Observed Changes in the Morphology and Phenotype of Breast Cancer Cells in Direct Co-Culture with Adipose-Derived Stem Cells

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Background: Regarding aesthetics and long-term stability, cell-assisted lipotransfer is a promising method for breast reconstruction. Here, autologous fat grafts enriched with autologous adipose-derived stem cells are transferred. However, as adipose-derived stem cells secrete high amounts of growth factors, potential risks of tumor reactivation remain. In this study, influences of adipose-derived stem cells on inflammatory breast cancer cells were evaluated in a direct co-culture system.

Methods: Human adipose-derived stem cells were isolated and cultivated either alone or in a direct co-culture with the inflammatory breast carcinoma cell line T47D. At different time points, cell morphology was observed by scanning electron microscopy, cell membranes were stained by immunofluorescence, and gene expression was analyzed by real-time polymerase chain reaction.

Results: In co-cultures, T47D breast carcinoma cells showed tumorsphere-typical growth surrounded by a monolayer of adipose-derived stem cells. Direct cell-to-cell contacts could be observed between the two different cell types. Immunofluorescence revealed vesicular exchange and fusion between carcinoma cells and adipose-derived stem cells. Expression levels of transcriptional genes for typical malignancy markers were substantially higher in co-cultures compared with single cultures.

Conclusions: Direct intercellular contact between carcinoma cells and adipose-derived stem cells by means of exosomal vesicular exchange was revealed. Breast cancer cells displayed a change towards a more malignant phenotype associated with higher rates of metastasis and worsened prognosis. As cell-assisted lipotransfer is often performed after breast cancer surgery, transfer of adipose-derived stem cells might lead to deterioration of prognosis in case of recurrence as it has been described for inflammatory breast cancer. (*Plast. Reconstr. Surg.* 134: 414, 2014.)

A new and promising technique for restoration of the breast following ablative surgery besides the standard lipotransfer technique is the so-called cell-assisted lipotransfer.[1] In this technique, one portion of the lipoaspirate is separated, digested, and centrifuged to obtain the stromal vascular fraction in which there is a resident population of mesenchymal stem cells (adipose-derived stem cells). This fraction is mixed with the other portion of untreated lipoaspirate and injected for breast reconstruction. Adipose-derived stem cells as a subtype of mesenchymal stem cells have been shown to possess a highly angiogenic potential and thus are thought to improve fat survival in transplant grafts.[2]

However, as these cell-based therapies are used more often and tumor cells might still

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Received for publication November 5, 2013; accepted May 28, 2014.
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Presented in part at the 17th Surgical Research Days, in Frankfurt, Germany, October 4 through 5, 2013; the Second Congress of the International Society of Plastic Regenerative Surgery, in Berlin, Germany, June 7 through 9, 2013; the 130th Congress of the German Society of Surgery, in Munich, Germany, April 30 through May 3, 2013; and the Seventh Fraunhofer Life Science Symposium, in Leipzig, Germany, November 29 through 30, 2012.

DOI: 10.1097/PRS.0000000000000525

Disclosure: *None of the authors has a financial interest in any of the products or devices mentioned in this article. This study received no external funding.*

reside in breast cancer patients, the question of the safety of such therapies arises concerning tumor induction or at least promotion of tumor growth.[3] Mesenchymal stem cells can support tumor growth indirectly by secreting angiogenic effectors such as vascular endothelial growth factor and hepatocyte growth factor. Mesenchymal stem cells might as well participate in building carcinoma-associated stroma, which plays a major role particularly in breast cancer, as carcinoma-associated stroma–resident fibroblasts promote tumor growth.[4] The surrounding stroma also has been found to support metastasis and invasiveness.[5] Recently, mesenchymal stem cells that are resident in tumor stroma have been described to favor tumor growth[6,7]; they may engraft and/or transdifferentiate to tumor tissue, directly promoting its growth. In recent studies, mesenchymal stem cells were discussed as the origin for certain sarcomas, indicating that mesenchymal stem cells are able to transform to tumor cells themselves and thus initiate tumorigenesis.[8]

In this study, we investigated mechanisms of stem cell–tumor cell interaction. Direct cell-to-cell contacts were visualized by scanning electron microscopy and follow-up of vesicular exchanges. In addition, we performed cell-cycle analysis to determine the fraction of proliferating cells, and secretion of inflammatory cytokines was measured by enzyme-linked immunosorbent assays. The gene expression of breast cancer markers, in particular, those associated with a bad prognosis, were determined by quantitative polymerase chain reaction.

PATIENTS AND METHODS

Isolation and Expansion of Adipose-Derived Stem Cells

Adipose-derived stem cells were isolated from human donors following dermolipectomy operations with the written consent of the patients as described earlier.[9] No approval was needed as stated by the Research Ethics Committee of the Medical School Hannover (Hannover, Germany). Cells were cultured in adipose-derived stem cell growth medium containing Dulbecco's Modified Eagle Medium/F12 medium without Ca^{2+}/Mg^{2+} (PAA Laboratories GmbH, Pasching, Austria), supplemented with 20% fetal calf serum, 1% gentamicin (10,000 µg/ml), and 0.1% ascorbate-2-phosphate (all from Biochrome AG, Berlin, Germany). The cells were characterized by flow cytometry for CD73, CD90, and STRO1 (positive); and CD11b and CD14 (negative) (all antibodies and Cytomics FC500 by Beckman Coulter Germany GmbH, Krefeld, Germany). In addition, representative samples were differentiated into adipogenic, chondrogenic, and osteogenic lineages as described elsewhere.[10]

The T47D cell line representing an estrogen receptor-α infiltrating ductal carcinoma cell line of the breast was obtained from LGC Standards GmbH (Wesel, Germany). T47D cells were cultured with Roswell Park Memorial Institute-1640 medium with 2 mM L-glutamine (T47D cell growth medium; PAA).

For all experiments, either adipose-derived stem cells from passage 2 or T47D cells from passage 12 were used. For co-cultures, a medium containing 50 percent adipose-derived stem cell growth medium and 50 percent T47D cell growth medium was used (co-culture medium), and the incubation time was 14 days except for analysis of vesicular exchange. Light microscopy was performed on an Olympus CK-40 inverse with an Olympus Color View camera and Olympus CellD acquisition software (Olympus Life Science Research Europa GmbH, Munich, Germany).

If not stated otherwise, experiments were carried out in duplicate at three different time points. Adobe Photoshop CS3 (Adobe Systems GmbH, Munich, Germany) was used for combining the panels to figures and adjustment of contrast and brightness.

Scanning Electron Microscopy

For scanning electron microscopy, round glass coverslips (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were placed in 12-well plates and used for adipose-derived stem cell and T47D cell co-cultures for 14 days. Specimens were fixed in sodium cacodylate buffer (Merck, Darmstadt, Germany) with a pH set at 7.3, containing 2.5% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) for 24 hours, dehydrated by increasing acetone dilutions and dried with a CPD030 (Bal-Tec, Balzers, Liechtenstein) by critical point drying, followed by gold sputtering with a scanning electron microscopy coating system (Polaron Instruments, East Grinstead, United Kingdom). Specimens were placed in a vacuum and viewed in a SEM500 microscope with a beam of 50 nm and a cathode potential of 10 kV cathode voltage (Philips, Hamburg, Germany). Photographs were taken with software developed by Gebert and Preiss.[11]

Cell Membrane Labeling

To visualize vesicular exchange as evidence for cell-to-cell contacts, cell membranes of either

adipose-derived stem cells or T47D cells were labeled with PKH26 (adipose-derived stem cells) or PKH67 (T47D cells) according to the manufacturer's instructions (both from Sigma-Aldrich). Thereafter, cells were incubated as co-cultures for 1, 2, 4, 7, and 14 days. After these time points, specimen were fixed for 20 minutes with 4% paraformaldehyde (Sigma-Aldrich) at room temperature and embedded in Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, Calif.) for cell nuclei staining. Specimen were then viewed with a Zeiss Axiovert 200 M inverse fluorescence microscope and photographed with Axiovivio Rel 4.6 acquisition software (Zeiss, Jena, Germany). Wavelengths used were 567 nm for visualizing PKH26 and 502 nm for visualizing PKH67.

Enzyme-Linked Immunosorbent Assay

Quantities of human inflammatory cytokines interleukin-1α, interleukin-β, interleukin-2, interleukin-6, interleukin-10, interferon-γ, and granulocyte-macrophage colony-stimulating factor were measured with the Multi-Analyte ELISArray Kit for human inflammatory cytokines according to the manufacturer's instructions (SABiosciences, Frederick, Md.).

Real-Time Polymerase Chain Reaction

For real-time polymerase chain reaction analysis, we isolated RNA using the RNA II kit (Macherey & Nagel, Düren, Germany) as recommended by the manufacturer. Two microliters of total RNA were reverse transcribed in a reaction containing reverse transcriptase at 42°C for 30 minutes. The resulting cDNA samples were measured using customized TaqMan Array Plates (Applied Biosystems, Carlsbad, Calif.). In brief, 20 ng of cDNA was mixed with TaqMan master mix included in the kit and loaded onto the plates. Plates were run on an iCycler real-time polymerase chain reaction cycler (BioRad Laboratories, Hercules, Calif.) set to 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Cell-Cycle Analysis

For cell-cycle analysis, adipose-derived stem cells and T47D cells were co-cultured for 14 days, detached with trypsin, and centrifuged at 300 *g* for 5 minutes. Cell number was set at 5×10^5 cells. After another centrifugation step, cells were fixed in 4 ml of 99% ethanol under vortex and stored at 4°C. Thereafter, cells were centrifuged again and resuspended in 1 ml of phosphate-buffered saline. Cell membranes were perforated by adding 1 μl of 0.1% Triton X-100 (Sigma-Aldrich, Bornem, Belgium) in phosphate-buffered saline for 5 minutes at room temperature, followed by digestion of RNA with 10 μl of RNase A (10 mg/ml; Sigma-Aldrich) to a final concentration of 100 μg/ml at room temperature for 5 minutes. Thereafter, staining was performed by incubation with 5 μl of propidium iodide (10 mg/ml; Invitrogen, Carlsbad, Calif.) to a final concentration of 50 μg/ml at room temperature in the dark for 1 hour. Flow cytometry and analysis were performed on a Cytomics FC500 cytometer.

RESULTS

In this study, we investigated the behavior of T47D cells and adipose-derived stem cells kept in direct co-culture, which led to characteristic morphologic and phenotypic changes. The co-culture system was established by seeding the cells in equal relations and keeping them together for up to 14 days. At regular time points, analyses were performed. Single cell cultures of both cell types were used as controls.

Growth Morphology

The morphologic appearance of co-cultures of adipose-derived stem cells and T47D cells was visualized by scanning electron microscopy. Whereas single cultures grew in monolayers, co-cultures built first flat, circular structures that grew into spherical formations after longer incubation periods for 14 days, which were assigned as tumorspheres (Fig. 1). On scanning electron microscopy, these tumorspheres were surrounded by flatly spread cells with typical adipose-derived stem cell morphology (Fig. 2, *above*). Whereas the adipose-derived stem cells were recognizable by their smooth surface, the tumorspheres had an irregular surface (Fig. 1, *below*, *left*). Interestingly, between adipose-derived stem cells and tumorspheres, gaps were spared (Fig. 1, *above*, *left*). Nevertheless, on higher magnifications, filopodia could be seen bridging the gaps between monolayers and spherical cell conglomerates (Fig. 1, *right*).

Intercellular Communication

These findings were confirmed by cell membrane labeling. Cells membranes were stably marked either with PKH26 in red (adipose-derived stem cells) or PKH67 in green (T47D cells) before being cultured together. Here, we

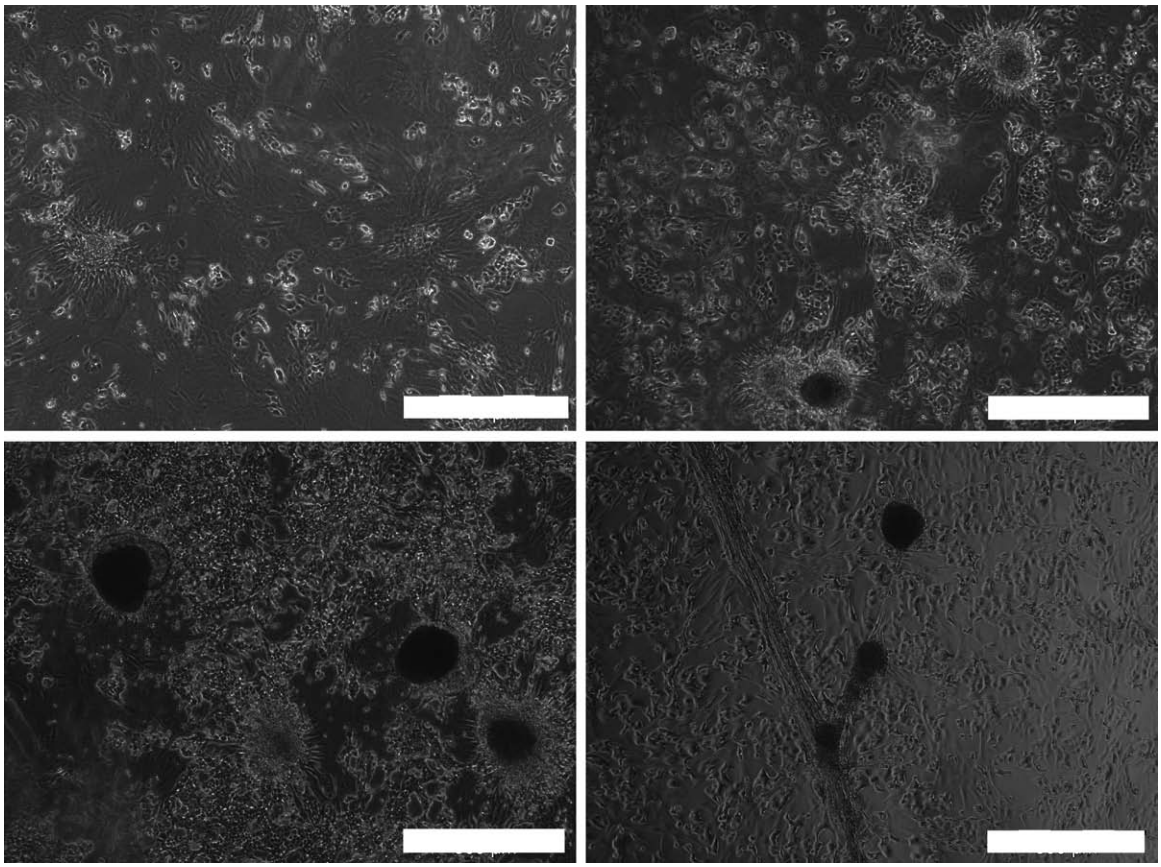

Fig. 1. Phase-contrast light microscopic photographs of T47D and adipose-derived stem cells kept in co-culture for 1 (*above*, *left*), 4 (*above*, *right*), 7 (*below*, *left*), and 14 days (*below*, *right*). Spheroid cell bodies are visible after 4 days (*arrows*) and continue to grow for the entire period of incubation (*above* and *below*, *left*, original magnification, × 100, *scale bar* = 500 μm; *below*, *right*, original magnification, × 200 magnification, *scale bar* = 200 μm).

could see filopodia stretching between the cells (Fig. 3, *above*, *left*) after 1 day of co-culture, indicating intercellular contacts. After 2 days of co-culture, vesicular exchange could be observed, as green vesicles appeared in the mainly red-stained cells and vice versa (Fig. 3, *above*, *right*). Interestingly, after 4 days of co-culture, yellow fluorescence was observed, indicating fusion of the two types of marked vesicles (Fig. 3, *below*, *left*). After being cultured together for 7 days, cells displayed a homogenous distribution of green, red, and yellow vesicles (Fig. 3, *below*, *right*), which remained for 14 days.

Visualizing Inflammatory Activity and Expression of Tumor Markers

The next step was to determine whether the cytokine spectra secreted by cells kept in direct co-culture systems differ from cells in single cultures. The values for the inflammatory cytokines interleukin-1α, interleukin-β, interleukin-2, interleukin-6, interleukin-10, interferon-γ, and granulocyte-macrophage colony-stimulating factor were increased in co-cultures compared with both types of single cell culture, although only the alterations of interleukin-1α, interleukin-2, and granulocyte-macrophage colony-stimulating factor were significant (Fig. 4).

Concerning expression of regulatory genes, we found that expression of C-terminal binding protein 1 (*CTBP1*) and mothers against decapentaplegic homologue 2 (*SMAD2*) were moderately higher in co-culture than in either monoculture (Fig. 5). Remarkably, typical markers for malignant cancer cell activity were significantly higher in co-cultures than in T47D single cultures; that is, epithelial cell adhesion molecule (*EPCAM*), erythroblastosis oncogene B2 (*ErbB2*), lymphoid-enhancer-binding factor 1 (*LEF1*), fibroblast growth factor receptor 4 (*FGFR4*), and synuclein gamma (breast cancer–specific protein 1; *SNCG*). The values for the cellular myelocytomatosis gene (*c-MYC*) were also higher in co-cultures compared with T47D single cultures.

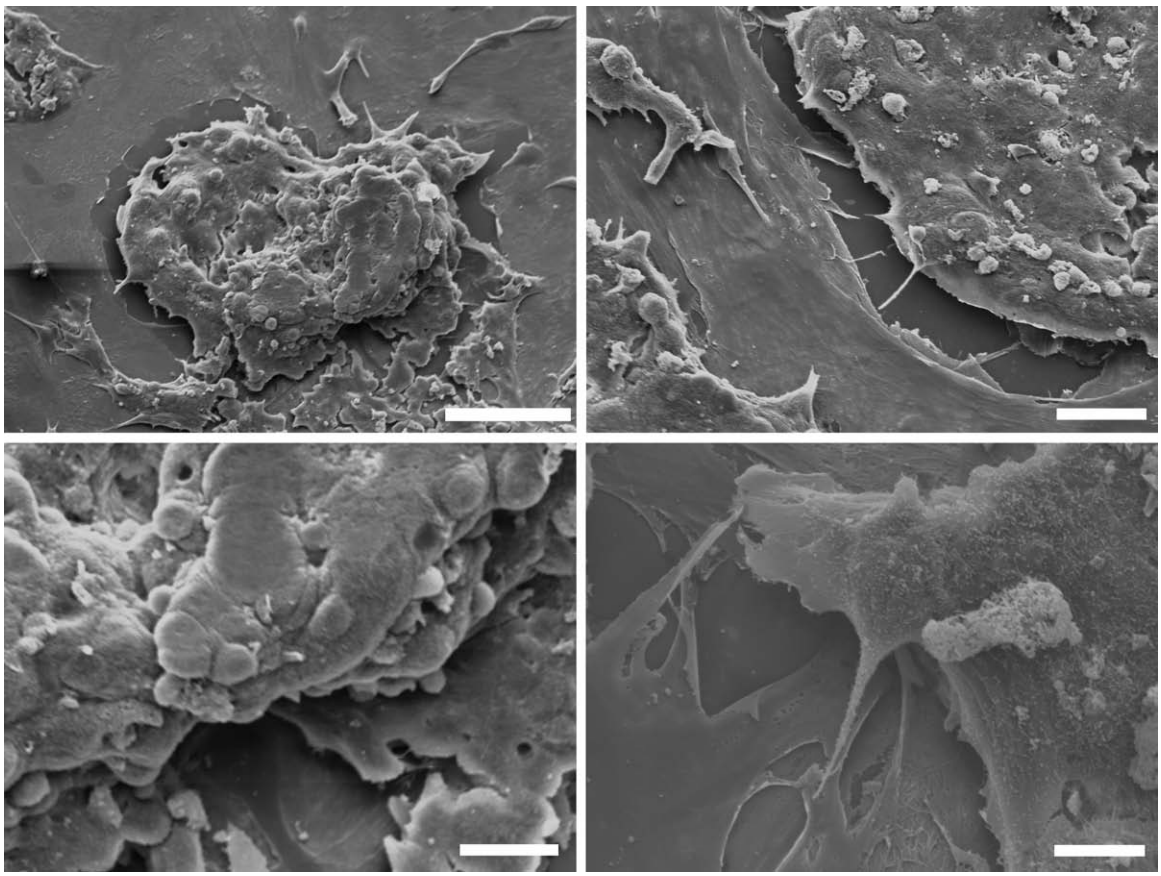

Fig. 2. Adipose-derived stem cells and T47D cells in a typical co-culture experiment visualized after 14 days by scanning electron microscopy. The resolution is 1333 × 1000 pixels for each photograph; cathode voltage, 10 kV; beam diameter, 50 nm. (*Above*, *left*) T47D tumorsphere with surrounding adipose-derived stem cell monolayer (original magnification, × 241; *scale bar* = 100 μm). (*Above*, *right*) The gap between the T47D tumorsphere and the adipose-derived stem cell monolayer is shown (original magnification, × 462; *scale bar* = 40 μm). (*Below*, *left*) The tumorsphere surface is shown (original magnification, × 965; *scale bar* = 10 μm). (*Below*, *right*) The tumorsphere-monolayer interface with filopodia bridging the gap is shown (original magnification, × 1860; *scale bar* = 5 μm).

Cell-Cycle Analysis

After 14 days of direct co-culture, we found an increase of cells in S phase in co-cultures compared with single cell cultures (i.e., 4.7 percent of cells for adipose-derived stem cells alone, 3.9 percent for T47D cells alone, and 8.6 percent for co-cultures) (Fig. 6). As S phase occurs before mitosis and is a necessity for it, it can be assumed that significantly more cells were proliferating in co-cultures, indicating again a stimulation of cell growth.

DISCUSSION

Morphologic Analysis and Intercellular Contacts

In direct co-culture systems, malignant tumorsphere formation could be found, indicating loss of contact inhibition or senescence (Fig. 1, *left*) surrounded by adipose-derived stem cell monolayers with concentric circles of space suggesting a unidirectional growth inhibition affecting the adipose-derived stem cells. Intercellular interactions could be seen with filopodia bridging the gaps between adipose-derived stem cell layers and T47D conglomerates (Fig. 1, *below*, *left*) and intercellular vesicular exchange after 2 days, indicating communication between adipose-derived stem cells and T47D cells. After four intracellular vesicles displayed a yellow membrane, which was probably caused by fusion of vesicular membranes originating from either adipose-derived stem cells (red) or T47D cells (green), respectively. The PKH membrane labeling dyes have been shown to be efficient for stable long-term staining of cell membranes.[12] In the literature, a regenerative stimulation mediated by exosomes has recently been described by Lai et al.[13] It could be demonstrated that transplantation of exosomes secreted by mesenchymal stem cells

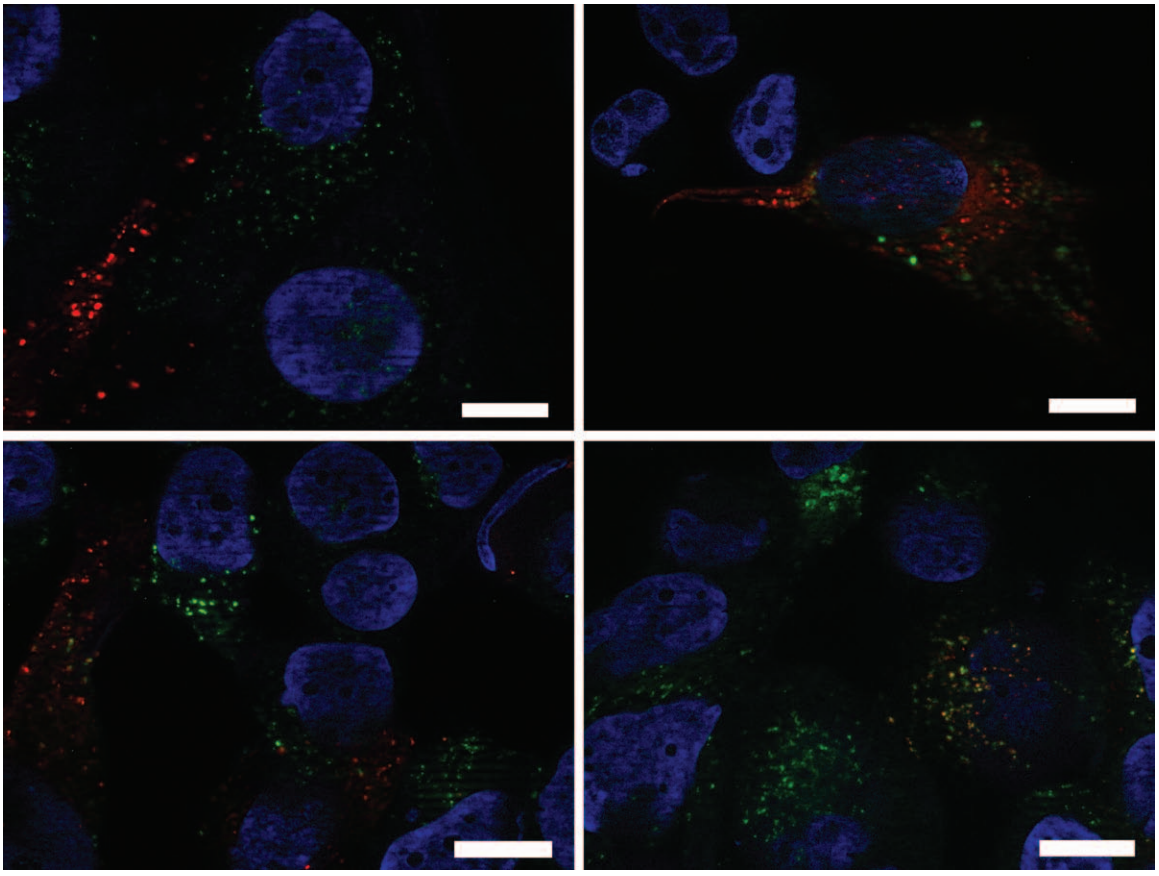

Fig. 3. Cell membrane labeling after individual membrane staining with either PKH26 (red fluorescence) in adipose-derived stem cells or PKH67 (green fluorescence) in T47D cells. Cells were kept in direct co-culture for (*above*, *left*) 1 day, (*above*, *right*) 2 days, (*below*, *left*) 4 days, and (*below*, *right*) 7 days (original magnification, × 400; *scale bar* = 50 μm).

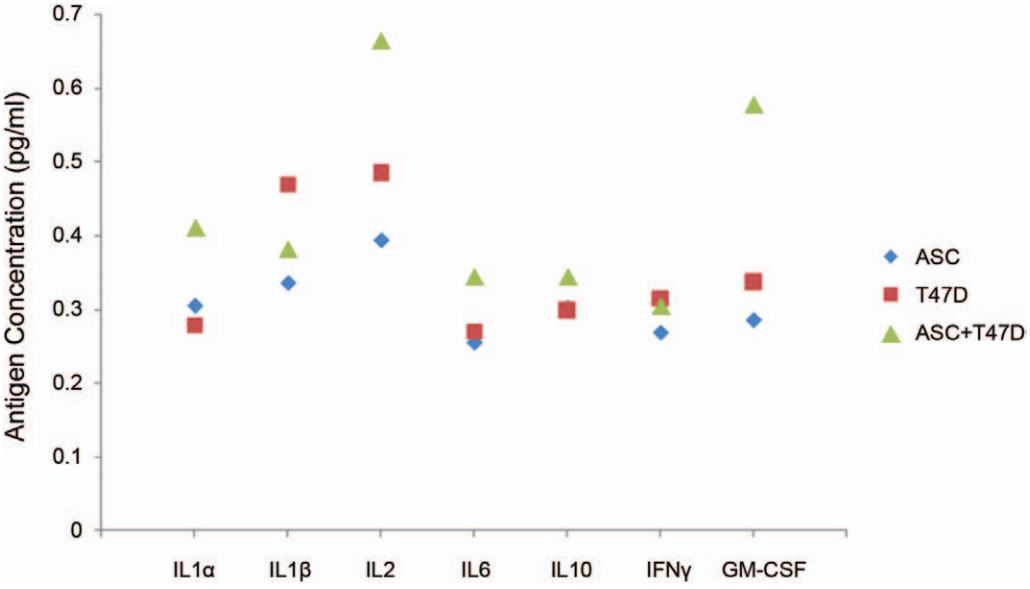

Fig. 4. Concentration of cytokines interleukin-1α, interleukin-1β, interleukin-2, interleukin-6, interleukin-10, interferon-γ, and granulocyte-macrophage colony-stimulating factor in conditioned medium (*$p < 0.05$). *IL*, interleukin; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *ASC*, adipose-derived stem cells.

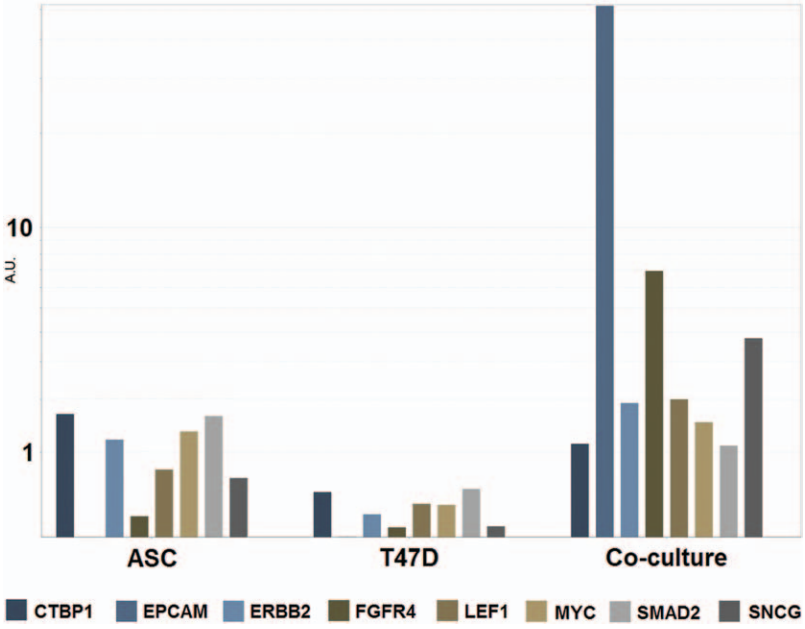

Fig. 5. Typical experiment for real-time polymerase chain reaction to determine the concentration of gene expression of *CTBP1*, *EpCAM*, *ErbB2*, *FGFR4*, *LEF1*, *MYC*, *SMAD*, and *SNCG* in arbitrary units on a logarithmic scale.

alone could reduce myocardial ischemia, displaying that at least some regenerative properties of mesenchymal stem cells are based on exchange of vesicles. These findings suggest a direct cell-to-cell contact-based stimulation of T47D cells by adipose-derived stem cells.

Function of Cytokines

The interaction between adipose-derived stem cells and T47D cells induced high levels of a number of cytokines, including interleukin-6, interleukin-2, interleukin-10, interleukin-1α, and granulocyte-macrophage colony-stimulating factor. An interleukin-6–mediated chemotaxis may facilitate the homing of mesenchymal stem cells to the sites of primary tumor growth, and induction of chemokine (C-X-C motif) ligand 7 (*CXCL7*) expression by these cells. Mesenchymal stem cell–derived *CXCL7*, in contrast, interacts with cancer cells through the chemokine (C-X-C motif) receptor 2.[14] Furthermore, Sethi et al. recently demonstrated that interleukin-6–mediated Jagged1/Notch signaling promotes breast cancer bone metastasis.[15] In breast cancer cell lines and in malignant breast tumors, expression of interleukin-1α is associated with a more malignant phenotype and lack of estrogen receptor-α expression and tumor growth.[16] Growing evidences suggest that interleukin-10 has an important role in initiation and progression of breast cancer,[17,18] although many of the intricate mechanisms involved have not been thoroughly investigated. Interleukin-2 is one of the most important cytokines for clonal expansion of antigen-activated T cells and is thus part of the cellular immune response during inflammation. For that reason, it can be discussed that the observed substantial increase was caused by stimulation of inflammatory T47D cells.

It is known that granulocyte-macrophage colony-stimulating factor induces mesenchymal stem cells to differentiate into myeloid-derived suppressor cells.[19] Myeloid-derived suppressor cells suppress T-cell differentiation and formation of natural killer cells. As a consequence, the natural immune response to the tumor is suppressed.

Function of Up-Regulated Markers

Epithelial-mesenchymal transition, a process in which epithelial cells develop a mesenchymal phenotype, is a prerequisite for dissemination from the primary tumor. Consequently, it leads to circulating tumor cells in the blood and metastasis, and therefore is commonly associated with poor prognosis. Cells undergoing this process are characterized by a typical set of marker genes, in part of epithelial and in part of mesenchymal origin. In the investigation presented herein, typical malignant tumor markers were raised substantially, for some markers up to 50-fold; most of them were typical markers for a very aggressive metastatic tumor growth.

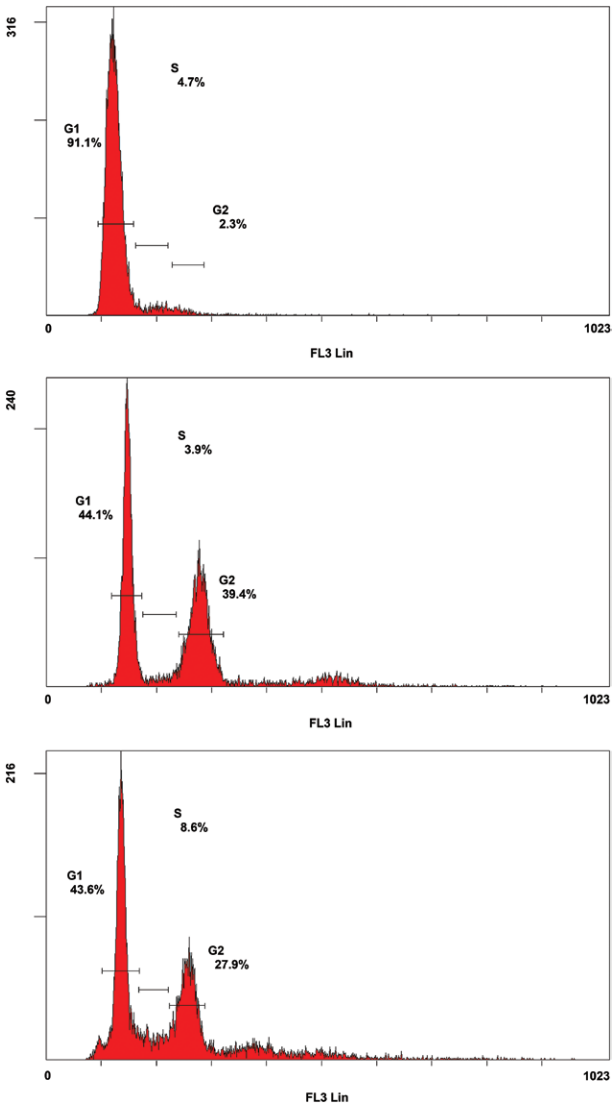

Fig. 6. Percentage of cells in G_1, S, and G_2 phases, determined by means of flow cytometry. (*Above*) adipose-derived stem cells, (*center*) T47D cells, and (*below*) co-culture of both after 14 days.

The epithelial cell adhesion molecule was identified as a surface antigen singularly expressed on epithelial cells.[20] The epithelial cell adhesion molecule has since become a clinical marker used for identification of circulating tumor cells, and the epithelial cell adhesion molecule plus circulating tumor cells are strongly associated with the formation of metastasis-initiating cells.[21,22] It is frequently overexpressed in invasive breast cancer or adenomatous squamous cancer and their metastases, and is associated with a poor prognosis.[23]

SNCG Promotes Tumor Progression in Breast Cancer and Colon Carcinoma

The tyrosine kinase-type cell surface receptor HER and thus *ErbB2* expression was detected in one-third of breast and ovarian cancers and in one-quarter of cancer-related deaths in women, correlating with chemoresistance.[24,25] *ErbB2* overexpression in breast cancer cells leads to an increase of tyr15 phosphorylation of *CDC2* and delayed M-phase entry protecting the cells against Taxol (Bristol-Myers Squibb, New York, N.Y.)-induced apoptosis.[26] Accordingly, increased expression has been associated with disease progression in breast cancer and poor prognosis.[27]

Furthermore, high values of *LEF1* are connected to disease progression and poor prognosis.[28] The expression of a mutated form of *FGFR4* is associated with cancer progression and tumor cell motility.[29] In approximately one-third of breast cancer cases, *FGFR4* amplification has been observed, although the prognostic value or any clues about its contribution to cancer progression are still unknown. However, in a recently published study, *FGFR4* has been associated with tumorigenesis.[30] *MYC* is a marker for uncontrolled cell proliferation and is found in aggressive tumors; its inhibition, in contrast, can be used for cancer therapy.[31]

CTBP1 is part of a transcriptional co-repressor complex repressing the activity of tumor suppressor genes, such as tumor invasion suppressor E-cadherin.[32] In accordance with its multiple pro-survival functions, *CTBP1* has been proposed as a possible target of breast cancer therapy.[33]

Mesenchymal Stem Cells and Tumor Growth

These findings are consistent with the findings of the cell-cycle analysis indicating a stimulation of proliferation, as a considerably higher percentage of cells were in S phase. For adipose-derived stem cells, stimulating and angiogenic properties, in particular, of a vascular network in cancer, have been described.[34] Because markers for inflammation and typical tumor markers were substantially increased, we proposed that mostly T47D cells were affected by stimulation of adipose-derived stem cells.

Our findings about vesicle exchange accompanied by phenotypic changes are in line with findings published recently that revealed the potential of mesenchymal stem cells to support tumor growth.[35,36] Nevertheless, in clinical studies, no significantly increased risk for general breast cancer neogenesis or recurrence could be shown.[3,37] In a recent study, there was no significantly higher risk after lipofilling for breast cancer recurrence; however, in the subgroup of intraepithelial neoplasia, a significantly higher risk could be proven.[38] Interestingly, the cell type used in our study was derived from an intraepithelial neoplasia. Because in

lipotransfer procedures more than one cell type is transferred, the role of other cells has to be considered as well. $CD34^+$ endothelial progenitor cells have been suspected of supporting tumor growth by neoangiogenesis.[39]

CONCLUSIONS

Stimulation of cell growth/inhibition of cellular death has been reported many times,[7,35,36,40] although this is the first study describing that intercellular communication between adipose-derived stem cells and breast cancer cells is existent and that activation of typical cancer markers and inflammatory activity occur. Although the question of whether tumor recurrences increase quantitatively or not remains uncertain, we could show qualitative changes of tumor cells in co-cultures with adipose-derived stem cells associated with a more malignant phenotype. The question remains of whether these results can be transferred to in vivo models needed to enlighten the interactions of tumor cells and adipose-derived stem cells in a systemic environment.

According to the proximity of the adipose-derived stem cell injection site to the site of the excised tumor, our results indicate a certain risk in postoncologic breast reconstruction by means of fat transplantation. Here, direct cell-to-cell stimulation and paracrine effects may appear. Consequently (in particular, if reconstruction with autologous fat is planned), a thorough validation of the complete excision of all tumor cells is mandatory. Also, follow-up investigations (i.e., regular magnetic resonance imaging) to detect recurrence or other irregularities should be performed according to the recommendations that have been issued by the U.S. Food and Drug Administration by the informational brochure "Breast Implants: Local Complications and Adverse Outcomes" (http://www.fda.gov/breastimplants).[41] Guidelines for fat transplantations have been published by the American Society of Plastic Surgeons and national societies.[42,43] However, there is a need for a consensus and guidelines concerning cell-assisted lipotransfer, as it may become a routine clinical procedure.

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ACKNOWLEDGMENTS

The authors thank Gerhardt Preiss for excellent technical assistance with the scanning electron microscope; Vincent Coger for helping in isolation of adipose-derived stem cells; and Susanne Schinner for fruitful discussion.

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