Human Adipose Tissue-Derived Stromal/Stem Cells Promote Migration and Early Metastasis of Head and Neck Cancer Xenografts

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Abstract

Background: Fat grafting has become popular for repair of postsurgical/postradiation defects after head/neck cancers resection. Fat graft supplementation with adipose tissue-derived stromal/stem cells (ASCs) is proposed to improve graft viability/efficacy, although the impact of ASCs on head/neck cancer cells is unknown.

Objectives: To determine whether ASCs affect growth, migration, and metastasis of human head/neck cancer.

Methods: Human Cal-27 and SCC-4 head/neck cancer cells were co-cultured human ASCs, or treated with ASC conditioned medium (CM), and cancer cell growth/migration was assessed by MTT, cell count, and scratch/wound healing assays in vitro. Co-injection of 3 × 10^6 Cal-27/green fluorescent protein (GFP) cells and ASCs into the flank of NUDE mice assessed ASC effect on tumor growth/morphology. Quantitation of human chromosome 17 DNA in mouse organs assessed ASC effects on micrometastasis. Primary tumors were evaluated for markers of epithelial-to-mesenchymal transition, matrix metalloproteinases, and angiogenesis by immunohistochemistry.

Results: Co-culture of Cal-27 or SCC-4 cells with ASCs from 2 different donors or ASC CM had no effect on cell growth in vitro. However, ASC CM stimulated Cal-27 and SCC-4 migration. Co-injection of ASCs from 2 different donors with Cal-27 cells did not affect tumor volume at 6 weeks, but increased Cal-27 micrometastasis to the brain. Evaluation of tumors sections from 1 ASC donor co-injection revealed that ASCs were viable and well integrated with Cal-27/GFP cells. These tumors exhibited increased MMP2, MMP9, IL-8, and microvessel density.

Conclusions: Human ASCs did not alter growth of human head/neck cancer cells or tumor xenografts, but stimulated migration and early micrometastasis to mouse brain.

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Over the last 2 decades, fat grafting has become an integral adjunct to aesthetic procedures performed by plastic surgeons. With about 70% to 80% of these procedures addressing the face, neck, or breasts, the popularity and utility of fat grafting continues to expand. The idea of dressing the face, neck, or breasts is appealing to patients and is much more favorable than replacing tissue deficits with foreign bodies such as implants or synthetic fillers.

While the most common use of fat grafting is in soft tissue facial rejuvenation as a result of aging or other pathology, fat grafting has been recently proposed as an important reconstructive modality following surgery and radiation treatments for head and neck cancer patients. These patients undergo morbid resectional procedures, often leading to severe deformity in the face and neck regions. Despite the advancements in reconstructive options for these patients, many of them are left desiring more acceptable cosmesis. Furthermore, fibrosis is an unfortunate sequela of radiation, rendering tissues in the head and neck chronically ischemic and hypoxic. This puts the patient at risk for poor wound healing, decreased tissue flexibility and mobility, and, ultimately, significant discomfort. Using autologous fat grafts in these patients not only facilitates an almost immediate improvement in facial contour, but the adipocytes that are injected have been show to demonstrate pro-angiogenic effects, which may help with vascularization of scarred, irradiated tissue.

Despite the recent popularity of fat grafting and the many benefits it purports, 1 of the most controversial aspects of this procedure is the ability of these grafts to survive. It is not uncommon that a patient who has undergone fat grafting has significant resorption or necrosis of the injected fat postoperatively. As such, various studies have been performed to modify the techniques in either the harvesting or injecting of the fat to improve its viability in vivo, yet there still remains little objective data on this topic. There are, however, many studies that have examined the role of adipose-derived stem cells (ASCs) in enhancing the efficacy of these fat grafts. These stem cells, which are pluripotent cells found in the stromal vascular fraction (SVF) of adult adipose tissue, exert a plasticity that may not only improve the outcomes of fat grafting, but also have a regenerative effect on surrounding tissues. Much of these data are based on the knowledge that these cells produce factors that are beneficial for both wound healing and regeneration. In regard to head and neck pathologies, studies have demonstrated the use of ASCs in fat grafts for postparotidectomy Frey Syndrome, as well as for tissue regeneration in the setting of velopharyngeal insufficiency. While this application of ASCs in the head and neck seems promising, the outcomes and consequences of these procedures are not well studied.

Interestingly, ASCs have also been implicated in the regulation of cancer growth, adding yet another layer of controversy to these methods. The growth factors and hormones involved in the differentiation of ASCs and adipogenesis, as well as those secreted by the ASCs themselves, have been shown in both human and animal studies to increase tumor invasion and metastasis. While there are several reports describing these potentially dangerous effects of ASCs on breast, lung, and prostate cancer in particular, there are no reports evaluating the effect of ASCs on head and neck cancer cells. Therefore, the objectives of this study were to determine the impact of ASCs on head/neck cancer cells and examine their interactions. The study compared the effects of human ASCs isolated from healthy female donors on Cal-27 and SCC-4 head/neck cancer cell lines in vitro and in vivo.

METHODS

Ethics Statement

ASCs were obtained from subcutaneous abdominal adipose tissue from healthy women with the patient’s written informed consent obtained by the plastic surgeons performing the elective surgery. The tissues were provided to the investigators under a protocol approved by the Institutional Review Board of the Pennington Biomedical Research Center Institution with all identifying information removed. The study was conducted in accordance with the guidelines set forth in the Declaration of Helsinki. Experiments using mice were approved by the Tulane University IACUC Committee under protocol #2941R2. The study was conducted from July 2010 to December 2013.

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Norcross, GA) unless otherwise specified.

Isolation, Collection, and Culture of Human ASCs

ASCs were isolated as described from subcutaneous abdominal adipose tissue from 2 healthy female donors (mean age 35.5 ± 8.5 and mean body mass index [BMI] 21.62 ± 3.4) during elective surgery with the patient’s informed consent under a protocol approved by the Institutional Review Board of the Pennington Biomedical Research Center Institution. Passage 0 (P0) ASCs were expanded in ASC growth medium [DMEM/F-12 Ham’s, 10% fetal bovine serum [FBS; Hyclone, Logan, UT, http://www.hyclone.com], 1% Penicillin-Streptomycin/0.25 g fungizone] and characterized for surface-marker expression as described. The mean percentage of ASCs that were positive for individual surface markers were as follows: CD29, 99.1 ± 0.1;
CD105, 98.0 ± 0.8; CD45, 13.8 ± 2.0; CD34, 94.8 ± 0.8; CD44, 14.3 ± 1.6; CD73, 89.8 ± 1.3; CD90, 93.6 ± 0.1. For all experiments, passage 1 ASCs were reconstituted from cryopreserved P0 ASCs. To collect growth conditioned medium, ASCs were cultured until 40% confluency in ASC growth medium, the medium was replaced with the same medium containing only 2% FBS, and the cells were cultured for an additional 3 days before collection of the growth conditioned medium (ADCM).

Adipogenic Differentiation of ASCs

Adipogenic differentiation of ASCs was performed as previously described. ASCs in ASC growth medium were cultured until 90% to 95% confluency and cells were trypsinized and replated in ASC growth medium (30,000 cells/cm²). The medium was removed 24 hours after plating and replaced with ASC adipogenic differentiation medium (Dulbecco’s modified Eagle’s-Ham’s F-12 medium supplemented with 3% or 10% FBS, 15 mM HEPES [pH 7.4], biotin [33 µM], pantothenate [17 µM], human recombinant insulin [100 nM, Boehringer Mannheim, Indianapolis, IN], dexamethasone [1 µM], 1-methyl-3-isobutylxanthine [IBMX; 0.25 mM], and rosiglitazone [1 µM]), and cells were cultured for 3 days. The medium was replaced with the same medium that did not contain IBMX and rosiglitazone for 6 days and the adipocyte differentiated conditioned medium (ADCM) was collected.

Cal-27 and SCC-4 Head/Neck Cancer Cell Lines

Cal-27 and SCC-4 squamous cell carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cal-27 cells were cultured in a 5% CO₂ humidified atmosphere at 37°C in Cal-27 culture medium (DMEM medium containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate supplemented with 10% FBS, 1% Pen-Strep [Invitrogen, Grand Island, NY}). SCC-4 cells were cultured in a 5% CO₂-humidified atmosphere at 37°C in SCC-4 culture medium (DMEM:F-12 [1:1] containing 2.5 mM L-glutamine, 0.5 mM sodium pyruvate, 15 mM HEPES, 1200 mg/L sodium bicarbonate, supplemented with 10% FBS, 1% Pen-Strep, 0.4 µg/mL hydrocortisone).

Preparation of ASC/RFP Cells

P0 ASCs from 2 separate donors were cultured in 6-well plates (5 × 10⁴ cells/well) in ASC growth medium for 24 hours. To increase lentivirus transduction efficiency, the medium was replaced with fresh medium containing 8 µg/mL polybrene. Renilla luciferase fluorescent protein (RFP)-lentiviral vector stock (5 µL), NL-Turbo-RFP [multiplicity of infection (MOI) in the range of >10⁷ TU/mL], was added to the ASCs for 24 hour. The medium containing lentiviral particles was removed and 2 mL fresh medium was added. ASC/RFP cells were cultured until >90% confluency (2-3 weeks) and RFP transduction was observed by fluorescent microscopy using a Nikon microscope with the filter for red fluorescence (TRITC).

Preparation of Cal-27/GFP Cells

The day before transduction, Cal-27 cells were plated in 6-well plates (1 × 10⁶/well) in Cal-27 culture medium. On the day of transduction, green fluorescent protein (GFP)-lentiviral vector stock aliquot, NL-eFloc-eGFP (MOI >10⁷ TU/mL), was thawed on ice. Medium from Cal-27 cells was removed and replaced with 1.5 mL fresh medium with polybrene at 8 µg/mL. Cells were transduced by adding 5 µL of GFP-lentiviral vector per well with gentle mixing. After a 24 hour incubation, medium containing lentiviral particles was removed and 2 mL fresh medium was added to each well. The cells were cultured and passaged for 2-3 weeks and GFP fluorescence was monitored using a Nikon microscope with the filter for green fluorescence (FITC) until >90% cells exhibited GFP fluorescence. The GFP-positive cells were sorted by fluorescence-activated cell sorting (FACS, BD Biosciences, San Jose, CA) and then cryopreserved for future in vitro and in vivo experiments.

Direct Co-culture of ASCs With Cal-27/GFP Cells or SCC-4 Cells

ASC/RFP cells (2.5 × 10⁶) were cultured in ASC growth medium for 24 hours prior to addition of 2.5 × 10⁶ Cal-27/GFP cells or 2.5 × 10⁶ SCC-4 cells to the same wells. Bright field and fluorescent microscopic photographs were taken on days 1 through 4 after addition of the Cal-27/GFP or SCC-4 cells. The average number of GFP⁺ cells counted in 4 separate fields was recorded from at least 3 independent sets of experiments.

Wound Healing/Scratch Assay to Measure Effect of ASC-Conditioned Medium on Cal-27 and SCC-4 Migration

Cal-27 or SCC-4 cells were cultured to 80% confluency for 24 hours. A single strip of cells was scraped off the surface of the plate with a P200 pipette tip and the medium was replaced with fresh medium containing 0%, 20%, or 50% GCM or ADMC. The cells were cultured for an additional 17 hours at 37°C. Wound closure was viewed under a light microscope and photographed at 0 and 16 hours after addition of GCM or ADMC. Percentage gap closure was calculated as: % gap closure = \( \frac{\text{Gap area at 0 hour time point} - \text{Gap area 6 hour time point}}{\text{Gap area at 0 hour time point}} \times 100\% \)
Animals
Female NUDE mice (BALB/c) aged 4 to 5 weeks obtained from Charles River (Indianapolis, IN) were housed in sterile cages and maintained in pathogen-free aseptic rooms with 12 hours/2 hours light/dark schedule. Mice were fed with autoclaved food pellets and water ad libitum. All mouse experiments were performed in accordance with approved IACUC protocol (#2941R2) from Tulane University.

Tumor Xenograft Studies
Xenograft procedures were performed as previously described by our laboratory.6-13 Briefly, exponentially growing Cal-27/GFP cells and ASC/RFP cells were harvested. Animals were divided into 2 groups (n = 5 mice/group, 10 tumors/group) by injecting either 3 × 10⁶ Cal-27/GFP cells alone, or 3 × 10⁶ Cal-27/GFP + 3 × 10⁶ ASC/RFP cells in 150 µL of phosphate-buffered saline (PBS)-Matrigel mixture (50 µL cell suspension in PBS was mixed with 100 µL of Matrigel) bilaterally in the flanks of female NUDE mice. Control injections were injection of 3 × 10⁶ ASC/RFP cells alone or 3 × 10⁶ BJ-5ta human fibroblasts (ATCC® CRL-4001™) + 3 × 10⁶ Cal-27/GFP cells. Tumor caliper measurements were taken twice/week, and tumor volume was calculated by the formula: 0.523 × L²M where L is large diameter and M is small diameter as described.10,12 Six weeks postinjection, mice were euthanized by exposure to CO₂, and tumors and mouse organs were removed for further evaluation.

Fluorescence Microscopy and Hematoxylin & Eosin (H&E) Staining of Tumors and Mouse Tissues
Tumors and mouse organs were removed. Immediately after removal from animals, fresh tumors and organs were placed on a Nikon AZ100 fluorescent microscope and photomicrographed with a Nikon DS-Qi1Mc camera using NIS-Elements software (Nikon, Melville, NY). Tumors and mouse organs were prepared for either paraffin embedding/sectioning and H&E staining by addition of 10% neutral buffered formalin, frozen sectioning, and fluorescent microscopy, or measurement of chromosome-17 by real-time reverse transcription-polymerase chain reaction (RT-PCR) from snap frozen tissues as described in our previous studies.9,14

Quantification of Micrometastases
Micrometastases were measured by quantitating human DNA in mouse organs by quantitative real time RT-PCR using primer and probes directed towards a human-specific α-satellite DNA sequence of the centromere region of human chromosome 17 as previously described by our laboratory.9 The cycle threshold (CT) value obtained for human chromosome 17 was normalized using primers and probe that detected both mouse and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a measure of total DNA for the samples. ΔCT = CT value of human chromosome-17 minus CT value of mouse/human GAPDH. For incidence of metastasis, a ΔCT value below 27 was scored positive for metastasis to the mouse organ/tissue. For quantitative comparison of metastasis to different organs/tissue between groups, the data were presented as fold change = 2^−ΔΔCT where Cal-27/GFP alone was set as 1.

\[ \Delta \Delta CT = \Delta CT \text{ of Cal-27/GFP + ASC/RFP cells} - \Delta CT \text{ of Cal-27/GFP alone} \]

Incidence of micrometastases was assessed by counting the number of organs in a tumor group that exhibited a positive signal for human chromosome 17 DNA after the CT value obtained for human chromosome 17 was normalized using primers and probe that detected both mouse and human GAPDH as a measure of total DNA for the samples. ΔCT = CT value of human chromosome 17 minus CT value of mouse/human GAPDH. For incidence of micrometastases, a ΔCT value below 27 was scored positive for metastasis to the mouse organ/tissue. For mouse tissues in which no CT value was recorded for human chromosome 17 when a concurrent CT value for GAPDH was substantial, the tissue was scored negative for micrometastases.

Statistical Analysis
Statistical analysis of the data was performed using Graphpad Prism v5.0 software (La Jolla, CA). Data were expressed as mean ± SD. P < .05 was considered significant. The mean and standard deviation were calculated using Microsoft Excel or GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Statistical significance was determined by 2-sample t-tests (P < .05) (two-tailed) and 1-way ANOVA followed by Newman-Keuls multiple comparison test.

RESULTS
ASC Effect on Growth and Migration of Head/Neck Cancer Cells In Vitro
To assess the effect of ASCs on Cal-27 head and neck cancer cell growth in direct co-culture, Cal-27/GFP cancer cells were co-cultured with or without ASCs for 4 days. Fluorescence microscopy was used to count the number of Cal-27/GFP cells in the culture dishes (Supplementary Figure 1A). In addition, FACS was used to count the number of Cal-27/GFP cells present at day 4 cultured with or without ASCs (Supplementary Figure 1B). There was no difference in the number of Cal-27/GFP cells during co-culture with or without ASCs for either assay. Conditioned
medium (20% or 50%) from ASCs cultured in GCM did not affect Cal-27 cell growth in vitro after 48 hours culture (Supplementary Figure 1C). Similar results for the conditioned medium experiments were found with the SCC-4 head and neck cancer cell line (data not shown). Direct co-culture of Cal-27/GFP cells with ASC/RFP cells showed ASCs surrounding clusters of Cal-27 cells in culture, but an intermixture of ASC/RFP cells with Cal-27/GFP was not observed (Figure 1). A similar distribution of ASCs surrounding clusters of SCC-4 cells was also observed (data not shown).

To assess whether ASCs could stimulate migration of Cal-27 and SCC-4 cells, conditioned medium (CM) from ASCs cultured in ASC growth medium (growth conditioned medium; GCM), or CM from ASCs undergoing adipocyte differentiation (adipocyte differentiation conditioned medium; ADCM) was added to cultured Cal-27 and SCC-4 cells in the wound healing (scratch) assay. 20% and 50% GCM from proliferating ASCs stimulated migration of both Cal-27 and SCC-4 cells (Figure 2 and Supplementary Figure 2). ADCM also stimulated migration of Cal-27 and SCC-4 cells. Similar results were observed using conditioned medium from a second ASC donor (data not shown).

**ASCs Did Not Affect Primary Tumor Growth of Cal-27 Xenografts**

Cal-27/GFP cells were co-injected with or without ASC/RFP cells (1:1 ratio) into the flank of NUDE mice to determine the effect of ASCs on tumor growth and metastasis in vivo. Injection of Cal-27/GFP alone formed tumors of up to 1000 mm³ by 6 weeks post injection (Figure 3A). Injection of ASC/RFP alone did not form palpable masses. Co-injection of ASC/RFP cells with Cal-27/GFP cells resulted in a modest effect on increasing primary tumor volume in the early stages, but there was no significant effect on tumor volume at the end of the experiment (6 weeks) (Figure 3A). A control experiment in which

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**Figure 1.** Co-culture of Cal-27 cells with adipose-derived stem cells (ASCs). 2.5 x 10⁴ cells/well Cal-27/GFP cells (GREEN) were cultured with or without ASC/RFP cells (RED) at a 1:1 ratio in 6 well plates for 4 days and bright field and fluorescent microscopy photographs were taken on day 4. (A) Bright field picture. (B) Immunofluorescence for GFP. (C) Immunofluorescence for RFP. (D) Merge of GFP and RFP fluorescence.
BJ5TA fibroblasts were co-injected with Cal-27/GFP cells showed a similar result (Figure 3A and Supplementary Figure 3). Similar results for Cal-27/GFP tumor volume were observed with a second ASC donor (Supplementary Figure 4A). The excised, fresh whole Cal-27/GFP tumors showed evidence of GFP fluorescence throughout the tumors (Figure 3C) but no RFP fluorescence (Figure 3D). In the Cal-27/GFP + ASC/RFP tumors, both GFP (Figure 3G) and RFP (Figure 3H) fluorescence was evident in the excised, fresh whole tumors.

ASCs Were Integrated Within the Cal-27/GFP Tumors

Tumor morphology for co-injection with 1 ASC donor was assessed by sectioning and H&E staining. Fluorescence microscopy was used to distinguish Cal-27/GFP cells from ASC/RFP cells. The Cal-27/GFP group and Cal-27/GFP + ASC/RFP tumors exhibited a similar tumor morphology as evidenced from 5 µM thick H&E stained sections (Figure 3J and N). The Cal-27/GFP tumors exhibited GFP expression that overlapped with the majority of DAPI positively stained nuclei with no RFP expression detected (Figure 3K-M). In the Cal-27/GFP + ASC/RFP tumors, both GFP and RFP expression was detected in the same sections (Figure 3O-Q). As with the GFP expression, the RFP expression was coincident with DAPI-positive nuclei and was distinct from the majority of GFP expression (Figure 3Q). The frozen sections were 10 µM thick and represented at least 2 cell layers so that some overlap of GFP and RFP fluorescence was detected. These data demonstrate that ASCs were viable in the Cal-27 tumor up to 6 weeks post injection and were well integrated with Cal-27 tumor cells.

ASCs Stimulated Early Metastasis of Cal-27 Tumors to the Brain

Tumor-bearing mice did not exhibit visible metastases nor GFP or RFP fluorescence in fresh, whole mouse organs at the termination of tumor experiments. To detect early micrometastasis, DNA was prepared from fresh mouse organs (brain, bone marrow from femurs, kidney, lung, and liver) to quantitate the amount of human DNA in the mouse organs by quantitative real-time RT-PCR directed towards an α-satellite sequence specific for human chromosome 17.11,13 The incidence of micrometastases to an organ was defined as detection of a positive signal for human chromosome 17 DNA signal (see Methods). In 3 tissues (brain, kidney, lung) there was an increase in the incidence of micrometastases for the Cal-27/GFP + ASC/RFP compared with the Cal-27/GFP group. For the Cal-27/GFP + ASC/RFP group, the incidence of micrometastases to brain was 100% (4/4), to kidney was 50% (2/4), and to lung was 50% (2/4). For the Cal-27/GFP group, the incidence of micrometastases to brains was 60% (3/5), to kidney was 20% (1/5), and to lung was 20% (1/5). However, only in the brain was a quantitative increase in human chromosome 17 DNA level detected (21 fold); there was no quantitative difference in level of chromosome 17 DNA between the 2 groups for any other mouse tissue examined (Figure 4). As a control experiment to assess whether other cells could increase micrometastases, Cal-27 cells were co-injected with BJ5TA fibroblasts. BJ5TA fibroblasts did not increase the incidence of micrometastases or the quantitative level of chromosome 17 DNA in any mouse tissues (Figure 4).
cells into the systemic circulation as a result of the injection technique (data not shown).

In experiments repeated with a second ASC donor, the incidence of micrometastases to the brain was 33\% (1/3) in the Cal-27/GFP group and 83\% (5/6) in the Cal-27/GFP + ASC/RFP. In the other tissues (femur, kidney, liver, and lung) there was no difference between the 2 groups in the incidence of micrometastases (Supplementary Figure 4B). The level of chromosome 17 DNA detected in mouse tissues in experiments with ASC donor 2 was too low.
to detect quantitative differences in relative levels between the 2 groups.

**ASCs Increased Matrix Metalloproteinase Expression and Angiogenesis in the Primary Tumors**

Our previous study demonstrated that MDA-MB-231 breast tumor xenografts formed by coinjection with ASCs exhibited increases in matrix metalloproteinase-9 (MMP9; a marker of matrix degradation), vimentin (a mesenchymal marker), IL-8 (interleukin-8; a pro-inflammatory cytokine), vascular endothelial growth factor (VEGF; a pro-angiogenic hormone), and cluster of differentiation-31 (CD-31; a marker of increased microvessel density).9 These markers are hallmarks of tumors that exhibit an increase in metastatic properties. In Cal-27 tumors that were co-injected with 1 ASC donor, a similar set of markers was measured. Cal-27 tumors formed by co-injection with ASCs exhibited increases in MMP2 (Figure 5A-C), MMP9 (Figure 5D-F), IL-8 (Figure 5G-I), and CD-31 (Figure 5J-L), with no changes observed in vimentin, VEGF, e-cadherin, or beta-catenin (data not shown).

**DISCUSSION**

Head/neck surgery involves deforming and highly morbid procedures, with fat grafting holding promise in cosmetic repair of cavity defects. The opportunity to improve fat grafting efficacy without graft necrosis by supplementation of grafts with ASCs is an especially promising strategy. The present study was undertaken to determine the effect of ASCs on in vitro growth and migration and in vivo tumor formation and metastasis of established head and neck cancer cell lines. Results demonstrated that ASCs had no effect on Cal-27 and SCC-4 cell growth in vitro or Cal-27 tumor xenografts in mice in vivo. However ASCs stimulated in vitro migration of both Cal-27 and SCC-4 cells and increased early micrometastasis of Cal-27 tumor xenografts to mouse brain in vivo. These data indicate that further evaluation of ASC effects on head and neck cancer cell migration and metastasis is warranted.

ASCs from 2 donors had no effect on growth of Cal-27 or SCC-4 cell in co-culture using several different in vitro assays. The majority of studies examining effects of ASCs on cancer cells in vitro have examined breast cancer cells15-23 and reported mixed effects of ASCs on tumor cell growth. The present study is the first to report the effects of ASCs on head and neck cancer cell lines in vitro. In vitro, the ASCs surrounded clusters Cal-27 and SCC-4 tumor cells in distinct clusters but did not freely intermix with the tumor cells suggesting that ASCs may provide a stromal support system for the tumors cells.

Similarly to the in vitro results that showed no effect of 2 ASC donors on growth of Cal-27 or SCC-4 cells, co-injection of an equal number of ASCs from 2 donors in separate experiments had no effect on Cal-27 xenograft tumor volume. These data are remarkable since the number of cells injected for the Cal-27 + ASC group was 2 times the number of cells injected for the Cal-27-alone group. Given that part of the tumor volume was comprised of viable ASCs, this may suggest that fewer Cal-27 cells comprised the Cal-27 + ASC tumors compared with the Cal-27 alone tumors. For one of the ASC donors, we compared tumors from the Cal-27 alone group and the Cal-27 + ASC group and found that the tumor architecture was similar. The fluorescence data from whole tumors and tumor sections demonstrated that ASCs were viable at the time of sacrifice (6 weeks) and were well integrated within the tumors, although no distinct structures could be attributed to the viable ASC/RFP cells (Figure 3).

ASC donor effect and identifying properties of ASC donors that stimulate tumor growth and metastasis are critical issues and important future directions for research in this area. Studies using other tumor xenograft models from breast cancer or lung cancer have shown variable effects of ASCs on primary tumor growth with some studies demonstrating that ASCs increased primary tumor growth17,24 and
another study showing no effect on primary tumor growth.25 Our previous study showed that ASC effect on MDA-MB-231 tumor xenograft volume was dependent on the ASC donor, with 1 donor having no effect on tumor volume and a second donor significantly increasing tumor volume.9 We found that the donor BMI had a significant effect on in vitro growth and osteogenic differentiation of ASCs.26 In the present study we found that neither ASC

Figure 5. Adipose-derived stem cell (ASC) effect on tumor markers. Immunohistochemistry was conducted as described in Methods for markers of tumor morphology, angiogenesis, and proteins known to be modulated by ASCs. Paraffin-embedded tumor sections from Cal-27/GFP (A, D, G, J) and the Cal-27/GFP + ASC/RFP (B, E, H, K) groups were stained for MMP2 (A, B), MMP9 (D, E), CD-31 (G, H), and IL-8 (J, K). Bright-field photomicrographs were taken, and representative images are presented. Quantitative representation of the staining for matrix metalloproteinase 2 (MMP2; C), MMP9 (F), cluster of differentiation-31 (CD-31; I), and IL-8 (L) is indicated. No differences in expression of e-cadherin, vimentin, beta-catenin, or vascular endothelial growth factor were observed (data not shown). *$P < 0.05$, ***$P < 0.001$. 

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donor had a significant effect on tumor volume at the end of the experiment (6 weeks). Similarly, a donor effect was not observed in the in vitro growth and migration experiments with neither ASC donor promoting in vitro growth of Cal-27 or SCC-4 cells, and both ASC donors equally promoting Cal-27 and SCC-4 migration. However, there was a donor effect on the degree of Cal-27 metastasis that was promoted.

Studying ASCs from only 2 donors does not permit an identification of particular characteristics of ACSs that are consistent with promoting tumor growth and metastasis. Other potential limitations of the study include the 2 ASC donors were different in age and BMI, the use of clonal head/neck cancer cell lines and not patient-derived biopsy samples for the xenografts, and the limited time of the in vivo study (6 weeks). All of these limitations could have contributed to the different effects on metastasis observed from the 2 ASC donors, and/or the absence of significant metastasis observed in other mouse tissues. The design of the present study only permits speculation on the step[s] in the metastatic process that is promoted by ASCs. Future studies would need to use model systems that assess multiple steps of metastasis. It is noted, however that the ACSs from the first donor induced a partial epithelial to mesenchymal transition and MMP expression in the tumors that could be mediated by soluble factors released from the ASCs. It is possible that this may be a mechanism underlying the increased quantitative micrometastases observed with this donor. It is also noted that conditioned medium from both donors induced migration of Cal-27 and SCC-4 cancer cells in vitro, again suggesting that the early steps of the metastatic process are promoted by ASCs. Further speculation on mechanism are not warranted until a more systematic study evaluating multiple ASC donors effects on tumor growth and metastasis can be initiated, or studies using ASC donors that are genetically depleted of candidate pathways.

Although ASCs did not alter the growth of Cal-27 or SCC-4 cells in vitro, or Cal-27 tumor xenografts in mice, conditioned medium from ASCs from 2 different donors increased the migration of Cal-27 cell in vitro. These experiments used conditioned medium from both proliferating ASCs as well as ASCs undergoing adipogenic differentiation. In addition to demonstrating that release of paracrine factors by ASCs, and not direct cell contact, was sufficient to stimulate migration of Cal-27 cells, these data also demonstrate that both ASCs in a nondifferentiated, proliferating state, as well as ASCs differentiated towards adipocytes secreted factors that were sufficient to stimulate cancer cell migration. We found a similar result for ASC-conditioned medium stimulation of breast cancer cell migration.9

Human Cal-27 tumor cells exhibit high migration potential in vitro compared with other head and neck cancer cell lines, however no studies have observed visual macrometastatic lesions of Cal-27/GFP xenograft tumors in mice. The present study did not identify visual metastatic lesions for any organs in mice bearing Cal-27/GFP tumors. Micrometastasis of Cal-27/GFP tumors in mice was assessed using a very sensitive assay that quantitates the amount of human DNA in mouse tissues. The results demonstrated that ASCs from 1 donor significantly increased the quantitative level of human DNA (micrometastases) in the brain but not other mouse organs, and ASCs from a second donor increased the incidence of micrometastases (positive DNA signal) in the brain but not other mouse organs (Figure 4 and Supplementary Figure 4B). Although visual metastases in mouse organs were not evident from either tumor group, or with either ASC donor, the elevated level and incidence of human DNA in brain for the Cal-27/GFP + ASC/RFP group indicates that more human cells were seeded in the brain as a result of co-injection with ASC/RFP cells. For 1 ASC donor, there was also an increased incidence of micrometastases to the kidney and lung in the Cal-27 + ASC group. We found similar results in our previous study in which ASC/RFP cells were co-injected with MDA-MB-231 breast cancer cells and this resulted in increased metastasis to several mouse organs that was donor dependent.9 The present experiments were terminated at 6 weeks when the tumor burden became too large for the animal to survive; it is possible that additional time may have led to development of overt metastases in the mouse organs, and possibly an increased incidence and level of human DNA in other organs besides the brain. The detection of only early micrometastases in mouse brain and not overt lesions suggests that ASCs may facilitate the early steps of the metastatic process, such as escape from the primary tumor site, survival through the vasculature, or seeding in distant organs, but may not facilitate later metastatic outgrowth in the organs. Although it remains to be determined whether both Cal-27 and ASCs contributed to the positive human DNA signal in mouse organs, our previous study using MDA-MB-231 breast tumor xenografts demonstrated that ASC/RFP cells (RFP fluorescence) were not detected in any mouse organs that exhibited metastases.9 Furthermore, in control injections of ASC/RFP cells alone from 1 donor, a positive signal for human chromosome 17 DNA was not detected in any mouse tissues (data not shown).

CONCLUSIONS

In conclusion, human ASCs increased migration and micrometastasis of Cal-27 head/neck cancer cells predominantly to mouse brain, without significantly altering cell or tumor growth. The applied use of ASCs for supplementation of fat grafts for reconstruction after head and neck cancer surgery should be approached with caution until further studies are undertaken to determine the impact of ASCs on head and
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