

In Vitro Effects of Adipose-Derived Stem Cells on Breast Cancer Cells Harvested From the Same Patient

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Introduction: Fat grafting for breast cancer (BrCa) reconstruction and breast augmentation has become increasingly more popular. A major area of debate and controversy is the effect of adipose-derived stem cells (ASCs) on remnant or undetected BrCa cells. We investigate the in vitro response of BrCa to ASCs in a coculture model with regards to cell migration.

Methods: The study was approved by the institutional review board. BrCa and adipose tissue specimens either from subcutaneous breast tissue or abdominal lipoaspirate were obtained from the same patient. BrCa cells and ASCs were harvested with either explant culture and/or enzymatic digestion. Tissues were grown in cell culture flasks until adequate cell libraries were established. Adipose-derived stem cells from adipose specimens were characterized with flow cytometry. Immunofluorescence (IF) staining of the initial cell population harvested from the BrCa specimens confirmed the presence of CD24, an epithelial marker of BrCa. A homogenous CD 24+/CD 90–BrCa cell population was obtained with flowcytometric cell sorting. The in vitro migration of BrCa cells was examined in coculture with and without ASCs.

Results: Adipose-derived stem cells harvested from the adipose specimens were positive for mesenchymal stem cell markers CD 105, CD 90, CD 73, and CD 44 and negative for lymphocyte cell marker CD 34 and leukocyte marker CD 45. The percentage of the CD 24+/CD 90–BrCa cells in the initial cell population harvested from BrCa specimens was 0.61%. The BrCa cells morphologically had large nuclei and small cytoplasm in clusters under the light microscope, suggesting a cancer cell phenotype. CD 24 expression on the surface of BrCa cells was confirmed with IF staining. The number of BrCa cells migrated in ASCs coculture was approximately 10 times higher than the number of BrCa cells migrated in BrCa cell only cultures.

Conclusions: Adipose-derived stem cells significantly increase the migration capacity of BrCa cells in vitro in cocultures. This should be taken into consideration when performing fat grafting to the breast especially in patients with a history of BrCa or strong family history of BrCa.

Key Words: fat grafting, breast cancer recurrence

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Breast cancer (BrCa) is the most common cancer in women and an increasing number of women are seeking postmastectomy breast reconstruction since the passage of the Women's Health and Cancer Rights Act in 1998.¹ In conjunction with the increased number of cases, autologous fat grafting to the breast has become increasingly popular for postmastectomy reconstruction as well as cosmetic augmentation.² However, debate and controversy between surgeons and scientist over the safety of autologous fat grafting to the breast with regards to BrCa recurrence has been a hot topic in the plastic surgery community, especially after the discovery of adipose-derived stem cells (ASCs) in fat tissue.^{3–5}

The approach of plastic surgeons to fat grafting also changed considerably in response to ongoing debate and new scientific findings.

In 1987 the American Society of Plastic Surgeons (ASPS) banned autologous fat grafting to the breasts with concerns over the ability to detect and differentiate BrCa from fat necrosis.⁶ In 2007, with advancements in BrCa screening technology, the ASPS revisited autologous breast fat grafting agreeing the procedure can be useful and safe, but results remained unpredictable.⁷ In 2009, the ASPS lifted the ban and set forth the Fat Graft Task Force to monitor the procedure warning surgeons to proceed with “caution when considering fat grafting procedure in patients at high-risk for BrCa.”⁸

As of today, there is still no consensus on the safety of breast fat grafting in terms of BrCa recurrence. The discrepancy between clinical and basic science studies with regards to BrCa recurrence after breast fat grafting is rampant in the literature.⁹ Although clinical studies have failed to show an increase in BrCa recurrence after fat grafting to the breasts, many basic science studies have shown potentially dangerous effects of ASCs on BrCa cells. On one hand, advocates of clinical fat grafting to the breast argue that the procedure is technically safe with low complication rates, can be performed in an outpatient setting with minimal donor site morbidity, and offers autologous tissue transfer without microsurgical expertise or resources. They further argue that the majority of basic science studies use purchased banked BrCa cell lines, which are more hardy and resilient than what is often clinically encountered. These studies are also limited by using adipose tissue and BrCa specimens harvested from different patients. On the other hand, scientists opposed to clinical fat grafting argue that the clinical studies are limited by few prospective trials, short overall follow-up time frames, and small study populations.^{10–17}

Our aim in this study is to investigate the in vitro response of BrCa cells to ASCs from the same patient in a coculture model with regards to cell migration in a more clinically applicable model, specifically eliminating the use of banked resilient cancer cell lines and the use of adipose and BrCa specimens from different patients. We strive to answer the question: Is ASC enriched fat grafting to breast safe following oncologic surgery of the breast with potential remnant BrCa and is this procedure safe for augmentation in young woman with a strong family history of BrCa with potentially undetected or dormant cancer cells in the breast?

METHODS

The study was approved by the institutional review board (approval 254494). Adipose tissue, in the form of lipoaspirate or subcutaneous tissue, and primary BrCa specimens, were obtained from the same patient, a 67-year-old white woman. Adipose-derived stem cells and BrCa cells were harvested from human tissues as follows.

Harvesting of ASCs

To harvest ASCs, adipose tissue was digested enzymatically using 0.15% (w/v) type I collagenase (Calbiochem, San Diego, CA) at 37°C with vigorous shaking. The effect of the enzyme was neutralized by addition of an equal volume of cell growth medium (Dulbecco Modified Eagle Medium [DMEM; Gibco, Grand Island, NY] containing 10% (v/v) fetal bovine serum [FBS; Corning, Manassas, VA], and 1% antibiotic-antimycotic solution [Sigma, St Louis, MO]). The mixture was centrifuged at 1500 rpm for 5 minutes. The resultant cell pellet was seeded onto 75-mm² tissue culture flasks (Nunc, Denmark) after cell counting using trypan blue. The cells in culture flasks were

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maintained in a 37°C incubator with 5% CO₂. Fresh medium was added every 3 to 4 days, and the cells were subcultured at 80% to 90% confluence using TrypLE (Gibco). Cells from passage II to IV were used for all experiments.

Harvesting of BrCa cells

Breast cancer cells were harvested after a modification of a previously published protocol.¹⁸ Briefly, BrCa tissue was minced using a crisscross motion with 2 disposable scalpels until finely chopped. The minced tissue was transferred to a 50-mL conical centrifuge tube containing 1× collagenase and/or hyaluronidase solution (Stem Cell Technologies, Vancouver, BC, Canada) at a final volume of 10 mL/g of tissue. Breast cancer tissue was digested at 37°C with vigorous shaking for

2 hours. The effect of the enzyme was neutralized by addition of an equal volume of cell growth medium (DMEM containing 10% (v/v) fetal bovine serum and 1% antibiotic-antimycotic solution). The mixture was centrifuged at 1500 rpm for 5 minutes. The resultant cell pellet was seeded onto 75-mm² tissue culture flasks after cell counting using trypan blue. The cells in culture flasks were maintained in a 37°C incubator with 5% CO₂. Fresh medium (DMEM containing 20% (v/v) FBS, and 1% antibiotic-antimycotic solution) was added every 3 to 4 days, and the cells were subcultured at 80% to 90% confluence using TrypLE.

Characterization of ASCs

We analyzed the immunophenotype of ASCs using BD Stemflow Human MSCs Analysis Kit (BD Biosciences, Franklin Lakes, NJ).

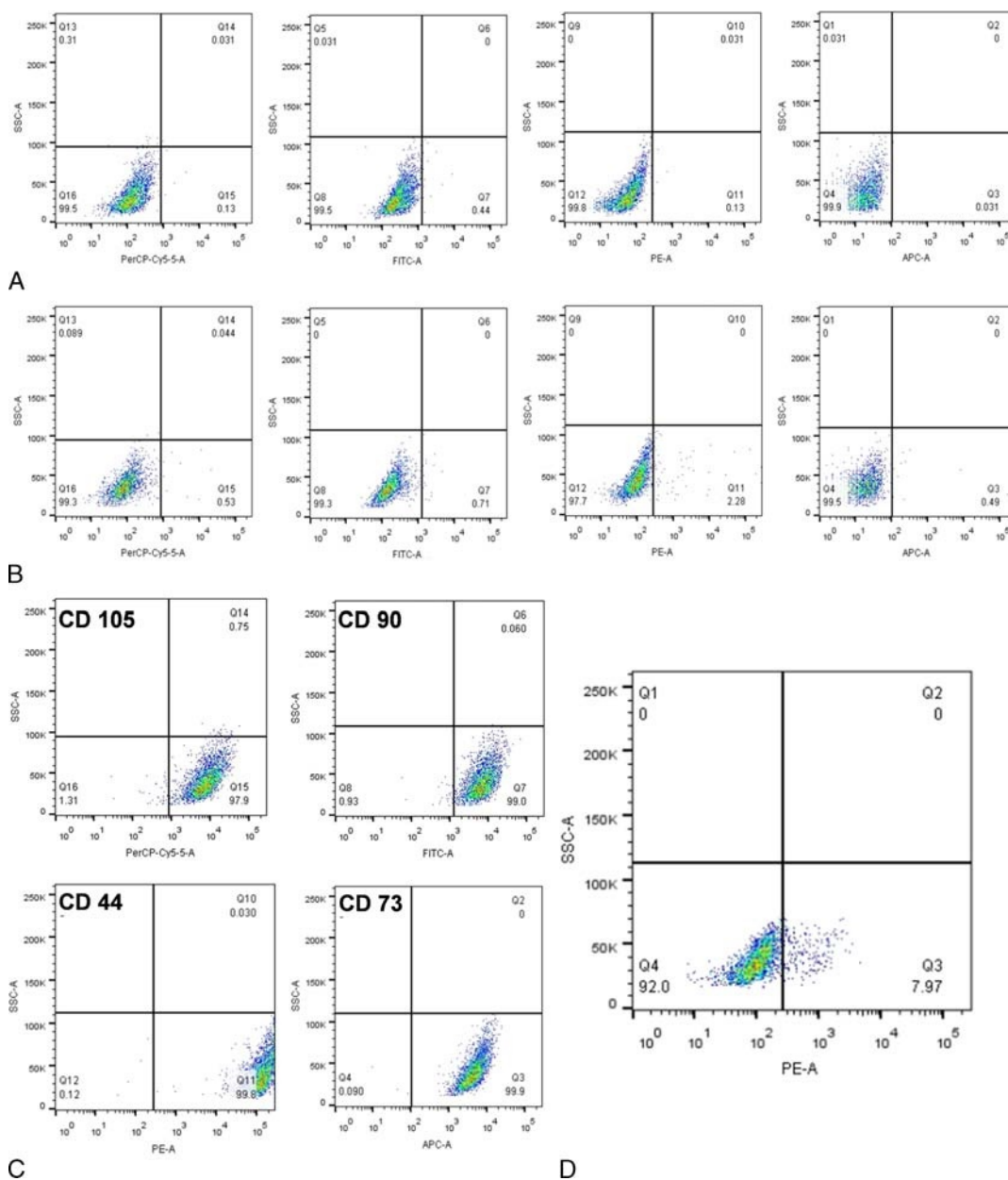


FIGURE 1. A, Unstained ASCs were negative for all fluorochromes used for flow cytometry. B, Isotype controls confirmed the lack of nonspecific binding of antibodies to cell surface markers. C, ASCs expressed mesenchymal stem cell markers strongly. D, Negative cocktail: ASCs did not express CD 34, CD 11b, CD 19, CD 45, and HLA-DR.

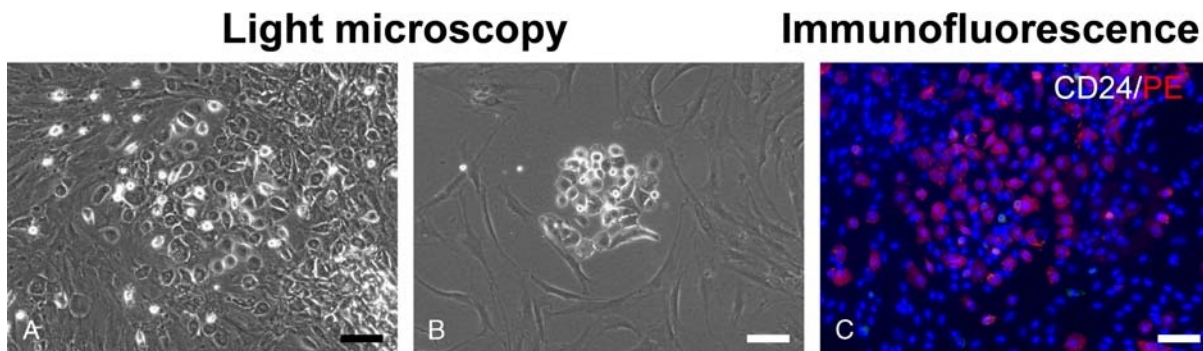


FIGURE 2. A and B, The BrCa cells in the primary cell cultures formed colonies and they were morphologically different from the surrounding ASCs under light microscopy as exhibited by round cytoplasm and bigger nuclei. C, BrCa cells also stained positive for epithelial BrCa marker CD24. Microbars, 50 μ .

Adipose-derived stem cells in suspension were incubated with Phycoerythrin (PE)-coupled antibody for rat CD44, fluorescein isothiocyanate-coupled antibody for CD90, and peridinin chlorophyll-Cy 5.5 coupled antibody for CD105, and APC-coupled antibody for CD73 in the dark, at room temperature for 30 minutes. PE mouse IgG2b, k antibody was used as isotype control and a negative cocktail containing CD 34-PE, CD 11b-PE, CD 19-PE, CD 45-PE, and HLA-DR-PE was used to document the lack of expression of lymphocyte, leukocyte, and hematopoietic stem cell markers. After primary incubation, the cells were washed with wash buffer (0.5% FBS in phosphate buffered saline) and were fixed in neutral 4% paraformaldehyde solution for 30 minutes. A Fortessa Flow cytometer (BD Biosciences) was used to detect the cellular markers expressed on the surface of ASCs.

Isolation and Characterization of a Homogenous Population of BrCa Cells

Immunofluorescence staining was performed using mouse anti-human CD24 antibody (R&D Systems, Minneapolis, MN) to verify the presence of CD 24, an epithelial marker for BrCa, in primary BrCa cell cultures. Antimouse AF488 (Abcam, Cambridge, MA) was used as a secondary antibody. After immunofluorescence staining, flow cytometric cell sorting was performed to isolate a CD24+/CD90- homogenous BrCa cell line using the primary cell cultures obtained by enzymatic digestion of patient samples. Briefly, cells were incubated with a mouse anti-human CD24 antibody conjugated with fluorescein isothiocyanate (AbCam) for 30 minutes at room temperature. Cells are washed with wash buffer and sorted with an Influx cell sorter (BD Biosciences). Sorted cells were plated in 24-well cultures dishes and expanded.

Coculture of ASCs and BrCa Cells

Breast cancer cells were plated on the semipermeable insert of the Cytoselect 24-well cell migration assay (Cell Biolabs Inc., San Diego, CA), whereas either cell growth medium alone or cell growth medium with ASCs were plated in the lower chamber. The system was incubated for 18 hours overnight. The inserts were washed with a lysis buffer in a clean well to detach migrated BrCa cells, and the fluorescence of migrated BrCa cells was detected using a fluorescence plate reader (SpectraMax i3x Multi-Mode Detection Platform; Molecular Devices, Sunnyvale, CA).

RESULTS

Isolation and Characterization of ASCs and BrCa Cells

Adipose-derived stem cells were positive for mesenchymal stem cell markers CD44 (99.8%), CD90 (99.0%), CD105 (97.9%), and CD73

(99.9%) and negative for lymphocyte, leukocyte and hematopoietic stem cell markers CD 34, CD 11b, CD 19, CD 45, and HLA-DR (Fig. 1).

The BrCa specimen was obtained from an infiltrating ductal breast carcinoma, estrogen receptor-/progesterone receptor-/Her2neu+. Breast cancer cells in the initial cell cultures obtained from patient samples formed colonies in the cell cultures flasks distinguished by round cells with large hyperchromatic nuclei resembling the well-known characteristics of malignant cells (Figs. 2A and B). Breast cancer cells also stained positive for CD24, whereas the ASCs were negative for CD24 (Fig. 2C). We were able to obtain a homogenous BrCa cell population from the initial cell cultures using flowcytometric cell sorting (Fig. 3). The BrCa cell population was 0.61% of the initial cell population.

Coculture of ASCs and BrCa Cells

The migration of BrCa cells increased significantly ($P < 0.05$) when cocultured with ASCs as shown by increased fluorescent signal

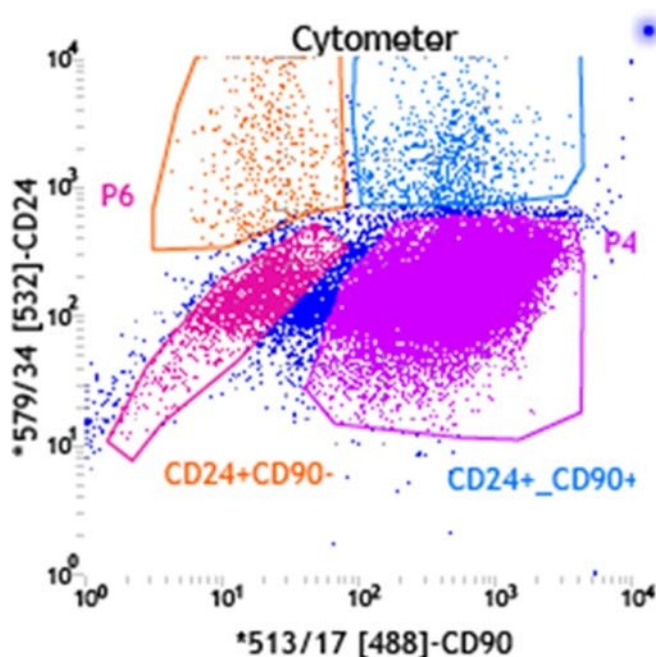


FIGURE 3. CD24+/CD90- BrCa cells (upper left quadrant) were sorted with flow cytometry.

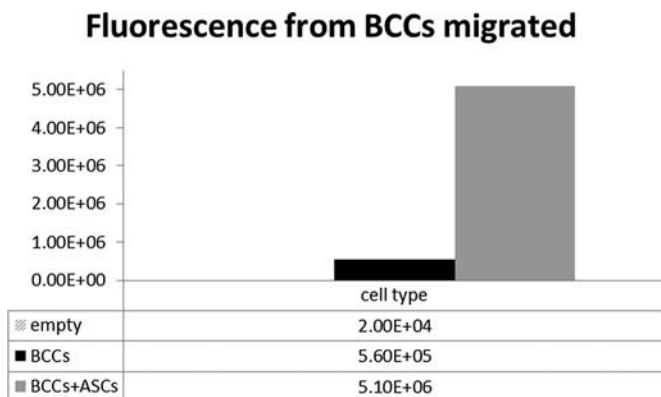


FIGURE 4. Cell migration assay showed that the migration of BrCa cells increased almost 10-fold when cocultured with ASCs.

from the wells containing BrCa cells and ASCs in comparison to wells containing only BrCa cells and cell growth medium (Fig. 4).

DISCUSSION

Basic science studies have established that ASCs not only communicate with BrCa cells through paracrine factors, but also make direct contact, thus increasing the expression of genes for inflammation, proliferation, migration, and invasion, suggesting an active role of ASCs in tumorigenesis.^{19–21} Additionally, xenograft murine models have demonstrated increased primary tumor growth and increased metastatic disease burden, specifically at first pass organs including the liver and lungs when BrCa cells are coinjected with ASCs.^{22,23} Coinjection of ASC and BrCa cells also lead to elevated levels of paracrine factors IL-8 and vascular endothelial growth factor. This finding suggests that these paracrine factors are involved with enhanced BrCa migration, potentially through increased angiogenesis.²² Additionally, Eterno et al²³ reported that hepatocyte growth factor/c-Met crosstalk between ASCs and BrCa cells may play a central role in enhanced BrCa cell migration, metastasis, and sustained tumor self-renewal. Other paracrine factors that were shown to have a role in ASCs BrCa cell interaction are: stromal cell-derived factor-1,²⁴ IGF, TGF β -1, Bcl-2, and IL-10.²⁵

Despite the replicable basic science data suggesting the potential hazards of ASCs in the presence of BrCa cells, clinical studies have failed to show an increased BrCa recurrence rate compared with the general population with 1 exception. Petit et al²⁶ published one of the larger retrospective reviews of 513 patients in 2011 looking at BrCa recurrence after breast fat grafting. We believe a more detailed analysis of other published patient series may yield a similar correlation with fat grafting and BrCa recurrence rates. The following year, 321 patients were analyzed against 1:2 matched cohorts for BrCa recurrence after breast fat grafting without a significant difference; however, a subset analysis of patients with intraepithelial neoplasm revealed a 10.8% local recurrence (4 of 37 patients) compared with no local recurrence in the cohort.²⁷ In 2013, Petit's team published a matched cohort study of 59 patients with intraepithelial breast neoplasm undergoing breast fat grafting and found a significantly higher 5-year cumulative risk of local recurrence at 18% compared with 3% in the matched cohort.²⁸

One major area of debate is the clinical applicability of the basic science research. Observing BrCa cell interaction to ASCs harvested from a different patient is different from the autologous fat grafting used in current practice. In addition, purchased banked BrCa cell lines tend to be more hardy and resilient than the average BrCa cells encountered clinically.²⁹ However, the biggest flaw with basic science studies is the high concentration of ASCs used in experiments compared with the intraoperative autologous fat graft harvested via lipoaspirate. Fat grafts have been shown to typically yield $4.0 \times 10^5 \pm 2.0 \times 10^5$ ASCs per

milliliter of lipoaspirate and $0.7 \times 10^6 \pm 0.1 \times 10^6$ stromal vascular cells per gram of adipose tissue.^{30,31} Even when comparing Yoshimura's technique of cell-assisted lipotransfer, which combines processed stromal vascular fraction with adipose lipoaspirate to create an ASC-rich fat graft, the ASC concentration is much smaller than ex vivo expansion techniques described in the basic science studies.^{16,32,33}

Recent Food and Drug Administration guidelines for human cells, tissues, and cellular and tissue-based products from adipose tissue: regulatory considerations, labeled 21 Code of Federal Regulations allows the intraoperative use of autologous fat grafting to the breast even after centrifuging as an exemption which would otherwise restrict its use.³⁴ An example to this exemption is Coleman technique of fat grafting after centrifuging the lipoaspirate to concentrate ASCs in the fat graft, which has been shown to be technically easy and safe as well as increasing graft retention.^{35–37} However, until further data can be obtained, we would caution surgeons using techniques that enrich the fat graft with ASCs. We feel fat grafting offers the plastic surgery patient many exciting and innovative potential advantages, but the safety of its use in BrCa reconstruction and augmentation in patients with a strong family history of BrCa remains unclear at this time. Although our data show that ASCs increase the migration potential of BrCa cells harvested from the same patient, we recognize that no substantial conclusions can be made from a single patient observation. Despite harvesting adipose and BrCa tissue from 10 patients in total, we were only successful in creating and expanding a viable cell line of ASCs and BrCa cells from 1 patient. We believe the great challenge in establishing viable cell lines from the same patient can be explained by advances in screening technology for BrCa, resulting in small, often subcentimeter, primary BrCa specimens, although we do not advocate for a cease of breast fat grafting, we would recommend using techniques that do not enrich the graft with ASCs in addition to including a discussion of the conflicting data of clinical and in vivo/in vitro studies in the informed consent.

CONCLUSIONS

Given the conflicting data in clinical and basic science studies concerning the oncologic safety of breast fat grafting, more data in the form of prospective randomized trials must be obtained before making a concrete decision on the practice of breast fat grafting. We feel it is paramount to include a discussion with patients during informed consent covering the lack of clinical data to suggest increased risk but the existence of basic science studies, demonstrating potentially adverse effects of ASCs on remnant or undetected BrCa cells. Although we cannot make any strong conclusions on the practice of breast fat grafting, we would advise against grafting ASC-enriched fat grafts until more data can provide a better understanding on the potential effects of fat grafting to the breast.

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