason P. Glotzbach, M.D., Michael Sorkin, M.D., Michael T. Longaker, M.D., M.B.A., and Geoffrey C. Gurtner, M.D.

Division of Plastic and Reconstructive Surgery, Department of Surgery, Stanford University, 257 Campus Drive West, Stanford, CA 94305

Abstract

Introduction—Adipose-derived stem cells (ASCs) have shown potential for cell-based therapy in the field of plastic surgery. However, the fate of ASCs after transplantation and the mechanism(s) of their biologic capabilities remain unclear.

Methods—We isolated and cultured ASCs from transgenic mice that express both luciferase and green fluorescent protein (GFP) and injected the cells into the inguinal fat pads of wild-type mice. We tested four experimental groups: ischemic fat pads with/without ASCs and control fat pads with/without ASCs.

Results—Transplanted ASCs were tracked with bioluminescence imaging. The luminescence gradually decreased over 28 days, indicating cell death after transplantation. More ASCs were retained in ischemic fat pads on day 7 compared to control fat pads. On day 14, adipose tissue vascular density was higher in the ASC transplantation groups compared to those without ASCs. On day 28, there was decreased atrophy of adipose tissue in ASC-treated ischemic fat pads. Transplanted ASCs were detected as non-proliferating GFP+ cells, whereas native endothelial cells adjacent to the transplanted ASCs were proliferative. Protein analysis demonstrated higher expression of hepatocyte growth factor and vascular endothelial growth factor in the ASC transplantation groups, suggesting a paracrine mechanism, which was confirmed by *in vitro* experiments with conditioned media from ASCs.

Conclusions—Transplanted ASCs are preferentially retained in ischemic adipose tissue, although most of the cells eventually undergo cell death. They exert an angiogenic effect on adipose tissue mainly through a paracrine mechanism. Increased understanding of these effects will help develop ASCs as a tool for cell-based therapy.

Keywords

adipose-derived stem cells; paracrine; angiogenesis; hepatocyte growth factor; vascular endothelial growth factor

INTRODUCTION

Adipose-derived stem cells (ASCs) are multipotent cells that can be readily isolated and cultured in large numbers from subcutaneous adipose tissue using the minimally invasive technique of liposuction.¹ They have shown potential for cytotrophy in a wide range of

Correspondence: Geoffrey C. Gurtner, M.D. Division of Plastic and Reconstructive Surgery, Department of Surgery, Stanford University, 257 Campus Drive West, Stanford, CA 94305, TEL: 650-724-6672, FAX: 650-724-9501, ggurtner@stanford.edu.

Conflict of Interest

The authors have no conflicts of interest to disclose.

medical conditions.² Several preclinical studies have described the application of ASCs to increase survival of fat grafts or soft tissue flaps.^{3–5} Furthermore, clinical use of ASCs to improve the success of fat grafting has been reported.⁶ Despite this early experience, the fate of ASCs after transplantation and the mechanisms of their effects remain unclear.

In vivo bioluminescence imaging of luciferase activity has been widely used in animal models to track implanted cells.^{7, 8} This technique has an advantage of monitoring cell survival within a living animal, and can be used to study spatiotemporal kinetics of transplanted cells. There is no need to sacrifice animals at each time point, which is required in conventional methods using fluorescent dye. However, application of this method to ASCs transplanted into adipose tissue has not been reported.

Although ASC transplantation is thought to have an angiogenic effect, underlying mechanisms are still controversial. Some groups have reported that ASCs can differentiate into endothelial cells.^{9, 10} On the other hand, there is evidence that ASCs secrete several angiogenic growth factors such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF),^{11, 12} suggesting that ASCs may exert angiogenic effects via paracrine mechanisms rather than by transdifferentiating into endothelial cells.^{13, 14} In this study, we isolated and cultured ASCs from transgenic mice that express both luciferase and green fluorescent protein (GFP), and tracked cell survival and tissue vascularization after transplantation into adipose tissue of wild-type mice. We found that paracrine effects predominate as a potential mechanism underlying ASC-mediated angiogenesis.

MATERIALS AND METHODS

Isolation of ASCs from Transgenic Mice

ASCs were harvested from transgenic mice (L2G85), which express both luciferase and GFP in all tissues.⁷ The adipose tissue was minced into 3-mm pieces, washed with phosphate buffered saline (PBS) and digested on a shaker at 37°C in PBS containing 0.1% collagenase A (Roche Diagnostics, Indianapolis, IN) for 60 minutes. Mature adipocytes and connective tissue were separated from pellets by centrifugation (400 × g, 5 minutes). The cell pellets were resuspended, filtered through a 100-μm cell strainer, and cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Primary cells were cultured for 5–7 days and were defined as "Passage 0". Surface marker expression specific for ASCs, including CD29, CD90, and CD105, was confirmed as previously described.¹⁵ ASCs at Passage 1 to 3 were used in the experiments.

Animal Experiments

Animal care was provided in accordance with institutional guidelines at the Stanford University School of Medicine. We utilized a severe ischemia model of adipose tissue as previously described.¹⁶ In brief, the inguinal fat pads of 6-week-old wild-type mice were surgically exposed along with the blood supply arising from the femoral vessels. The femoral artery and all communicating vessels between the skin and the fat pad were obliterated with electrocautery, permitting only reverse perfusion from the distal portion of the femoral artery. The fat pad with a sham operation (dissection without obliteration of the blood vessels) served as control. After preparation of the inguinal fat pad, 1×10^6 ASCs isolated from transgenic mice were suspended in 100 μl PBS and injected into the fat pad under direct visualization. The cells were evenly injected at 3 points in the fat pad. PBS alone was injected into the fat pads of the non-transplantation group. ASC transplantation was performed only once on the day of surgery. We tested four experimental groups: ischemic adipose tissue with/without ASCs and control (nonischemic) adipose tissue with/without ASCs (Fig. 1A).

Bioluminescence Imaging

Activity of luciferase was imaged using In Vivo Imaging System (IVIS Spectrum) (Xenogen, Alameda, CA). Quantification of the bioluminescence from a fixed region of interest was performed using Living Image 3.2 software (Xenogen). Mice were imaged on postoperative day 1, 3, 7, 14, and 28 ($n = 7$).

Immunohistochemistry

Harvested adipose tissue was zinc-fixed (Zinc Fixative; BD Biosciences, San Diego, CA) and paraffin-embedded. We performed immunostaining using the following primary antibodies: goat anti-GFP (Abcam, Cambridge, MA), rabbit anti-Ki67 (Abcam), and rabbit anti-perilipin A (Abcam) antibodies. For double fluorescence staining, Alexa Fluor 488 or 594-conjugated secondary antibodies appropriate for each primary antibody (Molecular Probes, Eugene, OR) were used. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Blood vessels were stained with Alexa Fluor 488-conjugated isolectin GS-IB₄ (lectin; Molecular Probes). To detect apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed using an *In Situ* Cell Death Detection Kit (Roche Diagnostics).

Whole-Mount Staining

Whole-mount staining was performed to detect GFP-positive cells and to distinguish dead cells from living cells in fresh adipose tissue. The fresh sample was minced into 3-mm pieces and incubated with Hoechst 33342 (Molecular Probes) and propidium iodide (PI; Molecular Probes) for 30 minutes. The sample was then washed and imaged with a TCS SP2 confocal microscope system (Leica, Heerbrugg, Switzerland).

Western Blotting

Adipose tissue specimens were homogenized in 1 ml of RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO) and centrifuged. The aqueous layer was collected and protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Equal amounts of protein (20 μ g) were loaded into each lane of a gel. The separated proteins were transferred to a nitrocellulose membrane, and immunostaining was performed using rabbit anti-HGF (Abcam), rabbit anti-VEGF (Abcam), and rabbit anti-beta-actin (Abcam) antibodies. Beta-actin signal served as internal control. Band densities were quantified with ImageJ software (National Institutes of Health, Bethesda, MD) ($n = 7$).

Harvest of Conditioned Media

ASCs were grown in a 10-cm dish with DMEM containing 10% FBS. At confluence, the media were switched to 10 ml of DMEM containing 2% FBS, and ASCs were placed in either normoxic (20% O₂) or hypoxic (1% O₂) conditions. Seventy-two hours later, the conditioned media were collected.

Proliferation Assay

Mouse bEND.3 endothelial cells (ATCC, Manassas, VA) were grown with DMEM containing 10% FBS. They were plated in a 12-well plate at 1×10^4 cells per well. Six hours later, the media were changed to DMEM containing 2% FBS (control) or conditioned media prepared as above. After 4 days, the cells were detached and counted ($n = 9$).

Network Formation Assay

Matrigel (BD Biosciences, San Jose, CA) was placed in a 4-well chamber slide and incubated at 37°C for 30 minutes. 3×10^4 mouse bEND.3 endothelial cells were suspended

in DMEM containing 2% FBS (control) or conditioned media, and plated on the prepared Matrigel. Six hours later, capillary-like network formation was evaluated under a light microscope ($\times 100$), and total network length per field was calculated. With Photoshop CS3 software (Adobe Systems, San Jose, CA), the network was traced and the length (pixel) of the trace was compared to that of the scale ($n = 4$).

Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean. Comparisons between two groups were performed with Welch's t-test. Comparisons of multiple groups were done by analysis of variance with Bonferroni's multiple t-test. A value of * $p < 0.05$ was considered significant.

RESULTS

Characterization of ASCs from Transgenic Mice

ASCs from the transgenic mice, which express both luciferase and GFP in all tissues, were successfully harvested and maintained in vitro as previously described.¹ Surface marker expression specific for ASCs, including CD29, CD90, and CD105, was confirmed (data not shown). Bioluminescence imaging showed a linear relationship between cell number and luminescence ($R^2 > 0.99$), indicating that the imaging method can be used to trace and quantify transplanted ASCs (Fig. 1B). Fluorescence microscopy demonstrated that transgenic ASCs expressed GFP uniformly within the cytosol (Fig. 1C).

Fate of Transplanted ASCs

Bioluminescent signal from the transplanted ASCs gradually decreased during the first 14 days after transplantation both in control and ischemic adipose tissue. Interestingly, more ASCs were retained in ischemic adipose tissue on day 7 compared to control adipose tissue. However, the luminescence had faded to background levels by day 28, indicating cell death and minimal long-term engraftment following transplantation (Fig. 2A). We observed similarly low ASC survival when we transplanted ASCs to immunocompromised nude mice (data not shown). Immunohistochemistry identified GFP+ cells within the samples out to day 14 both in control and ischemic adipose tissue. Double immunostaining for GFP and the adipocyte marker perilipin demonstrated that transplanted cells were located in the interstitial spaces between adipocytes, and that some of them formed clusters (Fig. 2B). However, there were almost no GFP-positive cells observed in the samples on day 28 both in control and ischemic adipose tissue.

Effects of ASC Transplantation

Although most of the transplanted ASCs disappeared as shown above, we observed higher adipose tissue vascularity at day 14 in the ASC transplantation groups compared to those without ASCs (Fig. 3A). Quantification of vessels using lectin staining confirmed that the difference in vascular density was statistically significant (Fig. 3B). Furthermore, the weight of adipose tissue on day 28 showed that ASC transplantation mitigated ischemia-induced atrophy of the ischemic adipose tissue (Fig. 3C). These data suggest that ASC transplantation has an angiogenic effect and helps to prevent atrophy in ischemic adipose tissue even although the transplanted cells do not persist within the ischemic tissue.

Cellular Events after Transplantation

To characterize the dynamics of ASC transplantation at the cellular level, we performed immunohistochemistry using the samples on day 7. ASC transplantation groups demonstrated a higher rate of TUNEL-positive cells, suggesting apoptosis of transplanted

ASCs (Fig. 4A). Cell death of the transplanted ASCs was confirmed by whole-mount staining of fresh adipose tissue. GFP-positive transplanted cells were detected and some of them were positive for propidium iodide, which is a marker for cell death (Fig. 4B). Double immunostaining for Ki67 and GFP showed that most GFP-positive cells were not proliferating, but some cells around the GFP-positive cells were positive for Ki67 (Fig. 4C). Combination of Ki67 and lectin staining demonstrated that some endothelial cells were proliferating after ASC transplantation (Fig. 4D). These data suggest that although transplanted ASCs die, the adjacent endothelial cells proliferate, which leads to increased vascularity in the ischemic adipose tissue.

Paracrine Mechanism of ASC-Mediated Angiogenesis

Since these *in vivo* data suggested a paracrine mechanism of ASC-mediated angiogenesis, we performed a protein analysis of adipose tissue lysates on day 7 using western blotting for HGF and VEGF, both of which have been shown to be major angiogenic growth factors that are secreted by ASCs. We observed significantly higher levels of HGF and VEGF protein in the groups with ASC transplantation (Fig. 5). Next, we performed *in vitro* studies using conditioned media from ASCs. The conditioned media increased proliferation of endothelial cells, especially when harvested after treatment of the ASCs with hypoxia (Fig. 6A). Furthermore, the conditioned media stimulated endothelial cells to form a capillary-like network (Fig. 6B). Neutralizing antibodies against HGF or VEGF inhibited the effects of the conditioned media both in proliferation and network formation of endothelial cells. These data indicate that ASCs can induce angiogenesis by activating endothelial cells through secreted humoral factors.

DISCUSSION

The cultured transgenic ASCs used in these studies were successfully traced by both bioluminescence imaging and GFP immunohistochemistry. To our knowledge, this is the first report of this approach used to study the fate of ASCs transplanted into adipose tissue. One of the interesting findings in this study is an unexpectedly low survival rate of transplanted ASCs. By day 28, almost all cells had undergone cell death. This result parallels the reported low survival of ASCs when transplanted into other tissues such as peripheral nerve¹⁷ or heart.¹⁸ Low survival after transplantation is a well-known impediment to successful implementation of cytotераpy. For example, bone marrow-derived mesenchymal stem cells (BMSCs) have also been reported to have minimal survival after transplantation.^{8, 18, 19} An immunological response could have played a role in the low survival we observed, primarily because transplantation in our study was not autologous. However, we also observed similarly low ASC survival rates when we transplanted ASCs to immunocompromised nude mice. Other researchers have reported that both syngeneic and allogeneic grafts of BMSCs reduced significantly in size after transplantation.⁸ For better engraftment of transplanted cells, improved tissue engineering methodologies, such as scaffolds for delivery and maintenance, should be the focus of future research.

Previous studies have shown that ASCs can survive and be activated under hypoxia.^{15,16} In the current study, we found that more ASCs were retained in ischemic adipose tissue on day 7 compared to control adipose tissue. Although these differences in cell survival did not persist past day 7, ASC transplantation showed an angiogenic effect in the ischemic adipose tissue, which led to decreased atrophy of the tissue. Furthermore, the higher protein levels of HGF and VEGF in the ASC transplantation groups as well as the results of *in vitro* experiments with conditioned media from ASCs supported a paracrine mechanism for the angiogenic effect. Previous studies have shown that ASCs activate not only endothelial cells but also cardiac progenitor cells²⁰ and dermal fibroblasts²¹ via paracrine mechanisms,

especially under hypoxia. Recent findings in BMSCs suggest that their effects on tissue repair are also mediated through a paracrine mechanism rather than through transdifferentiation.^{22–24}

From these observations, we believe that after transplantation, cultured ASCs perform a temporary proangiogenic function in ischemic adipose tissue primarily via paracrine mechanisms, although the possibility of ASC transdifferentiation into endothelial cells cannot be excluded completely based on our results. This paracrine mechanism of angiogenesis in ASC transplantation can be applied to autologous fat grafting. Fat graft includes not only adipocytes but also ASCs, and the grafted ASCs secrete angiogenic factors. Furthermore, we believe that ASCs are not the only cells that are able to stimulate angiogenesis. Some other cells like BMSCs, which secrete angiogenic factors,^{22–24} can have angiogenic effects in the same way as ASCs in cytotherapy. Among the potential angiogenic factors that ASCs might secrete in the setting of hypoxic tissue transplantation, only HGF and VEGF were examined in this study. Further studies will be necessary to define other important factors. Other candidates include stromal cell-derived factor 1, which has been recently shown to play a crucial role in ASC-mediated angiogenesis by promoting recruitment of endothelial progenitor cells to areas of ischemia.^{25, 26} It is important to emphasize that our findings reflect the biology of cultured cells and may not hold true for primary cells.

In conclusion, cultured ASCs are preferentially retained in ischemic adipose tissue acutely following transplantation, although most of the cells eventually undergo cell death. Despite this limited *in vivo* survival, ASCs exert an angiogenic effect on ischemic tissue, mainly through paracrine mechanisms. These properties suggest that ASCs have significant potential for use in cytotherapy to treat ischemic conditions.

Acknowledgments

Sources of Funding:

This study was supported by the U.S. National Institutes of Health grant RO1 DK074095-07 to G.C.G. H.S. was supported by the Naito Foundation and J.P.G. was supported by the U.S. National Institute of Health NRSA grant F32DK088448-01. We acknowledge the supports of the Oak Foundation and the Hagey Laboratory for Pediatric Regenerative Medicine to G.C.G. and M.T.L.

The authors would like to thank Ms. Yujin Park for her technical assistance.

References

1. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng.* 2001; 7:211–228. [PubMed: 11304456]
2. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res.* 2007; 100:1249–1260. [PubMed: 17495232]
3. Matsumoto D, Sato K, Gonda K, et al. Cell-assisted lipotransfer: Supportive use of human adipose-derived cells for soft tissue augmentation with lipoinjection. *Tissue Eng.* 2006; 12:3375–3382. [PubMed: 17518674]
4. Zu M, Zhou Z, Chen Y, et al. Supplementation of fat grafts with adipose-derived regenerative cells improves long-term graft retention. *Ann Plast Surg.* 2010; 64:222–228. [PubMed: 20098110]
5. Uysal AC, Mizuno H, Tobita M, et al. The effect of adipose-derived stem cells on ischemia-reperfusion injury: Immunohistochemical and ultrastructural evaluation. *Plast Reconstr Surg.* 2009; 124:804–815. [PubMed: 19730299]
6. Yoshimura K, Sato K, Aoi N, Kurita M, Hirohi T, Harii K. Cell-assisted lipotransfer for cosmetic breast augmentation: Supportive use of adipose-derived stem/stromal cells. *Aesthetic Plast Surg.* 2008; 32:48–55. [PubMed: 17763894]

7. Sheikh AY, Lin SA, Cao F, et al. Molecular imaging of bone marrow mononuclear cell homing and engraftment in ischemia myocardium. *Stem Cells*. 2007; 25:2677–2684. [PubMed: 17628019]
8. Westrich J, Yaeger P, He C, et al. Factors affecting residence time of mesenchymal stromal cells (MSC) injected into the myocardium. *Cell Transplant*. 2010; 19:937–948. [PubMed: 20350355]
9. Planat-Benard V, Silvestre JS, Cousin B, et al. Plasticity of human adipose lineage cells toward endothelial cells: Physiological and therapeutic perspectives. *Circulation*. 2004; 109:656–663. [PubMed: 14734516]
10. Cao Y, Sun Z, Liao L, et al. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. *Biochem Biophys Res Commun*. 2005; 332:370–379. [PubMed: 15896706]
11. Rehman J, Traktuev D, Li J, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation*. 2004; 109:1292–1298. [PubMed: 14993122]
12. Kilroy GE, Foster SJ, Wu X, et al. Cytokine profile of human adipose-derived stem cell; Expression of angiogenic, hematopoietic, and pro-inflammatory factors. *J Cell Physiol*. 2007; 212:702–709. [PubMed: 17477371]
13. Nakagami H, Maeda K, Morishita R, et al. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler Thromb Vasc Biol*. 2005; 25:2542–2547. [PubMed: 16224047]
14. Takahashi M, Suzuki E, Oba S, et al. Adipose tissue-derived stem cells inhibit neointimal formation in a paracrine fashion in rat femoral artery. *Am J Physiol Heart Circ Physiol*. 2010; 298:H415–H423. [PubMed: 19940081]
15. Thangarajah H, Vial IN, Chang E, et al. IFATS collection: Adipose stromal cells adopt a proangiogenic phenotype under the influence of hypoxia. *Stem Cells*. 2009; 27:266–274. [PubMed: 18974212]
16. Suga H, Eto H, Aoi N, et al. Adipose tissue remodeling under ischemia: Death of adipocytes and activation of stem/progenitor cells. *Plast Reconstr Surg*. 2010; 126:1911–1923. [PubMed: 21124131]
17. Erba P, Mantovani C, Kalbermatten DF, et al. Regeneration potential and survival of transplanted undifferentiated adipose tissue-derived stem cells in peripheral nerve conduits. *J Plast Reconstr Aesthet Surg*. 2010; 63:e811–e817. [PubMed: 20851070]
18. van der Bogot KE, Schrepfer S, Yu J, et al. Comparison of transplantation of adipose tissue- and bone marrow-derived mesenchymal stem cells in the infarcted heart. *Transplantation*. 2009; 87:642–652. [PubMed: 19295307]
19. Zimmermann CE, Gierloff M, Hedderich J, et al. Survival of transplanted rat bone marrow-derived osteogenic stem cells in vivo. *Tissue Eng Part A*. 2011; 17:1147–1156. [PubMed: 21142699]
20. Rubina K, Kalinina N, Efimenko A, et al. Adipose stromal cells stimulate angiogenesis via promoting progenitor cell differentiation, secretion of angiogenic factors, and enhancing vessel maturation. *Tissue Eng Part A*. 2009; 15:2039–2050. [PubMed: 19368510]
21. Lee EY, Xia Y, Kim WS, et al. Hypoxia-enhanced wound-healing function of adipose-derived stem cells: Increase in stem cell proliferation and up-regulation of VEGF and bFGF. *Wound Repair Regen*. 2009; 17:540–547. [PubMed: 19614919]
22. Phinney DG, Prockop DJ. Concise review: Mesenchymal stem/multipotent stromal cells: The state of transdifferentiation and modes of tissue repair—Current view. *Stem Cells*. 2007; 25:2896–2902. [PubMed: 17901396]
23. Psaltis PJ, Zannettino ACW, Worthley SG, et al. Concise review: Mesenchymal Stromal Cells: Potential for cardiovascular repair. *Stem Cells*. 2008; 26:2201–2210. [PubMed: 18599808]
24. Rustad KC, Wong VW, Sorkin M, et al. Enhancement of mesenchymal stem cell angiogenic capacity and stemness by a biomimetic hydrogel scaffold. *Biomaterials*. 2012; 33:80–90. [PubMed: 21963148]
25. Kondo K, Shintani S, Shibata R, et al. Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis. *Arterioscler Thromb Vasc Biol*. 2009; 29:61–66. [PubMed: 18974384]

26. Li M, Horii M, Yokoyama A, et al. Synergistic effect of adipose-derived stem cell therapy and bone marrow progenitor recruitment in ischemic heart. *Lab Invest.* 2011; 91:539–552. [PubMed: 21135814]

FIGURE 1.

Study design and ASCs from transgenic mice. (A) A schematic diagram of study design. ASCs were isolated from transgenic mice, expanded in culture, and transplanted into inguinal fat pads of wild-type mice. We made four experimental groups: ischemic fat pads with/without ASCs and control fat pads with/without ASCs. (B) Bioluminescence of ASCs isolated from transgenic mice. A linear relationship between cell number and luminescence was observed. (C) Fluorescence microscopy of ASCs isolated from transgenic mice. Most of the cells were positive for GFP. Scale bar = 200 μ m.

FIGURE 2.

Fate of transplanted ASCs. (A) Bioluminescence of transplanted ASCs. The signal decreased after day 3 and reached a background level until day 28 both in control and ischemic fat pads. On day 7, ischemic fat pads showed a significantly higher intensity than control. * $p < 0.05$ ($n = 7$). (B) Immunohistochemistry for GFP and perilipin on day 14. GFP-positive transplanted cells (green) were located in the interstitial spaces between perilipin-positive adipocytes (red), and some of them formed clusters. Nuclei were stained with DAPI (blue). Scale bars = 200 μm (left) and 50 μm (right).

FIGURE 3.

Effects of ASC transplantation. (A) Hematoxylin and eosin staining on day 14. ASC transplantation groups showed a higher vascularity. Scale bars = 50 μ m. (B) Vascular density on day 14. Blood vessels were stained with lectin and quantified. The vascular density was significantly higher in ASC transplantation groups. * $p < 0.05$ ($n = 5$). (C) Weight of fat pads on day 28. Ischemia lead to atrophy of adipose tissue, and the tissue showed significantly lower weight. ASC transplantation prevented the atrophy. * $p < 0.05$ ($n = 7$).

FIGURE 4.

Cellular dynamics after transplantation. (A) Apoptotic cells on day 7. TUNEL-positive apoptotic cells were more frequently observed in ASC transplantation groups. * $p < 0.05$ ($n = 3$). (B) Whole-mount staining of fresh adipose tissue on day 7. Some GFP-positive transplanted cells (green) were also positive for propidium iodide (PI, red), indicating cell death. White arrows show the double-positive cells. Scale bar = 50 μm . (C) Immunohistochemistry for Ki67 and GFP on day 7. Most of the GFP-positive transplanted cells (green) were not positive for Ki67 (red). There were some Ki67-positive proliferating cells around the GFP-positive cells. Scale bar = 25 μm . (D) Immunohistochemistry for Ki67 and lectin on day 7. Some lectin-positive endothelial cells (green) were positive for Ki67 (red), indicating proliferation. Scale bar = 25 μm .

FIGURE 5.

Western blotting for HGF and VEGF on day 7. Both HGF and VEGF showed significantly higher expression in ASC transplantation groups. * $p < 0.05$ ($n = 7$).

FIGURE 6.

Effects of conditioned media (CM) from ASCs on bEND.3 endothelial cells. (A) The number of endothelial cells was significantly higher with the CM, especially the one harvested under hypoxia, compared to control. Neutralizing antibodies against HGF or VEGF inhibited the effect. * $p < 0.05$ ($n = 9$). (B) The CM, hypoxic one in particular, significantly increased the total length of network that endothelial cells created. Neutralizing antibodies against HGF or VEGF inhibited the effect. * $p < 0.05$ ($n = 4$).