An Animal Model of Local Breast Cancer Recurrence in the Setting of Autologous Fat Grafting for Breast Reconstruction

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ABSTRACT

Autologous fat grafting after breast cancer surgery is commonly performed, but concerns about oncologic risk remain. To model the interaction between fat grafting and breast cancer cells, two approaches were employed. In the first approach, graded numbers of viable MDA-MB-231 or BT-474 cells were admixed directly into human fat grafts and injected subcutaneously into immunodeficient mice to determine if the healing graft is a supportive environment for the tumor. In the second approach, graded doses of MDA-MB-231 cells were suspended in Matrigel and injected into the mammary fat pads of mice. Two weeks after the tumor cells engrafted, 100 μL of human adipose tissue was grafted into the same site. Histologically, MDA-MB-231 cells seeded within fat grafts were observed and stained positive for human-specific pan-cytokeratin and Ki67. The BT-474 adipose tissue was grafted into the same site. Histologically, MDA-MB-231 cells seeded within fat grafts did not support the epithelial BT-474 cell growth, and support the mesenchymal MDA-MB-231 cell growth only at doses ten times greater than in Matrigel controls. Moreover, fat grafts in association with MDA-MB-231 cancer cells already present in the wound resulted in decreased tumor proliferation and increased fibrosis. These findings suggest that clinical fat grafting does not induce breast cancer cell growth, and may even have a suppressive effect.

SIGNIFICANCE STATEMENT

Injectable adipose cell and tissue therapies are revolutionizing breast reconstruction; however, concerns about oncologic consequences exist. In this study, the most clinically relevant therapy was tested for impact on cancer cell growth. In contrast to prior published scientific studies, which tested cell extracts not representative of the clinical therapy used in patients, the results of this study showed no increased risk of cancer growth.

INTRODUCTION

Autologous fat grafting (AFG) for breast augmentation and reconstruction after breast surgery has been gaining popularity [1, 2]. The demand for breast reconstruction is growing as the prevalence of breast cancer increases worldwide. Fat tissue is abundant in the body and AFG is an excellent option for a less-invasive breast reconstruction technique that produces a natural appearance. Breasts reconstructed with autologous fat grafts are free from implant related complications of rupture, malposition, and capsular contracture. Drawbacks of breast fat grafting include potential interference with mammography, and loss of 40%–60% of injected volume during the healing process. Another potential drawback of fat grafting is risk that the procedure could promote the growth of any retained tumor cells in the surgical field. This concern is based on the premise that bioactive adipose-derived stem cells (ASCs), which are known to play a role in graft healing [3], may secrete paracrine growth factors that increase the chances of breast cancer recurrence.

Despite increasing clinical use of AFG for breast reconstruction, the interaction between breast cancer cells and fat tissue is still not well elucidated [4]. Local breast cancer is reported to recur in about 5%–22% of patients without fat grafting, depending on the stage of breast cancer.
and follow-up period [5]. Most clinical reports of outcomes after fat grafting to the breast do not suggest a higher recurrence rate [6, 7]. Basic science studies examining the relationship between isolated adipose stem cells and cancer cells, however, are discordant with clinical data. In our previous rodent studies, we found that ASCs enhanced proliferation of human metastatic pleural effusion cells in vitro, and CD90+ metastatic pleural effusion cell tumorigenicity was enhanced when they were co-implanted with ASCs in vivo [8]. Rowan et al. co-injected MDA-MB-231 cancer cells and human ASCs into female nude mice, and reported that human ASCs markedly increased MDA-MB-231 cancer cell migration and metastasis possibly via increased angiogenesis [9]. In clinical studies, Petit et al. performed matched cohort studies and found recurrence rates to be the same overall. The authors initially noted that a subgroup of younger patients with ductal carcinoma in situ (DCIS) had a higher rate of recurrence after fat grafting. Reanalysis at a later time point, however, showed that this effect was no longer present because the rate of recurrences in the control group had equalized [6]. A retrospective study of 72 patients by Ihrai et al. showed that autologous fat transfer does not seem to alter the detection of breast cancer, and the local recurrence rate after AFG does not appear to be higher [10]. Brenelli et al. found local recurrence in three (5%) of 59 early breast cancer patients with prior breast conservative surgery who underwent AFG [11]. However, their study lacked a control group.

To better elucidate the relationship between fat grafting and local breast cancer growth, a new animal model is needed. Models assessing isolated ASCs and breast cancer cells do not adequately model the interaction between grafted fat and cancer cells, and such models are absent from the published literature. The aim of this study was to develop an animal model using human breast cancer cell lines, human fat tissue, and immune-deficient mice. We hypothesized that exposing cancer cells to grafted fat tissue, as opposed to isolated stem cells, would not exacerbate tumor growth.

**Materials and Methods**

**Experimental Design**

Two approaches to modeling the interaction between fat grafts and cancer cells were used. In the first approach (approach 1), varying doses of MDA-MB-231 or BT-474 cancer cells were seeded directly into human fat grafts, injected into the subcutaneous tissues of immune deficient mice and excised at 6 weeks. The same cell doses seeded into Matrigel were injected as positive controls. At 6 weeks, all specimens were harvested and assessed for mass, volume, and histology. The growth and active proliferation of cancer cells within the fat grafts was compared with the growth and active proliferation within the Matrigel controls. This approach ensured that cancer cells would be directly exposed to the healing fat grafts and could determine whether the fat graft was a supportive environment for cancer cell growth. It represented the “worst case scenario” of contact between the fat graft and the cancer cells.

In the second approach (approach 2), varying doses of MDA-MB-231 cells suspended in Matrigel were injected into the mammary fat pads of immune deficient mice. After allowing 2 weeks for the tumor cells to engraft, a simulated breast reconstruction was performed by injecting 100 µl of human fat graft to the same site. This scenario models breast reconstruction in the setting of retained tumor. Tumor specimens were excised 6 weeks after injection (4 weeks after fat grafting), and assessed for mass, volume, and histology.

**Cancer Cell Characterization**

BT-474 (order-# HTB-20) and MDA-MB-231 (order-# HTB-26) breast cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA), and cultured with Hybricare (ATCC) and L-15 media (ATCC) containing 10% fetal bovine serum and antibiotics, respectively. BT-474 cells were cultured at 5% CO2 at 37°C and MDA-MB-231 cells were cultured under 0% CO2 at 37°C (Fig. 1A).

Trypsinized single-cell suspensions were stained for multi-dimensional flow cytometry using monoclonal mouse anti-human antibodies to cell surface proteins (CD73-PE [BD Biosciences, San Jose, CA, Cat No. 550257], E-Cadherin-biotin [R&D Systems, Cat No. 1BA1M18381], CD44-PE-Cy7 [Abcam, Cambridge, MA, Cat No. ab46793], CD90-APC [BD Biosciences, San Jose, CA, Cat No. 559869], and streptavidin-ECD [Beckman Coulter, Brea, CA, Cat No. IM3326]. Cells were stained for surface markers according to a previously published procedure (2 µl each added to the cell pellet, 15–30 minutes on ice) and fixed with 2% formalin and 50% ice-cold methanol. Cells were then permeabilized with 1% saponin (Beckman Coulter) in phosphate buffered saline (PBS) with 0.5% human serum albumin (10 minutes at room temperature) and cell pellets were incubated with 5 µl of neat mouse serum for 5 minutes, centrifuged, and decanted. The cell pellet was disaggregated and incubated with 2 µl of anti-pan cytokeratin-fluorescein isothiocyanate (FITC) (Beckman Coulter, Brea, CA, Cat. No. IM2356) for 30 minutes. Cells pellets were diluted to a concentration of 10 million cells/400 µl of staining buffer and 4',6-diamino-2-phenylindole (DAPI) (Life Technologies, Grand Island, NY, Cat. D1306) was added 10 minutes before sample acquisition, to achieve a final concentration of 7.7 mg/ml and 40 µl/106 cells [12–14].

**Sample Acquisition**

Multi-dimensional flow cytometric acquisition was performed using a Gallios cytometer (Beckman Coulter, Miami, FL). An effort was made to acquire a total of 1 million events per sample at rates not exceeding 3000 events/second. For DAPI staining, photomultiplier tubes were gain optimized for linear (cell cycle) detection of 2N cells [12–14]. The cytometer was calibrated to predetermined photomultiplier target channels prior to each use using SpectrAlign beads (Dako, Cat. No. KO1111) and 8-peak Rainbow Calibration Particles (Spherotech, Libertyville, IL, Cat. No. RCP-30-5A). Offline compensation and analyses were performed using VenturiOne software designed for multidimensional rare event problems (Applied Cytometry, Dinnington, Sheffield, U.K.). Spectral compensation matrices were calculated for each experiment using single-stained mouse IgG capture beads (Becton Dickinson, Cat. No. 552843) for each tandem antibody and hard stained beads (Calibrite, BD) for single molecule dyes (Becton Dickinson, FITC, PE [Cat.No. 349502], APC [Cat.No. 340487]).

**Graft Preparation**

Fat tissues were obtained from patients undergoing elective surgical procedures at University of Pittsburgh Medical Center (UPMC), with the procedure for tissue collection approved by the Institutional Review Board. Patients were female gender, aged between 35 and 60 years old, non-diabetic, and body-mass index less than...
cells or directly into individual fat grafts (300 μl) at exact doses of 10, 100, $1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$ or $1 \times 10^6$ cells per injection. Using DAPI for exclusion, the exact number of viable cells was sorted using a three-laser MoFlo high-speed cell sorter (Beckman-Coulter) equipped with a class I biosafety cabinet.

The fat grafts containing BT-474 and MDA-MB-231 cells were repeatedly passed back and forth between two Luer-Lok syringes (BD) interlocked by a connector to obtain homogenous mixtures. For the Matrigel group, the exact number of cancer cells suspended in 25 μl of buffer was mixed with 25 μl of Matrigel for an injection volume of 50 μl.

In approach 2, MDA-MB-231 cancer cells were sorted directly into 25 μl of medium containing $1 \times 10^3$ irradiated feeder cells at exact doses of 3, 10, 30, or 100, cells per injection. The different cell doses, each suspended in 25 μl of buffer, were then mixed with 25 μl of Matrigel for an injection (total injection of 50 μl).

**Animal Experiments**

All animal experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and approved protocols at the University of Pittsburgh. Eight to 10-week-old female non obese diabetic (NOD) scid gamma (NSG) mice (NOD.Cg-Prkdcscid il2rgtm1Wjl/Sjl) (Harlan Laboratories, Indianapolis, IN) were used. All injections were performed under general anesthesia. For approach 1, 25 μl of Matrigel (BD Biosciences, San Jose, CA) plus 25 μl of cell suspension was injected into the subcutaneous tissues using an insulin syringe (BD) with MDA-MB-231 cell doses of 0 (control, n = 5), 10 (n = 5), 100 (n = 5), $1 \times 10^3$ (n = 5), or $1 \times 10^4$ (n = 5), cells per injection, and BT-474 cell doses of 0 (n = 5), 10 (n = 5), 100 (n = 5), $1 \times 10^3$ (n = 5), $1 \times 10^4$ (n = 5), $1 \times 10^5$ (n = 5), or $1 \times 10^6$ (n = 5). Aliquots of 300 μl of human lipoaspirate plus 25 μl of cancer cells admixed were injected using a 16G blunt cannula (Coviden, Mansfield, MA) subcutaneously in mice with the same cancer cell dose groups as described above. The mice implanted with MDA-MB-231 or BT-474 cells were euthanized at 6 weeks. The grafts were explanted and their weights and volumes were measured using a balance (Sartorius Weighing Technology, Goettingen, Germany) and AccuPyc II 1340 Gas Pycnometer (Micromeritics, Norcross, GA), respectively.

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**Histological Assessments**

Grafts were fixed with 10% formalin for 24 hours and immediately embedded in paraffin. Lungs, livers, and spleens were collected from every mouse and paraffin blocks were made. Tissues were sectioned to 5 μm, deparaffinized, hydrated, and stained with

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**Figure 1.** (A): Microscopic imaging of BT-474 and MDA-MB-231 cells in culture. Each scale bar indicates 200 μm. (B): Phenotypic characterization of BT474 (left column) and MDA-MB231 (right column) cell lines. The top panels show identification of cytokeratin+ epithelial cells and DAPI+ nucleated cells. These were further phenotyped (green box) for CD44, CD90, CD73, and E-cadherin expression (bottom panels). Direct comparison of BT474 and MDAMB231 phenotypes confirmed that BT474 is a purely epithelial breast cancer cell line (Cytokeratin+, CD44+, CD90−, E-cadherin+, and CD73−), while the MDA-MB-231 cell line co-expresses mesenchymal markers CD44 and CD73 (Cytokeratin−, CD44+, CD90−, E-cadherin−, and CD73+). A small subset of the MDA-MB-231 cell line also co-expresses CD90 (<0.5%). Abbreviation: DAPI, 4′,6-diamino-2-phenylindole.

30 kg/m². Aspirated fat tissues were placed in 10 ml-syringes and centrifuged at 3,000 rpm for 5 minutes. Upper oil layers and lower aqueous layers were discarded, and the middle fat layer was used.

Cancer cells were counted using a Beckman Coulter Act 10 Hematology Analyzer (Beckman Coulter, Indianapolis, IN) and Vi-CELL Cell Viability Analyzer (Beckman Coulter, Indianapolis, IN) followed by DAPI staining (1:100) and resuspension in sterile auto-MACS Running Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany).

In approach 1, MDA-MB-231 Cancer cells were sorted directly into 25 μl of medium containing $1 \times 10^3$ irradiated feeder cells or directly into individual fat grafts (300 μl) at exact doses of 10, 100, $1 \times 10^3$, $1 \times 10^4$, or $1 \times 10^5$ cancer cells per injection. BT-474 cells were sorted directly into 25 μl of medium containing $1 \times 10^4$ irradiated feeder cells or directly into individual fat grafts (300 μl) at exact doses of 10, 100, $1 \times 10^3$, $1 \times 10^4$, or $1 \times 10^5$ cancer cells per injection. Using DAPI for exclusion, the exact number of viable cells was sorted using a three-laser MoFlo high-speed cell sorter (Beckman-Coulter) equipped with a class I biosafety cabinet.

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H&E. Paraffin sections were stained against monoclonal mouse anti-human pan-cytokeratin (dilution of 1:50, Clone MNF116, Dako, Glostrup, Denmark), and monoclonal mouse anti-human Ki-67 antigen (dilution of 1:100, Clone MIB-1, Dako). Described briefly, for antigen retrieval, sections were put in a sodium citrate buffer pH 6.0 and heated on a steamer to 95°C for 20 minutes. After washing, sections were blocked using an avidin/biotin blocking kit (Vector, Burlingame, CA), endogenous blocking kit (Dako), protein blocking kit (Dako), and 5% goat serum. Tissue sections were incubated with a primary antibody overnight at 4°C. For negative controls, PBS was used instead of a primary antibody. Slides were washed and then incubated with a secondary antibody (EnVision + Dual Link System-HRP, Code K4063, Dako) for 30 minutes at room temperature. After washing, tissue sections were incubated with an ABC kit (Vector) at room temperature for 15 minutes, washed, and developed with a deaminobenzidine.
also similar to that of fat grafts without cancer cells (Fig. 3A). The appearance of the BT-474 plus Matrigel grafts was also whitish and angiogenesis around the grafts was obvious (Fig. 3B, 3C). Fat grafts injected 2 weeks after engraftment of the MDA-MB-231 cells plus Matrigel, used in approach 2, had obvious increased vascularity upon harvest (Fig. 4A).

**Density Calculations of Lipo Grafts Seeded with Tumor Cells**

The average density of tumor in Matrigel was 1.2336 g/cm³. The average density of a lipo graft without tumor cells was 0.85729 g/cm³. Tumor mass and fibrosis in harvested lipo specimens that had been seeded with tumor cells was calculated using a formula below.

\[
\text{Tumor mass} = \text{graft mass} \times (\text{graft density} - 0.85729) / (1.2336 - 0.85729)
\]

Regression analysis showed no effect on tumor mass and fibrosis when human lipo was co-injected with MDA-MB-231 cells compared to Matrigel grafts (p = .231) (Fig. 2C). As more tumor cells were seeded within the lipo grafts, greater tumor mass and fibrosis were obtained (p < .001). There was no tumor mass or fibrosis when the cell dose was 0.

In the BT-474 grafts, higher cell doses were injected and heavier grafts were obtained (p < .001). Human lipo had a significant effect on tumor and fibrosis, as determined by density measurements, compared to the Matrigel grafts (p < .001) (Fig. 3D).

Comparison between MDA-MB-231 plus Matrigel and MDA-MB-231 plus Matrigel followed by human lipo showed that human lipo had a significant effect on tumor and fibrosis mass (p < .001). Cell dose dose-dependently affected tumor and fibrosis mass (p < .001) (Fig. 4C).

**Presence of Tumor Cells Within Specimens on Harvest**

In approach 1, in which tumor cells were admixed with fat grafts and Matrigel controls, MDA-MB-231 cells engrafted in 100% of the Matrigel controls at every cell dose. H&E staining showed multiple dark and big nuclei in cancer cells plus Matrigel grafts (Figs. 2D, 2E, 3F, 4D, 4E). Human-specific pan-cytokeratin was positive in the cytosol, which revealed they were cancer cells. When the MDA-MB-231 cells were seeded within lipografts, however, they only engrafted at a cell dose one order of magnitude higher than in Matrigel. BT-474 cells seeded into lipo grafts failed to engraft at any dose, with, neither pan-cytokeratin-positive nor Ki67-positive cells seen (Figs. 3E, 4E). BT474 cells engrafted and survived in Matrigel at cell doses starting at 100 cells per graft. Fat grafts, therefore, prevented a much less favorable environment for tumor cell engraftment and survival than the Matrigel controls and, in the case of BT474 cells, an unfavorable environment. In approach 2, MDA-MB-231 cells suspended and Matrigel engrafted at every dose.

**Tumor Cell Proliferation**

In the MDA-MB-231 plus 300 μl human lipo grafts, grafts in which 1 × 10⁴ and 1 × 10⁵ MDA-MB-231 cells were injected showed 100% positivity with cytokeratin/Ki67 positive tumor (Table 1). Ki67 positivity was 25.3% ± 4.9%, 8.8% ± 2.8%, 1.2% ± 0.6%, 0.0% ± 0.0%, and 0.0% ± 0.0% in the 10 × 10⁵, 1 × 10⁵, 100, 10,
Figure 3. Macroscopic appearance of BT-474 plus lipo; $1 \times 10^6$ and $100 \times 10^3$ BT-474 cancer cells were co-injected subcutaneously with 300 µl of lipo on right and left side of back (A). Macroscopic appearance of BT-474 plus Matrigel; $1 \times 10^6$ and $100 \times 10^3$ BT-474 cells were subcutaneously injected with 50 µl of Matrigel and media on the right and left abdominal side (B). $10 \times 10^3$, $1 \times 10^3$, 100, and 10 BT-474 cancer cells were co-injected with 50 µl of Matrigel plus media on upper left, upper right, lower left, and lower right of the back (C). (D): Regression analysis on graft mass and weight of tumor and fibrosis (g) between BT-474 plus lipo group and BT-474 plus Matrigel group. Regression analysis depicts the significant effect on tumor mass when lipo was co-injected ($p < .001$). As more tumor cells were injected, bigger tumor plus fibrosis masses were obtained ($p = .000$). Histological observations of BT-474 plus lipo (E) and BT-474 plus Matrigel (F) grafts. H&E (I), human specific pan-cytokeratin (II), Ki67 (III), and negative control (IV) are shown from left to right. Each scale bar depicts 200 µm. (G): Regression analysis of the effect of lipo on pan-cytokeratin/Ki67 positive BT-474 cancer cells. In the BT-474 plus Matrigel group, pan-cytokeratin/Ki67 positive tumors were obtained dose-dependently. No BT-474 plus 300 µl human lipo grafts showed pan-cytokeratin/Ki67 positivity ($p = .033$).
and 0 cancer cell-injected sites, respectively. In the MDA-MB-231 plus Matrigel group, pan-cytokeratin/Ki67 positive tumors were all 100% positive except for the 0 cancer cell-injected site. Ki67 positivity was 17.0% ± 2.6%, 21.2% ± 5.4%, 11.9% ± 6.3%, 19.4% ± 11.9%, and 0% ± 0%, respectively.

In the BT-474 plus Matrigel group, pan-cytokeratin/Ki67 positive tumors were obtained dose-dependently. No BT-474 plus 300 μl human lipo grafts showed pan-cytokeratin/Ki67 positivity (Table 2).

In the MDA-MB-231 plus Matrigel grafts, tumors were obtained from every site injected (Table 3). Pan-cytokeratin/Ki67 positive tumors were 86.7%, 93.3%, 53.3%, and 40.0% in the 100, 30, 10, and 3 cancer cell-injected sites, respectively. Ki67 positivity was 17.3% ± 3.1%, 26.1% ± 3.8%, 7.2% ± 3.1%, and 3.5% ± 2.3% in the 100, 30, 10, and 3 cancer cell-injected sites, respectively. In the MDA-MB-231 plus Matrigel followed by 100 μl human lipo grafts, the percentage of cytokeratin/Ki67 positive tumors was 80%, 80%, 40%, and 20% in the 100, 30, 10, and 3 cell-injected sites, respectively. Ki67 positivity was 11.5% ± 4.5%.

Table 1. Experimental groups and tumorigenicity: MDA-MB-231 plus lipo versus MDA-MB-231 plus Matrigel

| Survival time point (weeks) | Cell dose | Scaffold (± 10k irradiated feeder cells) | N | Obtained grafts | Cytokeratin+/Ki67+ | Ki67 positivity (Mean% ± SEM) |
|-----------------------------|-----------|----------------------------------------|---|-----------------|-------------------|-------------------------------|
| MDA-MB-231                  | 6         | 10,000                                 | 5 | 5               | 5 (100%)          | 25.3 ± 4.9                   |
|                             |           | 1,000                                  | 5 | 5               | 5 (100%)          | 8.8 ± 2.8                    |
|                             |           | 100                                    | 5 | 5               | 4 (80%)           | 1.2 ± 0.6                    |
|                             |           | 10                                     | 5 | 5               | 0 (0%)            | 0 ± 0                        |
|                             |           | 0                                      | 10| 10              | 0 (0%)            | 0 ± 0                        |
| MDA-MB-231                  | 6         | 10,000                                 | 5 | 4               | 4 (100%)          | 17.0 ± 2.6                   |
|                             |           | 1,000                                  | 5 | 4               | 4 (100%)          | 21.2 ± 5.4                   |
|                             |           | 100                                    | 5 | 3               | 3 (100%)          | 11.9 ± 6.3                   |
|                             |           | 10                                     | 5 | 2               | 2 (100%)          | 19.4 ± 11.9                  |
|                             |           | 0                                      | 5 | 0               | 0 (0%)            | 0 ± 0                        |

Table 2. Experimental groups and tumorigenicity: BT-474 plus lipo versus BT-474 plus Matrigel

| Cancer cell line | Survival time point (weeks) | Cell dose | Scaffold (± 10k irradiated feeder cells) | N | Obtained grafts | Cytokeratin+/Ki67+ | Ki67 positivity (Mean% ± SEM) |
|------------------|----------------------------|-----------|----------------------------------------|---|-----------------|-------------------|-------------------------------|
| BT-474           | 6                          | 1,000,000 | 300 μl human lipo                      | 3 | 3               | 0 (0%)            | 0 ± 0                        |
|                  | 100,000                    | 3         | 3                                      | 0 (0%)            | 0 ± 0                        |
|                  | 10,000                     | 4         | 4                                      | 0 (0%)            | 0 ± 0                        |
|                  | 1,000                      | 4         | 4                                      | 0 (0%)            | 0 ± 0                        |
|                  | 100                        | 4         | 4                                      | 0 (0%)            | 0 ± 0                        |
|                  | 10                         | 4         | 4                                      | 0 (0%)            | 0 ± 0                        |
|                  | 0                          | 8         | 8                                      | 0 (0%)            | 0 ± 0                        |
| BT-474           | 6                          | 1,000,000 | Matrigel                               | 5 | 5               | 3 (60%)           | 1.9 ± 0.9                    |
|                  | 100,000                    | 5         | 5                                      | 4 (80%)           | 3.9 ± 2.2                    |
|                  | 10,000                     | 5         | 4                                      | 4 (100%)          | 0.8 ± 0.7                    |
|                  | 1,000                      | 5         | 3                                      | 2 (66.7%)         | 0.4 ± 0.3                    |
|                  | 100                        | 5         | 2                                      | 1 (50%)           | 0 ± 0                        |
|                  | 10                         | 5         | 1                                      | 0 (0%)            | 0 ± 0                        |
|                  | 0                          | 5         | 0                                      | 0 (0%)            | 0 ± 0                        |
cells also resulted in decreased tumor cell proliferation index (% positive Ki67 stained tumor cells) (Fig. 2C). These cells showed that injection of more cancer cells correlated with bigger tumor masses (p < .001) (Fig. 4C). Additionally, lipo injection had a significant increasing effect on tumor and fibrosis mass (p = .000) (Fig. 4C). Histologically, MDA-MB-231 cancer cells are residing and proliferating (Fig. 3D), but the proliferation index by Ki67 is significantly reduced (Fig. 3G), suggesting that the fat grafting is associated with downregulation of proliferation in local tumor cells. Multiple studies show that mesenchymal stem cells may have a relationship with cancer cells and possibly enhance tumor growth. Muehlberg et al. reported that murine tumor growth was enhanced when mouse ASCs were co-injected rather than intravenously injected [15]. They also showed that intravenously injected ASCs homed to the tumor site. McAllister et al. reported that bone marrow-derived mesenchymal stem cells (BM-MSCs) are activated in the presence of tumors [16]. They found that activated BM-MSCs mimic the systemic effects imparted by instigating tumors when co-injected with indolent cells. The clinical significance of these studies is still unknown, and prompted our group to develop animal models that more accurately represent AFG in the setting of occult tumor. From our regression analysis data (Figs. 2F, 3G), lipo significantly reduces cancer cell proliferation. In this study, Matrigel contains several types of extracellular matrices and growth factors. However, it is not clinically relevant because it was originally isolated from the Engelbreth-Holm-Swarm mouse tumor. In this study, Matrigel was used for positive controls. Both BT-474 and MDA-MB-231 tumors were shown to be dose-dependent on number, weight, and volume. Additionally, even three MDA-MB-231 cells could form a tumor in Matrigel.

**DISCUSSION**

In approach 1, we showed that MDA-MB-231 cells resided and proliferated in human fat grafts 6 weeks after co-injection (Fig. 2D). These cells showed positive for human-specific pan-cytokeratin and Ki67, verifying that the cancer cells survived and proliferated in fat grafts. Breast cancer cells are usually traveling in the body once invasive carcinoma arises. This is the setting that traveling cancer cells migrate into fat grafts. The tumor mass in the fat graft mass was calculated using the formula. Regression analysis showed that as more MDA-MB-231 cancer cells were injected, a bigger graft mass was obtained (p < .001) (Fig. 2C). However, there was no significant difference on tumor plus fibrosis mass when lipo was co-injected with MDA-MB-231 (p = .231) (Fig. 2C). These results revealed that fat grafting did not accelerate MDA-MB-231 cancer cell proliferation and, importantly, tumor cell Ki67 staining was reduced. Fat graft decreased MDA-MB-231 cancer cells.

Histology showed BT-474 cancer cells could proliferate in Matrigel (Fig. 3F), but BT-474 cancer cells did not engraft and proliferate in human fat grafts (Fig. 3E). Therefore, the increased mass in the BT-474 plus lipo group was derived from fibrosis, not tumor formation. This fibrosis may be due to an inflammatory response elicited by the dying tumor cells. BT-474 could not survive in fat graft. The first approach was conducted to confirm that the cancer cells are adequately and consistently in direct contacting with the fat grafts. The interactions between cancer cells and healing fat grafts were then assessed. This approach represents a scenario in which the cancer cells are directly exposed to the regenerating fat grafts.

In approach 2, we successfully modeled the scenario of fat grafting in the setting of retained tumor in a rodent model. Grafts showed that injection of more cancer cells correlated with bigger tumor masses and sustainability of fat grafts. When tumors are formed, cancer cells are viable and proliferating. We have previously established a

| Cancer cell line | Survival time point (weeks) | Cell dose | Scaffold (1:10k irradiated feeder cells) | N | Obtained grafts | Cytokeratin+/Ki67+ | Ki67 positivity (Mean% ± SEM) |
|------------------|-----------------------------|-----------|----------------------------------------|---|----------------|-------------------|-----------------------------|
| MDA-MB-231       | 6                           | 100       | Matrigel followed by100 µl human lipo  | 5 | 5              | 4 (80%)           | 11.5 ± 4.5               |
|                  | 30                          | 5         | 5                                      | 4 (80%)           | 13.8 ± 6.6       |
|                  | 10                          | 5         | 5                                      | 2 (40%)           | 5.1 ± 3.5        |
|                  | 3                           | 5         | 5                                      | 1 (20%)           | 2.7 ± 0.9        |
| MDA-MB-231       | 6                           | 100       | Matrigel                               | 15 | 15             | 13 (86.7%)        | 17.3 ± 3.1               |
|                  | 30                          | 15        | 15                                     | 14 (93.3%)        | 26.1 ± 3.8       |
|                  | 10                          | 15        | 15                                     | 8 (53.3%)         | 7.2 ± 3.1        |
|                  | 3                           | 15        | 15                                     | 6 (40.0%)         | 3.5 ± 2.3        |
|                  | 0                           | 15        | 1                                      | 0 (0%)            | 0 ± 0            |

Table 3. Experimental groups and tumorigenicity: MDA-MB-231 plus Matrigel followed by lipo versus MDA-MB-231 plus Matrigel.
fat graft model in the mouse in which we quantified blood vessel growth using CD31 staining [20]. Taken together, 6 weeks are enough time to assess interaction between cancer cells and healing fat grafts in this study.

Sorting the exact number of cancer cells and directly seeding them into fat tissue or Matrigel is a novel method to develop an animal model for breast cancer recurrence in fat grafting. Tumors were obtained from three measured MDA-MB-231 cells plus Matrigel and 10 measured BT-474 cells plus Matrigel at minimum cell dosing. Histologically, human-specific pan-cytokeratin and Ki67 positivity shows that injected cancer cells were proliferating. This novel method enabled us to sort an exact and small number of breast cancer cells and obtain tumors from a small number of cancer cells. We successfully established new animal model in the

Histological imaging

D: MDA-MB-231 plus Matrigel followed by 100 μL human lipo

E:

I: H&E II: Pan-cytokeratin III: Ki67 IV: Negative control

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setting of retain tumor. Even in this worst-case scenario, fat grafting is not stimulating tumor growth.

**CONCLUSION**

This study is the first to accurately simulate the scenario of breast reconstruction with AFG in the setting of retained tumor in reproducible animal model. Additionally, it also tested the “worst case scenario” of tumor cells exposed to autologous fat grafts by actually seeding the grafts directly with tumor cells. This study showed that autologous fat grafts are not a supportive environment for the growth of tumor cells, as compared with Matrigel positive controls, and may even have a suppressive effect on tumor cell proliferation.

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**AUTHOR CONTRIBUTIONS**

W.T.: participated in designing the study, performed animal experiments, and wrote the first draft of this manuscript; J.E.V.: participated in designing the study and performed animal experiments; K.G.M.: designed the study, commented on the manuscript; A.D.D.: did flow cytometry, analyzed the data, did statistical analysis and supervised this study; V.S.D.: did flow cytometry, analyzed the data, and supervised the study; J.P.R.: designed the study, supervised this study, commented on and amended the draft. All the authors read and approved the final manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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