Promotion of Primary Murine Breast Cancer Growth and Metastasis by Adipose-Derived Stem Cells Is Reduced in the Presence of Autologous Fat Graft

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Background: Cell-assisted lipotransfer involves enrichment of autologous fat with supraphysiologic numbers of adipose-derived stem cells to improve graft take. Adipose-derived stem cells have been shown to promote cancer progression, raising concerns over the safety of adipose-derived stem cells and cell-assisted lipotransfer in postoncologic breast reconstruction. The authors compared the effect of adipose-derived stem cells alone, cell-assisted lipotransfer, and conventional fat grafting on breast cancer growth and metastasis.

Methods: Proliferation and migration of murine 4T1 breast cancer cells cultured in control medium or mouse adipose-derived stem cell– or fat graft–conditioned medium were assessed by flow cytometry and scratch assay, respectively. Transcription levels of arginase-1, transforming growth factor-β, and vascular endothelial growth factor were assessed in adipose-derived stem cells and fat graft by quantitative reverse transcription polymerase chain reaction. An orthotopic mouse tumor model was used to evaluate breast cancer progression and metastasis. 4T1 cells were injected into the mammary pad of female BALB/c mice. Six days later, tumors were injected with saline, adipose-derived stem cells, fat graft, or cell-assisted lipotransfer ($n = 7$ per group). Two weeks later, primary tumors were examined by immunohistochemistry and lung metastasis was quantified.

Results: Adipose-derived stem cell–conditioned medium increased cancer cell proliferation ($p = 0.03$); migration ($p < 0.01$); and transcription of arginase-1, transforming growth factor-β, and vascular endothelial growth factor compared to fat graft–conditioned or control medium ($p < 0.02$). Tumor-site injection with adipose-derived stem cells alone led to increased primary tumor growth and lung metastasis compared to control, fat graft, or cell-assisted lipotransfer groups ($p < 0.05$). Adipose-derived stem cell injection increased $CD31^+$ vascular density in tumors ($p < 0.01$).

Conclusion: Adipose-derived stem cells alone, but not conventional fat graft or cell-assisted lipotransfer, promote breast cancer cell proliferation and invasiveness in vitro and in vivo. (*Plast. Reconstr. Surg.* 143: 137, 2019.)

Autologous fat grafting is an invaluable technique whereby lipoaspirate from a donor site is purified and injected into a soft-tissue defect.[1] To improve graft take, researchers have supplemented harvested fat

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with various agents that promote revascularization, including platelet-rich plasma, vascular endothelial growth factor, and insulin.[2–4] Still others have isolated the stromal vascular fraction from a portion of lipoaspirate and then combined it with conventional fat graft before injection. The stromal vascular fraction contains multiple cell populations, including endothelial cells, immune cells, smooth muscle cells, pericytes, and adipose-derived stem cells. Although adipose-derived stem cells represent only 1 to 10 percent of the stromal vascular fraction, they can be enriched and expanded ex vivo.[5] Preclinical research has pointed to several mechanisms whereby adipose-derived stem cells lead to improved graft take, including adipocyte differentiation and graft repopulation,[6,7] secretion of proangiogenic factors,[8] and differentiation into vascular endothelium.[7,9,10] Enrichment of fat graft with stromal vascular fraction or supraphysiologic levels of adipose-derived stem cells is referred to as cell-assisted lipotransfer. Prospective randomized clinical trials have demonstrated improved fat graft take with cell-assisted lipotransfer compared to conventional fat grafting.[11–13] Together, this evidence has led to the development of commercial cell separation systems for adipose-derived stem cell enrichment of fat grafts in the clinical setting.[14]

Although evidence increasingly points to the safety of conventional autologous fat grafting in breast reconstruction,[15–17] the oncologic risk associated with cell-assisted lipotransfer is poorly defined. Adipose-derived stem cells secrete cytokines with immunomodulatory, proangiogenic, antiapoptotic, and trophic effects that have been shown to promote cancer recurrence and metastasis in several experimental studies.[18–22] To date, only the RESTORE-2 trial has examined cell-assisted lipotransfer in the setting of correction of lumpectomy defects.[23] Although no local recurrence was observed in the 67 patients included in the study, a relatively short follow-up of 12 months makes it difficult to draw any firm conclusions with regard to the oncologic safety of adipose-derived stem cell enrichment of fat grafts in breast cancer patients. The current study aims to examine for the first time the effect of adipose-derived stem cells and fat graft individually or in combination (thereby simulating cell-assisted lipotransfer) on cancer progression and metastasis both in vitro and in a mouse model of breast cancer.

MATERIALS AND METHODS

Animals

Institutional ethics approval was obtained for the study. All experiments were performed in accordance with the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care. Eight-week-old female BALB/c mice were used for all experiments (Charles River Laboratories, Saint Constant, Quebec, Canada).

Cell Culture

Murine 4T1 breast cancer cells (ATCC, Manassas, Va.) were cultured at 37°C and 5% carbon dioxide in complete medium consisting of Dulbecco's Modified Eagle Medium (Corning Cellgro, Manassas, Va.) containing 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 units/ml penicillin (Corning Cellgro).

Adipose-Derived Stem Cell Isolation and Culture

Mouse adipose-derived stem cells were isolated as described previously.[24,25] Bilateral inguinal fat pads were infiltrated with 300 μl of lactated Ringer solution. As described previously,[26] tissue was placed in Krebs-Ringer bicarbonate buffer and incubated for 4 minutes. Tissue was minced with a no. 10 scalpel and collected in a 3-ml syringe before serial dispersion through a 16-gauge needle. The fat was then digested for 1 hour at 37°C in a digestion buffer composed of 0.1% collagenase VII (Sigma-Aldrich Co., Oakville, Ontario, Canada), 1% fetal bovine serum, and 2 mM calcium chloride (Roche Diagnostics, Laval, Quebec, Canada) in phosphate-buffered saline until the suspension had a smooth consistency. The suspension was centrifuged at 300 *g* for 5 minutes to separate mature adipocytes from the stromal vascular fraction. The stromal vascular fraction was washed in phosphate-buffered saline before culture for 1 week in complete medium to expand the adipose-derived stem cell population. The identity and purity of the resulting expanded $CD34^+CD105^+CD45^-CD31^-$ adipose-derived stem cells was confirmed by flow cytometric analysis as described previously.[25]

Generation of Conditioned Media

For fat-conditioned medium, minced fat was treated with digestion buffer and washed. Fat cells (4.5×10^5 cells/well) were suspended in the lower chambers of six-well Transwell plates (Corning Cellgro) in 2.5 ml of complete medium per well.

Fat was held under medium by placing the upper chamber Transwell insert into each well with 1.5 ml of additional medium. For adipose-derived stem cell–conditioned medium, single-passage adipose-derived stem cells (approximately 1.4×10^6 cells) were grown in 12 ml of complete medium. Conditioned media were collected after 48 hours.

Cancer Cell Proliferation Assay

A carboxyfluorescein diacetate *N*-succinimidyl ester (Life Technologies, Burlington, Ontario, Canada) dilution assay was performed to examine proliferation of 4T1 breast cancer cells in the presence of complete medium, fat graft-conditioned medium, or adipose-derived stem cell–conditioned media. As cells divide, the fluorescent carboxyfluorescein diacetate *N*-succinimidyl ester intravital dye is divided between sister cells, resulting in a drop in mean fluorescence intensity inversely proportional to cell proliferation.[25] Murine 4T1 breast cancer cells were labeled with 5 μM carboxyfluorescein diacetate *N*-succinimidyl ester for 10 minutes at 37°C and washed in Dulbecco's Modified Eagle Medium supplemented with 25 mM glucose and 10% fetal bovine serum. Cells were plated in triplicate in six-well plates (10^5 cells/well) with complete medium, fat graft-conditioned medium, or adipose-derived stem cell–conditioned medium. Media were replaced every 12 hours. Increased proliferation determined by mean fluorescence intensity was examined after 48 hours by flow cytometry.

Cancer Cell Migration Assay

Murine 4T1 breast cancer cells were plated onto 24-well plates at 80 percent confluence for 24 hours and then serum-starved for 24 hours. Cells were then treated with 10 μg/ml mitomycin C for 2 hours to halt cell proliferation. A sterile 100-μl pipette tip was then used to make a vertical scratch mid well. After washing the wells with phosphate-buffered saline, the cells were cultured in complete medium, fat graft–conditioned medium, or adipose-derived stem cell–conditioned medium. Conditioned medium was generated by collected supernatants from fat graft or adipose-derived stem cells cultured in complete medium for 48 hours. Images of the wells were taken using a Zeiss Axioplan II microscope (Carl Zeiss, Thornwood, N.Y.) at 10× magnification and analyzed using Axiovision software version 4.6 (Carl Zeiss) immediately after the scratch, 6, 12, 18, and 24 hours later. ImageJ software (version 1.48h; National Institutes of Health, Bethesda, Md.) was used to calculate the scratch surface area in pixels at various time points. Percentage scratch open for each treatment group was calculated by dividing the area of the scratch at the designated time point by the original scratch area.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated using a QIAGEN RNeasy Plus Mini Kit according to the manufacturer's instructions (Qiagen, Toronto, Ontario, Canada). Complementary DNA was generated using 500 ng of RNA with the Wisent Advanced cDNA Synthesis Kit (Wisent Bioproducts, St. Bruno, Quebec, Canada). Quantitative reverse-transcription polymerase chain reaction was performed in triplicate with 1 μl of cDNA using Quantifast SYBR Green (Qiagen). Amplification was performed using an RG-600 Rotor-Gene (Corbett Research, Sydney, New South Wales, Australia) and data were analyzed using the 2-ΔΔCt relative quantification technique and expressed relative to an internal normalizing mRNA. High-stringency primer pairs were obtained for vascular endothelial growth factor (VEGF), transforming growth factor-β (TGFβ), IL-6, arginase (Arg-1), IL-1β, and platelet-derived growth factor (PDGF) (Invitrogen, Camarillo, Calif.). 18S ribosomal RNA served as internal normalizing standards. [**See Table, Supplemental Digital Content 1**, which shows the custom primer sequences for gene transcripts of interest. High-stringency primer pairs used for quantitative reverse-transcription polymerase chain reaction reactions to assess transcription levels of Arg-1, IL-1β, IL-6, PDGF, TGFβ, VEGF, and 18S (internal control), ***http://links.lww.com/PRS/D184***.]

Metastatic Murine Breast Cancer Model

The 4T1 murine breast cancel model parallels human metastatic breast cancer by producing weakly immunogenic tumors that spontaneously metastasize to distant sites, including the lungs.[27,28] Female BALB/c mice were inoculated subcutaneously in the fourth mammary pad with 4T1 cells (5×10^5 cells in 50 μl of phosphate-buffered saline). Six days later, the tumor site, confirmed by palpation, was injected with one of the following: adipose-derived stem cells alone (5×10^5 cells resuspended in 50 μl of phosphate-buffered saline); purified fat (50 μl); purified fat (50 μl) supplemented with 5×10^5 adipose-derived stem cells; or phosphate-buffered saline alone (50 μl). The adipose-derived stem cell concentration used

corresponded to 750 times physiologic levels based on previous mouse studies.[29,30] Tumor growth was monitored every 3 days beginning at day 8 after tumor inoculation. Tumor volume was estimated using microcaliper measurements (volume = 0.5 × width^2 × length) as described previously.[31] Eleven days after fat grafting at the tumor site, mice were killed. Primary tumors were explanted, weighed, and submitted for histologic analysis.

Lung metastasis was examined using a clonogenic plating assay.[27] Each harvested lung was dissociated by mechanical dispersion through a sterile wire mesh and cultured on a petri dish in the presence of 60 μM 6-thioguanine. After 12 days, plates were fixed with methanol and stained with 0.03% methylene blue to enumerate metastatic 4T1 colonies.

Histologic Analysis

Tumor samples were fixed in 4% paraformaldehyde for 24 hours, followed by immersion in 70% ethanol at 4°C for 24 hours. Samples were subsequently embedded in paraffin, and 5-μm-thick sequential sections were cut.

To distinguish between 4T1 cells and potential intratumoral adipocytes in the explanted specimens, paraffin sections were stained with hematoxylin and eosin. The proportion of tumor cells relative to adipocyte coverage was determined using ImageJ as described previously.[25,32] As most of the adipocyte cell body is composed of lipid vacuole, this fraction accurately represents adipocyte coverage of the sectioned graft.[4] Mean tumor cell and adipocyte coverage was determined based on three sections per tumor and then averaged between groups.

To determine vascular density, additional paraffin sections underwent heat-mediated antigen retrieval before staining with polyclonal rabbit anti-mouse CD31 (Abcam, Toronto, Ontario, Canada). CD31$^+$ endothelial immunostaining was visualized with a 3,3′-diaminobenzidine-linked secondary system (Vector Laboratories, Burlington, Ontario, Canada). Six random high-power fields were selected by a blinded observer per tumor. The percentage of CD31$^+$ staining vessels was calculated per high-power field using ImageJ.

Statistical Analysis

Data are expressed as means ± SEM. A nonparametric two-tailed Mann-Whitney *U* test was used for comparison between two groups. Comparisons between more than two groups were made using a Kruskal-Wallis nonparametric analysis of variance with a Dunn post hoc test. Statistical significance was set at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, Calif.).

RESULTS

Proliferation of Breast Cancer Cells Is Increased by Conditioned Medium from Adipose-Derived Stem Cells But Not Fat Graft

Murine 4T1 breast cancer cells were cultured in vitro in the presence of complete medium and supernatants from adipose-derived stem cell and fat graft cultures to examine whether soluble factors from adipose-derived stem cells or conventional fat graft could affect cancer cell proliferation (Fig. 1). Increased proliferation of cancer cells, as evidenced by lower dye intensity, was observed in the presence of adipose-derived stem cell–conditioned medium (mean fluorescence intensity, 541.7 ± 29.8) when compared to complete medium alone (mean fluorescence intensity, 3311 ± 102.7; $p = 0.03$). No difference in proliferation was observed between fat graft–conditioned medium (1172 ± 236.3) and complete medium.

Adipose-Derived Stem Cell–Conditioned Medium Increases Breast Cancer Cell Migration

The effect of soluble factors from adipose-derived stem cells and fat graft on breast cancer

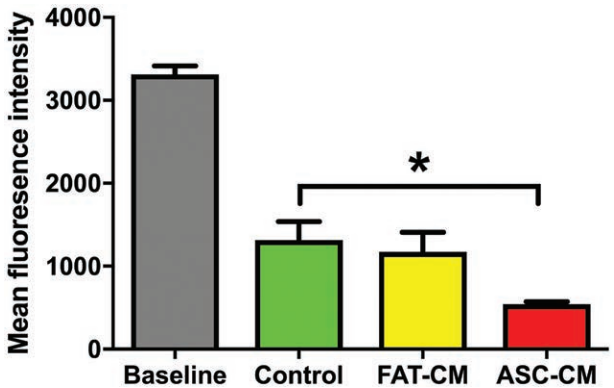

Fig. 1. Conditioned medium from adipose-derived stem cells, but not fat graft, increases murine breast cancer cell proliferation. A carboxyfluorescein diacetate *N*-succinimidyl ester dilution assay was used to assess proliferation of murine 4T1 breast cancer cells in the presence of control medium, adipose-derived stem cell–conditioned medium (*ASC-CM*), and fat graft–conditioned medium (*FAT-CM*). Increased proliferation is inversely proportional to mean fluorescence intensity. Each condition was tested six times per experiment. Data are representative of three independent experiments (*$p < 0.05$).

cell migration was investigated using a scratch assay (Fig. 2). A defect was produced in monolayers of murine 4T1 breast cancer cells which were then treated with adipose-derived stem cell–conditioned medium, fat graft–conditioned medium, or complete medium. At various time points, the degree of closure of the resulting defect was assessed and compared to the original defect area. At all time points, cells cultured in the presence of adipose-derived stem cell–conditioned medium demonstrated more rapid migration than cells treated with complete medium or fat graft–conditioned medium ($p < 0.05$). Apart from the 12-hour time point ($p < 0.01$), no difference was observed in migration of cancer cells treated with complete medium or fat graft–conditioned medium.

Adipose-Derived Stem Cells Demonstrate Increased Prometastatic Gene Transcription Compared to Fat Graft

To identify potential adipose-derived stem cell gene products responsible for the observed increase in cancer cell proliferation and migration, cultured adipose-derived stem cells and fat graft from which conditioned media were collected were submitted to quantitative reverse-transcription polymerase chain reaction (Fig. 3). Adipose-derived stem cells exhibited increased transcription of the immunomodulatory protein Arg-1 (2.8 ± 0.07) compared to fat graft (0.5 ± 0.03; $p < 0.001$). In addition, mRNA levels of TGFβ, a potent promoter of cancer cell invasion, were higher in adipose-derived stem cells (2.9 ± 0.3) than in fat graft (0.4 ± 0.01; $p < 0.001$). Finally, VEGF transcription was noted to be higher in adipose-derived stem cells (5.2 ± 1.0) compared to fat alone (0.8 ± 0.2, $p = 0.013$). No differences in PDGF, IL-1β, or IL-6 were noted between adipose-derived stem cells and fat graft.

Adipose-Derived Stem Cells Alone Promote Primary Tumor Growth

Tumors became palpable by day 8, but no differences in tumor volumes (Fig. 4) were noted between experimental groups until 14 days after tumor inoculation. At day 14, tumors co-injected with adipose-derived stem cells exhibited greater tumor volumes (448 ± 86 mm^3) relative to phosphate-buffered saline controls (242 ± 20 mm^3; $p = 0.005$). By day 17, the volumes of tumors in the adipose-derived stem cell group (709 ± 77 mm^3) were significantly greater than volumes in all other groups ($p < 0.05$). No statistical differences were noted in volumes between cell-assisted lipotransfer, fat graft alone, or phosphate-buffered saline control groups.

As depicted in Figure 5, explanted primary tumor weights in the adipose-derived stem cell group (0.77 ± 0.05 g) were significantly higher than those

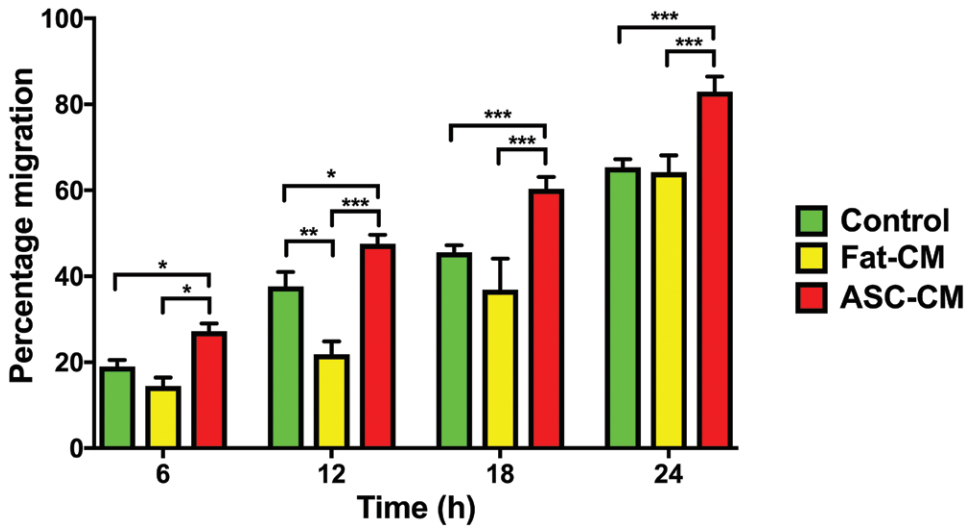

Fig. 2. Conditioned medium from adipose-derived stem cells, but not fat graft, increases murine breast cancer cell migration. In a scratch assay, mitomycin C–treated 4T1 breast cancer cells were grown in adipose-derived stem cell–conditioned medium (*ASC-CM*), fat graft-conditioned medium (*Fat-CM*), or control medium. At all time points, the scratch area in wells of the cancer cells treated with adipose-derived stem cell–conditioned medium was significantly smaller than cells grown in control medium or cells treated fat graft–conditioned medium (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Each condition was tested six times per experiment. Data are representative of three independent experiments.

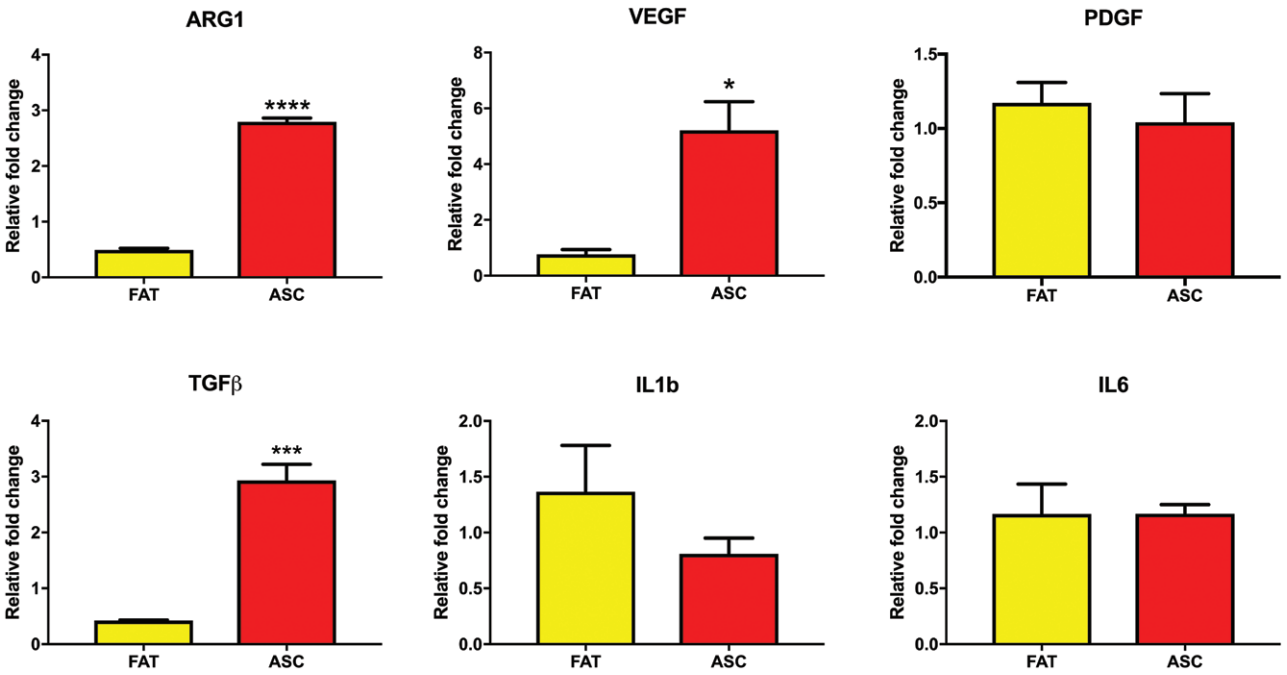

Fig. 3. Adipose-derived stem cells demonstrate increased transcription of genes promoting cancer spread. Complementary DNA was generated from conventional fat graft (*FAT*) and adipose-derived stem cells (*ASC*). Results are displayed as fold increase compared to the housekeeping gene 18S ribosomal RNA. Adipose-derived stem cells demonstrated increased transcription of ARG-1 (****$p < 0.0001$), VEGF (*$p < 0.05$), and TGFβ (***$p < 0.001$). No significant differences in transcription levels were noted in PDGF, IL-1β, or IL-6. Each condition was tested three times per experiment. Data are representative of four independent experiments.

observed in the phosphate-buffered saline control group (0.48 ± 0.07 g; $p = 0.02$). No differences were noted in primary tumor weights between adipose-derived stem cell–enriched fat graft, fat graft alone, or phosphate-buffered saline control groups.

To ensure that the increased primary tumor volumes and weights in the adipose-derived stem cell treatment group were not attributable to more adipocytes in the tumor mass, tumor sections were processed for surface area analysis (Fig. 6). No statistical differences in mature adipocyte contributions to the cross-sectional areas of the tumors were noted between groups. In keeping with tumor volume and weight data, the surface areas of primary tumors in the adipose-derived stem cell group ($4.17 \pm 0.29 \times 10^6$ arbitrary units) were greater than those in either the cell-assisted lipotransfer ($2.84 \pm 0.41 \times 10^6$ arbitrary units; $p = 0.0065$), fat graft alone ($2.44 \pm 0.33 \times 10^6$ arbitrary units; $p = 0.0006$), or phosphate-buffered saline ($2.46 \pm 0.28 \times 10^6$ arbitrary units; $p = 0.006$) group.

Adipose-Derived Stem Cells Alone Promote Lung Metastasis

Using a clonogenic assay, the number of 4T1 breast cancer colony-forming units in the lungs were enumerated at day 17 after inoculation (Fig. 7). Significantly more colony-forming units were observed in the lungs of adipose-derived stem cell treatment group mice (15,544 ± 1030) compared to adipose-derived stem cell–enriched fat graft (10,210 ± 826; $p = 0.023$), fat graft alone (9865 ± 885; $p = 0.024$), or phosphate-buffered saline control (8432 ± 1043; $p = 0.0018$) mice. No differences were noted in colony-forming units between the cell-assisted lipotransfer, the fat graft alone, or the phosphate-buffered saline control group.

Adipose-Derived Stem Cells, But Not Cell-Assisted Lipotransfer, Are Associated with Increased CD31$^+$ Vessel Density in Primary Breast Cancer Tumors

Primary tumor sites injected with adipose-derived stem cells exhibited greater CD31$^+$ immunostaining (3.15 ± 0.39 percent) compared with those treated with cell-assisted lipotransfer (0.52 ± 0.06 percent; $p = 0.003$) (Fig. 8). No significant differences in CD31$^+$ immunostaining were noted compared to the fat graft alone (1.69 ± 0.24 percent) or the phosphate-buffered saline control (1.67 ± 0.20 percent) group.

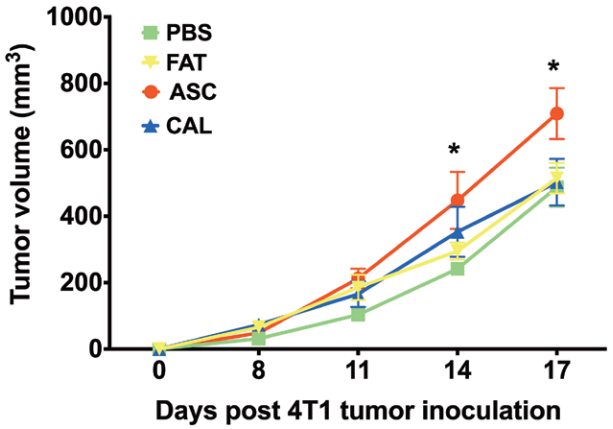

Fig. 4. Adipose-derived stem cells alone, but not fat graft– or adipose-derived stem cell–enriched fat graft, led to increased primary breast tumor volumes. Female BALB/c mice were inoculated subcutaneously in the fourth mammary pad with murine 4T1 breast cells (5×10^5 cells in 50 μl of phosphate-buffered saline). Six days later, the tumor site was injected with one of the following ($n = 7$ per group): adipose-derived stem cells alone (5×10^5 cells), purified fat, purified fat supplemented with 5×10^5 adipose-derived stem cells, or phosphate-buffered saline alone. Tumor volume was monitored every 3 days by digital caliper beginning at day 8 after tumor inoculation. Two weeks after tumor inoculation, tumors injected with adipose-derived stem cells were significantly larger than controls (*$p < 0.05$). At 17 days, adipose-derived stem cells were significantly larger than tumors in all other groups (*$p < 0.05$). *PBS*, phosphate-buffered saline; *ASC*, adipose-derived stem cells; *FAT*, fat graft; *CAL*, cell-assisted lipotransfer.

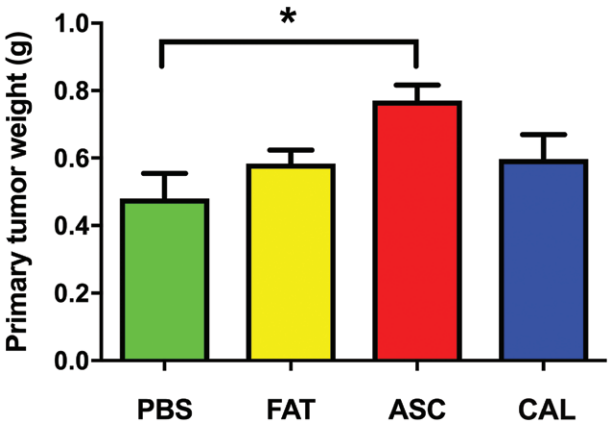

Fig. 5. Adipose-derived stem cells alone, but not fat graft– or adipose-derived stem cell–enriched fat graft, led to increased primary breast tumor weights. Weights of primary tumors injected with adipose-derived stem cells were significantly heavier than tumors in the phosphate-buffered saline control group ($n = 7$ per group) (*$p < 0.05$). No such differences were noted between tumors injected with purified fat alone or adipose-derived stem cell–enriched fat graft (cell-assisted lipotransfer). *PBS*, phosphate-buffered saline; *ASC*, adipose-derived stem cells; *FAT*, fat graft; *CAL*, cell-assisted lipotransfer.

DISCUSSION

This is the first study to directly compare the oncologic safety of adipose-derived stem cells alone, conventional fat grafting, and cell-assisted lipotransfer. Using an animal model of breast cancer, we demonstrate that adipose-derived stem cells alone promote primary tumor growth and lung metastasis, whereas conventional fat grafting and cell-assisted lipotransfer do not appear to carry additional cancer risk.

Our in vitro results point to possible mechanisms whereby adipose-derived stem cells promote breast cancer growth and spread. In keeping with earlier findings using human adipose-derived stem cells and breast cancer cell lines,[33] we found that conditioned medium from adipose-derived stem cell cultures promoted murine 4T1 breast cancer cell growth and migration in vitro. Unlike earlier studies, however, our current work compared adipose-derived stem cell–conditioned and fat graft-conditioned media, and found that the latter did not promote cancer cell proliferation or migration. We next examined expression of gene products from fat graft and adipose-derived stem cells that could account for their differential effects on 4T1 cancer cells. We observed that adipose-derived stem cells had higher transcription levels of TGFβ, a cytokine shown to increase breast cancer invasiveness and promote epithelial-to-mesenchymal transition,[34] a critical step in the development of metastasis. In addition, we found that adipose-derived stem cells had higher meRNA levels of the immunosuppressive protein Arg-1 than fat graft. Arginase-1 inhibits antitumor immune responses by breaking down L-arginine, an amino acid essential for T-lymphocyte function.[35] Although we did not examine the effect of adipose-derived stem cells and fat graft on tumor immune surveillance in the current report, differences in Arg-1 expression by adipose-derived stem cells and fat graft warrant further future investigation, as Arg-1 has been shown to be expressed at higher levels in the sera of early-stage breast cancer patients compared to healthy controls.[36] We observed elevated transcription levels of VEGF in adipose-derived stem cells compared with fat graft alone. This finding is consistent with earlier work showing that adipose-derived stem cells contain a $CD45^-CD34^+$ endothelial progenitor cell subpopulation that is a rich source of VEGF and is particularly effective at inducing angiogenesis.[10,37] Although we only examined gene expression in fat graft and adipose-derived stem cells, a recent report showing adipose-derived stem cell

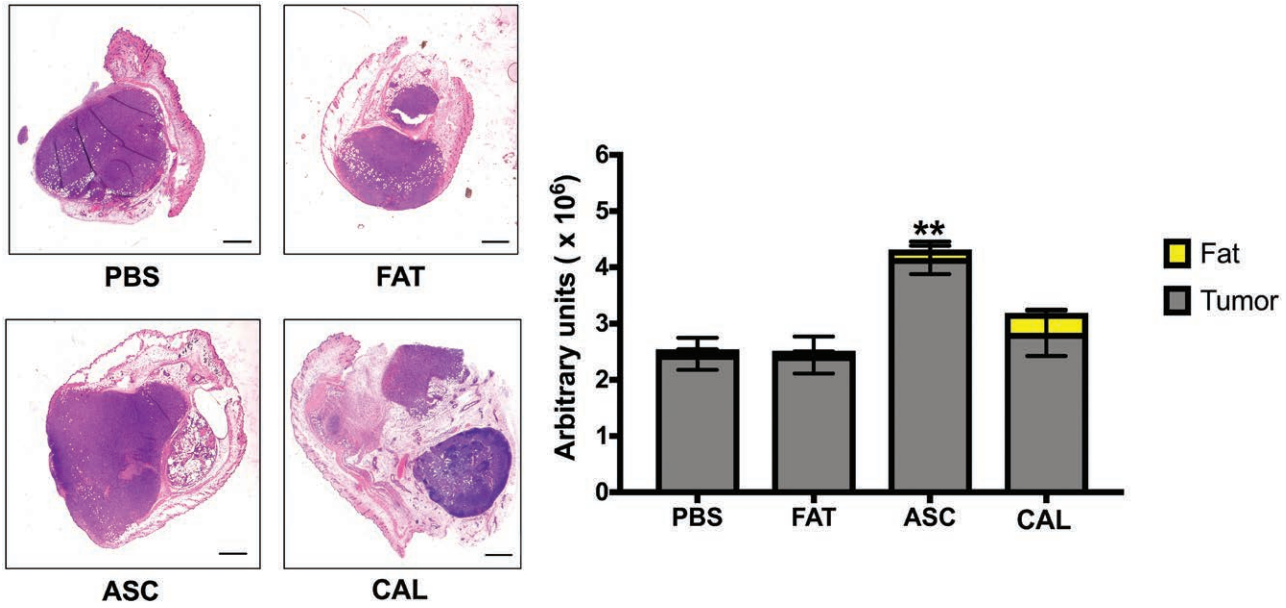

Fig. 6. Representative images and quantification of cancer cell and adipocyte coverage. Differences in tumor volumes and weights are not attributable to intratumoral fat engraftment. To ensure that the increased primary tumor volumes and weights were not attributable to increased adipocytes in the tumor mass, sections were submitted for surface area analysis. Mean tumor cell and adipocyte coverage was determined based on three sections per explanted tumor and then averaged between groups. No statistical differences in mature adipocyte contributions to the cross-sectional areas of the tumors were noted between groups. In keeping with the tumor volume and weight data, the surface areas of primary tumors in the adipose-derived stem cell group were greater than those in either the cell-assisted lipotransfer, fat graft alone, or phosphate-buffered saline control group (**$p < 0.01$). *Scale bar* = 1 mm. *PBS*, phosphate-buffered saline; *ASC*, adipose-derived stem cells; *FAT*, fat graft; *CAL*, cell-assisted lipotransfer.

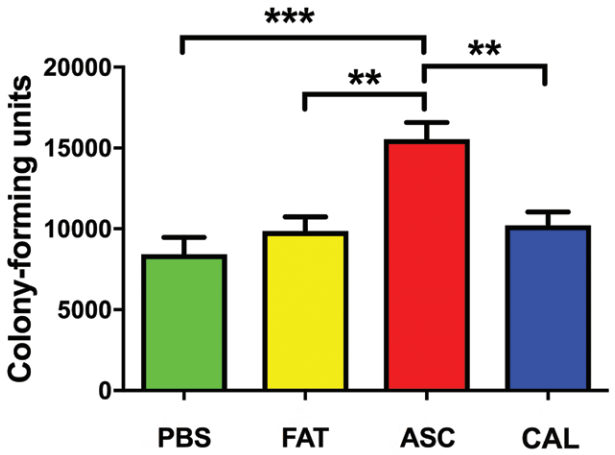

Fig. 7. Adipose-derived stem cells alone, but not fat graft or adipose-derived stem cell–enriched fat graft, led to increased lung metastasis. A clonogenic assay was used to determine the number of murine 4T1 colony-forming units in the lungs of mice killed 2 weeks after tumor-site injection with phosphate-buffered saline, purified fat, adipose-derived stem cells alone, or adipose-derived stem cell–enriched fat (cell-assisted lipotransfer) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). For each group, $n = 7$. *PBS*, phosphate-buffered saline; *ASC*, adipose-derived stem cells; *FAT*, fat graft; *CAL*, cell-assisted lipotransfer.

up-regulation of VEGF expression when co-cultured with breast cancer cells points to cross-talk between adipose-derived stem cells and cancer cells.[38] Interestingly, despite its established role in promoting tumor progression, we observed no differences in IL-6 levels between adipose-derived stem cells and fat graft through our quantitative reverse-transcription polymerase chain reaction analysis. The lack of a difference in this critical cytokine may be attributable to its secretion at high levels by both adipose-derived stem cells and cancer-associated mature adipocytes, or to its post-translational regulation.[39]

We compared the impact of autologous fat graft, cell-assisted lipotransfer, and adipose-derived stem cells alone on breast cancer progression in vivo. We observed increased primary tumor growth and metastasis when adipose-derived stem cells alone were injected into breast cancers, consistent with earlier reports. Kamat et al.[21] demonstrated increased tumor weights and metastasis following inoculation with increasing ratios of human adipose-derived stem cells to cancer cells in athymic mice. Similarly, Rowan et al.[33] also

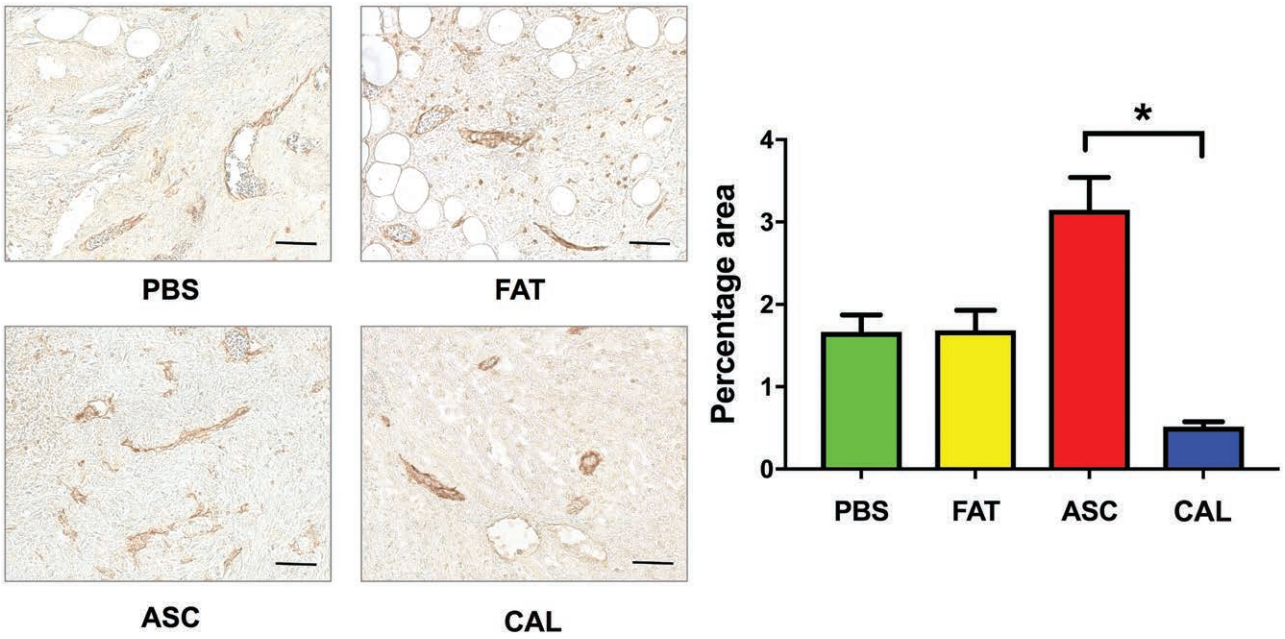

Fig. 8. Injection with adipose-derived stem cells is associated with higher vascular density in primary tumors. Representative images and CD31$^+$ immunostaining in primary tumors injected with saline (phosphate-buffered saline), purified fat, adipose-derived stem cells, and adipose-derived stem cell–enriched fat (cell-assisted lipotransfer). Six high-power fields were captured per specimen. Percentage of CD31$^+$ vessels per high-power field was performed using ImageJ software (*$p < 0.05$). For each group, $n = 7$. *Scale bar* = 100 μm. *PBS*, phosphate-buffered saline; *ASC*, adipose-derived stem cells; *FAT*, fat graft; *CAL*, cell-assisted lipotransfer.

demonstrated increased spread of human MDA-MB-231 breast cancer cells in a murine model after local co-injection of human adipose-derived stem cells. Unlike these previous studies, in our in vivo model, mouse breast cancer cells were inoculated into immunocompetent mice, allowing for development of normal host antitumor immune responses.

Interestingly, when the same number of adipose-derived stem cells were co-injected with fat graft at tumor sites to parallel the clinical technique of cell-assisted lipotransfer, no increase in primary tumor size or metastasis was observed compared to controls. We speculate that the co-injected fat may exert a paracrine influence on adipose-derived stem cells, causing them to preferentially undergo adipogenesis as opposed to angiogenesis, a theory supported by our finding of lower vessel density in tumors co-injected with adipose-derived stem cell–enriched fat relative to those injected with adipose-derived stem cells alone (Fig. 8). Alternatively, the fat may act as a barrier that prevents soluble factors produced by adipose-derived stem cells from reaching cancer cells and promoting their growth and spread. More simply, the addition of fat may serve to reduce the relative concentration of adipose-derived stem cells in the tumor microenvironment to a level below which they cannot as effectively promote cancer cell proliferation. What that level is remains unclear. Although Kamat et al.[21] have demonstrated a dose-dependent increase in primary tumor growth and metastasis following inoculation with higher ratios of adipose-derived stem cells to breast cancer cells, no study has examined whether increasing supraphysiologic levels of adipose-derived stem cells in fat grafts leads to a similar dose-dependent effect in cancer progression. Improved fat graft take has been observed with adipose-derived stem cell doses ranging from as high as 6000 times[30] to as low as 375 times[40] physiologic levels. For our cell-assisted lipotransfer experimental group, we used an adipose-derived stem cell dose of 750 times physiologic levels and did not observe increased primary tumor growth or metastasis. Future research should aim to determine the minimum adipose-derived stem cell dose required to improve fat graft take in cell-assisted lipotransfer without increasing cancer risk.

No study is without limitations, and the current report is no exception. First, we used a murine model of breast cancer, which may not perfectly reflect tumor biology in humans. In our model, we used lean, young mice whose adipose tissue likely had higher tissue partial pressure of oxygen and less inflammation than is typically

seen in human subjects at risk for breast cancer. Second, in clinical practice, cell-assisted lipotransfer for breast reconstruction would presumably occur after lumpectomy with or without adjuvant radiotherapy. The chance of cancer recurrence or the progression of residual microscopic disease in this setting is presumably lower than with a preexisting tumor mass. To this end, our model does not simulate cancer recurrence per se, but instead uses the presence of an existing tumor to demonstrate "proof-of-concept" that supraphysiologic levels of adipose-derived stem cells alone, but not in combination with fat graft, stimulate cancer progression. Overall, our study lends support for the oncologic safety of conventional fat grafting and cell-assisted lipotransfer.

CONCLUSIONS

Our research contributes to the current body of evidence by demonstrating that supraphysiologic levels of adipose-derived stem cells are capable of stimulating proliferation and metastatic spread of cancer cells in vitro and in vivo. We show that when the same number of adipose-derived stem cells are co-injected with fat (simulating the clinical technique of cell-assisted lipotransfer), this cancer-promoting effect is not observed. Although this study lends support for the oncologic safety of conventional fat grafting and cell-assisted lipotransfer in breast reconstruction, further experimental and clinical studies are required to determine the concentration of adipose-derived stem cell enrichment required to improve fat graft without increasing cancer risk.

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