Human adipose-derived mesenchymal stromal cells may promote breast cancer progression and metastatic spread

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Abstract: BACKGROUND: Stem cell-enriched fat grafting has been proposed as a potential therapy for reconstructive, restorative, or enhancement-related procedures of the breast. Its role in postoncologic breast reconstruction is still emerging, with concerns about safety. The authors investigated the dose-dependent interaction between human adipose-derived mesenchymal stromal cells (AD-MSCs) and human breast cancer cell (BCC) lines [MDA-MB-231 (MDA) and MCF-7 (MCF)] focusing on tumor microenvironment, tumor growth, and metastatic spread. METHODS: Adipose-derived mesenchymal stromal cell influence on viability and factor expression [regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor- , and eotaxin) of breast cancer cells was studied in vitro using direct and indirect co-culture systems. Groups were formed according to adipose-derived mesenchymal stromal cell-to-cancer cell number ratio [MDA/MCF only, AD-MSC/(MDA/MCF), and AD-MSC/(MDA/MCF)]. A humanized orthotopic murine cancer model was used to evaluate breast cancer progression and metastasis (n = 10/group). Cells were injected into the mammary pad in different ratios and animals were monitored over 42 days. Microdialysis was performed to analyze RANTES levels in the tumor microenvironment (days 21 and 42). Primary and metastatic tumors were weighed and analyzed for oncogene, growth factor, and metastatic marker expression. RESULTS: MDA cell viability increased from 45.5 percent to 95.5 percent in presence of adipose-derived mesenchymal stromal cells in vitro. In vivo, animals with AD-MSC showed increased mean tumor weight (MDA, $p < 0.01$; MCF versus controls, $p < 0.05$) and metastatic occurrence (40 percent in MDA; 30 percent in MCF versus 0 percent in controls). Cytokine analysis revealed switching of MCF tumor phenotype to a more malignant type in the presence of adipose-derived mesenchymal stromal cells. CONCLUSION: Human adipose-derived mesenchymal stromal cells may promote progression and metastatic spread in breast cancer through a switch to a more malignant phenotype with worse prognosis.

DOI: https://doi.org/10.1097/PRS.0000000000001321

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Human Adipose-Derived Mesenchymal Stromal Cells May Promote Breast Cancer Progression and Metastatic Spread

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Background: Stem cell–enriched fat grafting has been proposed as a potential therapy for reconstructive, restorative, or enhancement-related procedures of the breast. Its role in postoncologic breast reconstruction is still emerging, with concerns about safety. The authors investigated the dose-dependent interaction between human adipose-derived mesenchymal stromal cells (AD-MSCs) and human breast cancer cell (BCC) lines [MDA-MB-231 (MDA) and MCF-7 (MCF)] focusing on tumor microenvironment, tumor growth, and metastatic spread.

Methods: Adipose-derived mesenchymal stromal cell influence on viability and factor expression [regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor-α, and eotaxin) of breast cancer cells was studied in vitro using direct and indirect co-culture systems. Groups were formed according to adipose-derived mesenchymal stromal cell–to–cancer cell number ratio [MDA/MCF only, AD-MSC^{low}/(MDA/MCF), and AD-MSC^{high}/(MDA/MCF)]. A humanized orthotopic murine cancer model was used to evaluate breast cancer progression and metastasis (n = 10/group). Cells were injected into the mammary pad in different ratios and animals were monitored over 42 days. Microdialysis was performed to analyze RANTES levels in the tumor microenvironment (days 21 and 42). Primary and metastatic tumors were weighed and analyzed for oncogene, growth factor, and metastatic marker expression.

Results: MDA cell viability increased from 45.5 percent to 95.5 percent in presence of adipose-derived mesenchymal stromal cells in vitro. In vivo, animals with AD-MSC^{high} showed increased mean tumor weight (MDA, $p < 0.01$; MCF versus controls, $p < 0.05$) and metastatic occurrence (40 percent in MDA; 30 percent in MCF versus 0 percent in controls). Cytokine analysis revealed switching of MCF tumor phenotype to a more malignant type in the presence of adipose-derived mesenchymal stromal cells.

Conclusion: Human adipose-derived mesenchymal stromal cells may promote progression and metastatic spread in breast cancer through a switch to a more malignant phenotype with worse prognosis. (*Plast. Reconstr. Surg.* 136: 76, 2015.)

The breast has a high cellular turnover for gland renewal under hormonal influence, with stem cells differentiating into ductal, alveolar, and myoepithelial cells.[1,2] This highly active environment is prone to the risk of failure,

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Received for publication September 26, 2014; accepted January 15, 2015.

DOI: 10.1097/PRS.0000000000001321

Presented at 11th Meeting of the International Federation for Adipose Therapeutics and Science, in New York, New York, November 21 through 24, 2013 (awarded Best Paper Plenary Session); and the 24th Annual Meeting of the European Association of Plastic Surgeons, in Ischia, Italy, May 29 through 31, 2014.

Disclosure: *The authors have no financial interest in any of the products or devices mentioned in this article.*

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making breast cancer the most frequent malignancy in women today.

For reconstructive purposes, treatment with fat grafting has been suggested as an option for cancer patients.[3,4] Fat grafting is also considered for aesthetic breast augmentation. However, despite extensive experience with fat grafting for scar treatment and as a filler, it is indeed not considered a reproducible and predictable technique. Moreover, safety guidelines for these procedures are currently based on recommendations derived from cancer biology, individual risk analysis, and basic science evidence.[5] Newest approaches are based on the use of stem cell–enhanced fat grafting for improved outcome, regardless of the lack of scientific evidence for the procedure's safety.[6,7] Adult human mesenchymal stem cells are a suitable cell type and can be isolated from white adipose tissue, which makes adipose-derived mesenchymal stromal cells interesting because of ease of harvest. These cells have multiple functions, including the secretion of soluble factors through which they participate and contribute to immunomodulation, antiapoptosis, angiogenesis, and regeneration.[8–10] Because of these functionalities, local and migrating stem cells have been suspected to endorse breast cancer development.[11,12] Suggested by reports, adipose-derived mesenchymal stromal cells, and more in general mesenchymal stem cells, can promote progression and metastasis of breast cancer[13–17] through homing of stem cells to tumors as an evident process.[18–20] Cytokines and chemokines might play a role in breast cancer growth,[21,22] metastasis, or recurrence, with growing evidence that growth and progression of breast tumors depend on their microenvironment as well.[23,24] In this study, we investigated the interactions of human breast cancer cells with human adipose-derived mesenchymal stromal cells more in detail, focusing on the influence of the adipose-derived mesenchymal stromal cell–to–breast cancer cell ratio and its effects on cancer promotion and metastasis.

MATERIALS AND METHODS

Cell Cultures

Human adipose-derived mesenchymal stromal cells were isolated from female abdominal lipoaspirates (postmenopausal, no family history of cancer, nonsmoking, nondiabetic) after informed consent and cantonal ethics committee approval. Adipose-derived mesenchymal stromal cells were isolated according to previously established protocols in our laboratory. Briefly, the lipoaspirates were digested in equal volume of mixture of 0.1% collagenase type I (Sigma-Aldrich, St. Louis, Mo.), 1% bovine serum albumin (Sigma-Aldrich), and 2 mM calcium chloride in phosphate-buffered saline; centrifuged; and resuspended in Dulbecco's Modified Eagle Medium/F-12 (Invitrogen, Life Technologies Europe, Zug, Switzerland) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Zug, Switzerland) for cell culture at 37°C with 5% carbon dioxide.

Flow cytometry was performed on adipose-derived mesenchymal stromal cells (passage 2) for mesenchymal characterization. (**See Figure, Supplemental Digital Content 1**, which shows characterization of human adipose-derived mesenchymal stromal cells. Primary isolated human adipose-derived mesenchymal stromal cells were stained for CD34, CD73, CD90, and CD105 and characterized by fluorescence-activated cell sorting. Bar graphs show mean ± SD, ***http://links.lww.com/PRS/B319***.)

The cancer cell lines MDA-MB-231 (MDA) and MCF-7 (MCF) were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. On confluence, cells were detached with trypsin (0.25%)/ethylenediaminetetraacetic acid (1 mM), counted with a hemocytometer with trypan blue exclusion, and aliquoted to the cell number required.

Co-culture Systems

Different adipose-derived mesenchymal stromal cell–to–breast cancer cell ratios were used for co-cultures, seeding 5×10^4 adipose-derived mesenchymal stromal cells (AD-MSC) and different amounts of breast cancer cells (BCC) per well: 5×10^5 (AD-MSC^{low}/BCC), 5×10^4 (AD-MSC^{equal}/BCC), and 5×10^3 (AD-MSC^{high}/BCC). Breast cancer cells and adipose-derived mesenchymal stromal cells alone served as controls.

Indirect co-cultures were conducted in six-well plates with transparent 3-μm-pore membrane inserts (ThinCert; Greiner Bio One, St. Gallen, Switzerland). Adipose-derived mesenchymal stromal cells were seeded into inserts and breast cancer cells in the bottom. Adipose-derived mesenchymal stromal cells and breast cancer cells were allowed to share the same media (Dulbecco's Modified Eagle Medium/F-12, 10% fetal bovine serum, 1% penicillin/streptomycin) over 8 days. The percentage of viable breast cancer cells was analyzed by means of propidium iodide (0.1 μg/ml) uptake using flow cytometry. Viability assay was performed only for MDA cells because of slow growth of MCF cells and no influence on viability.

Similarly, direct co-cultures were conducted in six-well plates with adipose-derived mesenchymal stromal cell/breast cancer cell mixtures as described above. Cells were cultured for 8 days and the supernatant was collected for single-run multiplex assay analysis.

Animal Model

Animal experiments were performed according to national guidelines for the care and use of laboratory animals and with approval of the local animal ethics committee. Six-week-old nu/nu mice (Charles River, Sulzfeld, Germany) were injected with 2.5×10^6 MDA cells suspended in 100 μl of serum-free medium into the right fourth mammary pad. Two million (AD-MSC^{high}/MDA group, $n = 10$) or 0.2×10^6 AD-MSC (AD-MSC^{low}/MDA group, $n = 10$) were injected at the same site. As a control, one group was not injected with adipose-derived mesenchymal stromal cells (MDA group, $n = 10$). Similarly, for the MCF groups, animals were injected with 0.5×10^6 MCF cells into the right fourth mammary pad coupled to 1×10^6 AD-MSC (AD-MSC^{high}/MCF group, $n = 10$), 0.1×10^6 AD-MSC (AD-MSC^{low}/MCF group, $n = 10$), or no AD-MSC as a control (MCF group, $n = 10$). All animals were observed for 42 days, after which they were euthanized and inspected for tumors. Primary tumors at the injection site and abdominal metastases were harvested and weighed.

Microdialysis

Microdialysis was performed on days 21 and 42 for regulated on activation, normal T cell expressed and secreted (RANTES) measurement in the tumor microenvironment ($n = 3$ per group). Anesthesia and antidote reversion were achieved using triple-mix injections as described previously.[9,10] Microdialysis microprobes (CMA20, 100 kDa; Polyethersylfone; CMA Microdialysis, Stockholm, Sweden) were inserted into the peritumoral tissue and perfused with Ringer/hydroxyethyl starch 6% (Fresenius, Oberdorf, Switzerland) using a microinjection pump (CMA/100; Polyethersylfone). The perfusion rate was set to 1 μl/minute as described previously.[25,26] The probes were perfused for 30 minutes for equilibration before sample collection. Dialysates were stored at −80°C and analyzed for RANTES.

Multiplex Assays

Multiplex assays were performed on tumor lysates and dialysates. A cell lysis kit was used according to the manufacturer's instructions to obtain tumor lysates (Bio-Rad, Cressier, Switzerland). Human cytokine 27-plex kit and cancer panel I 16-plex kit (M50-0KCAF0Y and 171-AC500M; Bio-Rad) were used according to the manufacturer's instructions to measure different markers.

Histologic Analysis

The tumors were fixed in 4% formaldehyde and embedded in paraffin. Four-micron sections were stained for hematoxylin and eosin and CD31 and CD90 (polyclonal antibodies; Lifespan Biosciences, Inc., Seattle, Wash.).[27] For immunofluorescence, secondary antibodies conjugated with fluorescent dyes (anti-rabbit Alexa 488; Life Technologies, Switzerland) and anti-goat Cy3 (Millipore; Switzerland) were used. After secondary antibody incubation, sections were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich, Buchs, Switzerland).

Statistical Analysis

GraphPad Prism (GraphPad Software, Inc., San Diego, Calif.) was used for statistical analysis. Nonparametric Kruskal-Wallis test with Dunn's multiple comparison was performed to observe differences between the groups. Values of $p < 0.05$ were chosen for statistical significance.

RESULTS

Co-Cultures

A higher adipose-derived mesenchymal stromal cell–to-MDA ratio favored breast cancer cell survival through cytokine exchange over the permeable membrane in indirect co-cultures. (**See Figure, Supplemental Digital Content 2**, which shows indirect co-culture. MDA and adipose-derived mesenchymal stromal cells were indirectly co-cultured at different ratios and stained with propidium iodide to assess cell viability after 8 days, ***http://links.lww.com/PRS/B320***.) MDA cells cultured with AD-MSC^{low} and AD-MSC^{equal} were 50.1 percent and 47.9 percent viable after 8 days, whereas in those cultured with AD-MSC^{high}, the MDA cell viability increased to 95.5 percent. In the absence of AD-MSC, MDA cells showed 54.5 percent viability.

Supernatants from direct adipose-derived mesenchymal stromal cell/breast cancer cell co-cultures were assessed for RANTES, tumor necrosis factor (TNF)-α, and eotaxin. (**See Figure, Supplemental Digital Content 3**, which shows direct co-culture. Direct co-culture of adipose-derived mesenchymal stromal cells with MDA or MCF cells was performed and supernatants

were tested for RANTES, TNF-α, and eotaxin expression, ***http://links.lww.com/PRS/B321***.) An increasing AD-MSC/MDA ratio enhanced RANTES expression (358 pg/ml, 242 pg/ml, and 237 pg/ml for AD-MSC^{high}, AD-MSC^{equal}, and AD-MSC^{low}, respectively; 130 pg/ml for MDA). Increased RANTES expression was determined for decreasing AD-MSC/MCF ratios (28 , 170, and 158 pg/ml for AD-MSC^{high}, AD-MSC^{equal}, and AD-MSC^{low}, respectively; 47 pg/ml for MCF). RANTES expression for AD-MSC was 47 pg/ml.

TNF-α expression showed a trend similar to that for RANTES. It increased under AD-MSC influence for MDA (37 pg/ml for AD-MSC^{high}, 30 pg/ml for AD-MSC^{equal}, and AD-MSC^{low}; 27 pg/ml for MDA) and decreased in MCF cultures, increasing the AD-MSC dose (13, 24, and 21 pg/ml for AD-MSC^{high}, AD-MSC^{equal}, and AD-MSC^{low}, respectively; and 8 pg/ml for MCF). TNF-α was 14 pg/ml for adipose-derived mesenchymal stromal cells.

Eotaxin was highly expressed in MDA cells with AD-MSC^{high} (81 pg/ml) and decreased with fewer adipose-derived mesenchymal stromal cells (27 and 31 pg/ml for AD-MSC^{equal} and AD-MSC^{low}, respectively; 38 pg/ml for MDA). For MCF cells, eotaxin was 14 pg/ml (AD-MSC^{high}), 20 pg/ml (AD-MSC^{equal}), and 14 pg/ml (AD-MSC^{low}), and 1 pg/ml for MCF, whereas adipose-derived mesenchymal stromal cells exhibited the highest levels (97 pg/ml).

Tumor Progression

Primary and metastatic tumors were isolated and weighed on day 42 (Fig. 1). Adipose-derived mesenchymal stromal cells promoted larger tumors. MCF primary tumors had a volume of 0.80 ± 1.79 mm^3, whereas tumor volume was 7.79 ± 9.27 mm^3 for AD-MSC^{low} (not significant) and 9.31 ± 5.76 mm^3 for AD-MSC^{high} ($p < 0.01$ versus MCF). MDA tumors were 27 ± 56 mm^3, 55 ± 82 mm^3 with AD-MSC^{low}, and 79 ± 182 mm^3 with AD-MSC^{high} (not significant). Total tumor weight including primary tumor and metastases increased significantly in MCF tumors with AD-MSC^{high} [20 ± 21 mg versus 4 ± 7 mg (MCF) and 5 ± 6 mg (AD-MSC^{low}); $p < 0.05$]. MDA tumors were larger (68 ± 131 mg) and promoted by AD-MSC^{low} and AD-MSC^{high} (179 ± 244 mg and 368 ± 491 mg; $p < 0.01$ versus MDA).

Besides increased progression, adipose-derived mesenchymal stromal cells influenced cancer cells by increasing metastatic spread. Two mice from AD-MSC^{high}/MDA died prematurely (days 34 and 40) because of advanced metastatic disease. In MDA-only animals, no metastases were found. Conversely, 20 percent of the animals developed metastases with AD-MSC^{low}, whereas occurrence increased to 40 percent with AD-MSC^{high}. A similar metastatic behavior was found in MCF tumors, even though occurrence of metastases was comparatively lower, being a low-malignancy breast cancer cell line. Animals with MCF only and AD-MSC^{low} had no metastases, whereas the rate increased to 30 percent with AD-MSC^{high}.

Tumor Lysates

Tumor samples were processed to determine the concentration of cancer and angiogenesis biomarkers. Each group consisted of 10 animals,

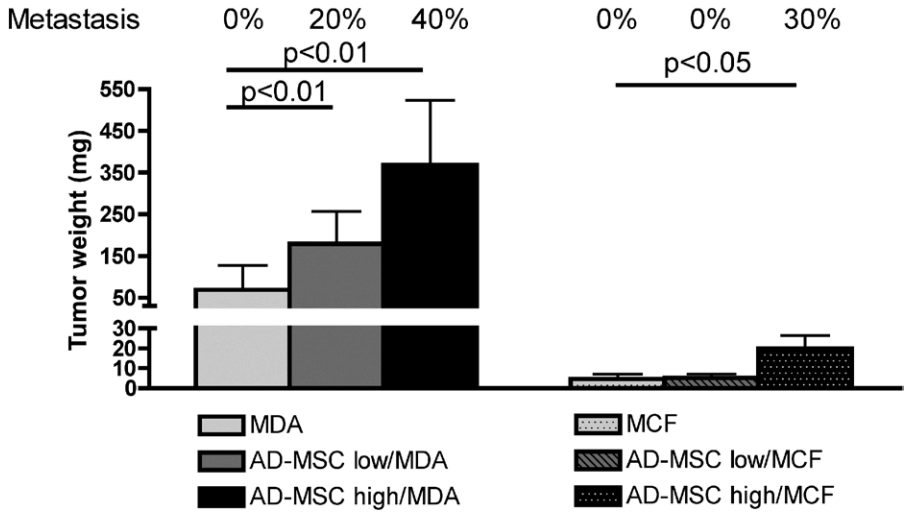

Fig. 1. Tumor weight measurements and metastatic spread. Primary and metastatic tumors were measured in all animals after 6 weeks. Bar graph depicts the mean and SD of tumor size and indicates the percentage of metastatic tumor for each group. *AD-MSC*, adipose-derived mesenchymal stromal cells.

but not all in the breast cancer cell–only groups developed tumors; thus, the number of samples in these was reduced. In MDA animals, sHER-2/neu levels were 1011 ± 202 pg/ml and decreased under adipose-derived mesenchymal stromal cell influence (732 ± 277 pg/ml and 619 ± 445 pg/ml for AD-MSC^{high} and AD-MSC^{low}, respectively; not significant). [**See Figure, Supplemental Digital Content 4**, which shows cancer markers in tumor tissue samples. sHER2/neu (MDA and MCF), follistatin (MDA), and osteopontin (MDA) expression were determined in tumor lysates by multiplex assay. Each data point represents one animal. Statistical analysis was performed using the Kruskal-Wallis test with Dunn's multiple comparison, ***http://links.lww.com/PRS/B322***.] In MCF animals, sHER-2/neu was 62.04 ± 20 pg/ml and increased with adipose-derived mesenchymal stromal cells to 515.8 ± 751 pg/ml (AD-MSC^{high}) and 1335 ± 789 pg/ml (AD-MSC^{low}; $p < 0.05$ versus MCF). (**See Figure, Supplemental Digital Content 4**, ***http://links.lww.com/PRS/B322***.)

Follistatin in MDA animals showed a trend similar to that for sHER-2/neu, with no significant differences between the groups (484 ± 145 pg/ml and 404 ± 217 pg/ml for AD-MSC^{high} and AD-MSC^{low}, respectively; 578 ± 42 pg/ml for MDA). (**See Figure, Supplemental Digital Content 4**, ***http://links.lww.com/PRS/B322***.) In MCF animals, follistatin levels were 1687 ± 881 pg/ml and significantly lower with adipose-derived mesenchymal stromal cells (470.6 ± 274 and 434.3 ± 139 for AD-MSC^{high} and AD-MSC^{low}, respectively; $p < 0.05$ versus MCF) (Fig. 2).

Osteopontin levels were 1200 ± 22 pg/ml for MDA tumors (**see Figure, Supplemental Digital Content 4**, ***http://links.lww.com/PRS/B322***) and decreased to 961.8 ± 214 pg/ml (AD-MSC^{high}) and 893.4 ± 365 pg/ml (AD-MSC^{low}) with adipose-derived mesenchymal stromal cells (not significant); whereas in MCF-only tumors, osteopontin was significantly higher (2639 ± 1337 pg/ml) compared with AD-MSC^{high} and AD-MSC^{low} (1177 ± 320 pg/ml and 1085 ± 144 pg/ml, respectively; $p < 0.05$ versus MCF) (Fig. 3).

Angiogenesis within the tumor microenvironment was determined by analyzing tumor lysates for soluble Tie-2 (sTie-2) and platelet-derived growth factor (PDGF) AB/BB. (**See Figure, Supplemental Digital Content 5**, which shows tumor angiogenesis markers. sTie-2 and PDGF AB/BB levels in tumor lysates were determined by multiplex assay. Each data point represents one animal. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison, ***http://links.lww.com/PRS/B323***.) sTie-2 levels showed significant differences between MDA and MCF animals depending on adipose-derived mesenchymal stromal cell dosage. Adipose-derived mesenchymal stromal cells diminished sTie-2 levels in MDA tumors (587 ± 109 pg/ml and 407 ± 145 pg/ml for AD-MSC^{high} and AD-MSC^{low}, respectively; 723 ± 23 pg/ml for MDA; $p < 0.05$ versus MDA). MCF animals (1800 ± 865 pg/ml) had significantly higher levels than AD-MSC/MCF animals (507 ± 178 and 460 ± 115 pg/ml for AD-MSC^{high} and AD-MSC^{low}, respectively; $p < 0.05$ versus MCF). PDGF AB/BB values were not different between the MDA groups (209 ± 78 and 211 ±

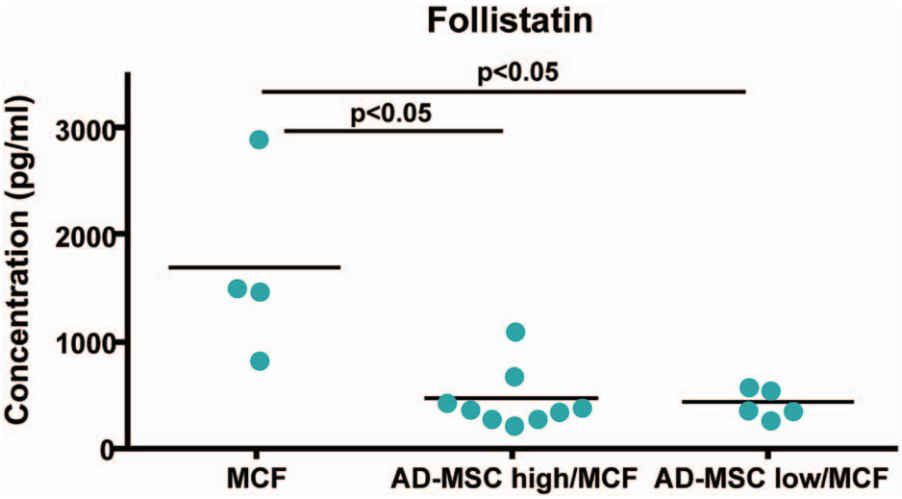

Fig. 2. Follistatin expression in MCF tumors. Follistatin expression was determined in tumor lysates by multiplex assay. Each data point represents one animal. Statistical analysis performed using Kruskal-Wallis test with Dunn's multiple comparison.

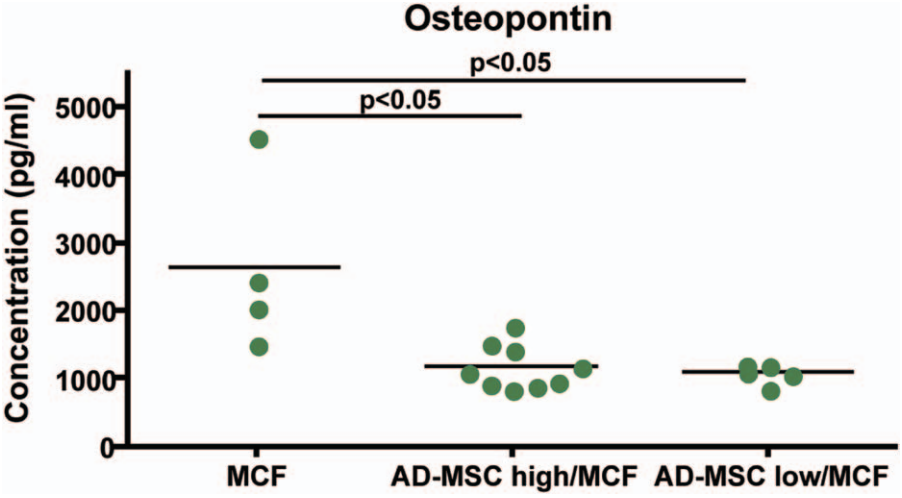

Fig. 3. Osteopontin expression in MCF tumors. Osteopontin expression was determined in tumor lysates by multiplex assay. Each data point represents one animal. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison.

166 pg/ml for AD-MSC^{high} and AD-MSC^{low}, respectively; 262 ± 2 g/ml for MDA). MCF tumors had 1248 ± 959 pg/ml PDGF AB/B, which decreased significantly with AD-MSCs (221 ± 149 and 186 ± 52 pg/ml for AD-MSC^{high} and AD-MSC^{low}, respectively; $p < 0.05$ versus MCF).

Tumor Dialysates

Dialysates from the tumor microenvironment were assessed for RANTES to account for tumor invasion potential. (**See Figure, Supplemental Digital Content 6**, which shows the tumor microenvironment. Samples from the tumor microenvironment were collected on days 21 and 42 to determine expression of RANTES. Bar graphs represent mean and SD, ***http://links.lww.com/PRS/B324***.) Results are grouped as AD-MSC/MDA and AD-MSC/MCF, without distinction between ratios. For animals injected with MDA cells only, levels were maintained over time (8 ± 2 pg/ml on day 21 and 7 ± 1 pg/ml on day 42). RANTES expression was 7 ± 0 and 6 ± 1 pg/ml, respectely, for MCF tumor. In animals receiving adipose-derived mesenchymal stromal cells, RANTES expression was increased in both breast cancer cell lines (10 ± 4 and 9 ± 1 pg/ml for AD-MSC/MDA; and 9 ± 1 and 8 ± 1 pg/ml for AD-MSC/MCF).

Histologic Analysis

Hematoxylin and eosin–stained tumor sections revealed a heterogeneous distribution of different cell types and clusters in the nodules. [**See Figure, Supplemental Digital Content 7**, which shows Immunostaining of tumor tissue samples. Samples from the tumor were fixed in paraffin and stained as described earlier under Materials and Methods. Hematoxylin and eosin staining of tumor node (*above, left*), CD31 staining in an animal injected with MDA only (*above, right*) and in one injected with MDA and adipose-derived mesenchymal stromal cells (*below*). *Scale bar* = 40 μm, ***http://links.lww.com/PRS/B325***.] There was a relatively clear margin zone at the interface between human cancer and mouse subdermal tissue, and no difference was observed between the different groups. Samples from animals that were injected with MDA cells showed CD31 positivity confined along the vasculature (**see Figure, Supplemental Digital Content 7**, *above, right*, ***http://links.lww.com/PRS/B325***). In contrast, when adipose-derived mesenchymal stromal cells were combined with MDA, single large mononuclear CD31$^+$ cells were found in tissue other than vessels (**see Figure, Supplemental Digital Content 7**, *below*, ***http://links.lww.com/PRS/B325***). To evaluate whether the infiltrating CD31$^+$ cells were differentiated adipose-derived mesenchymal stromal cells, samples were double-stained with CD90 antibody, which revealed no overlapping cells, suggesting the infiltrated CD31$^+$ cells were not differentiated stem cells (Fig. 4). Indeed, single CD90$^+$ cells were found in proximity to blood vessels or the previously mentioned CD31$^+$ cells.

DISCUSSION

Our study highlights the influence of adipose-derived mesenchymal stromal cells on phenotypic and metastatic characteristics of breast cancer cells and confirms that adipose-derived mesenchymal stromal cells predispose tumor cells toward growth, metastasis, and dedifferentiation. Our

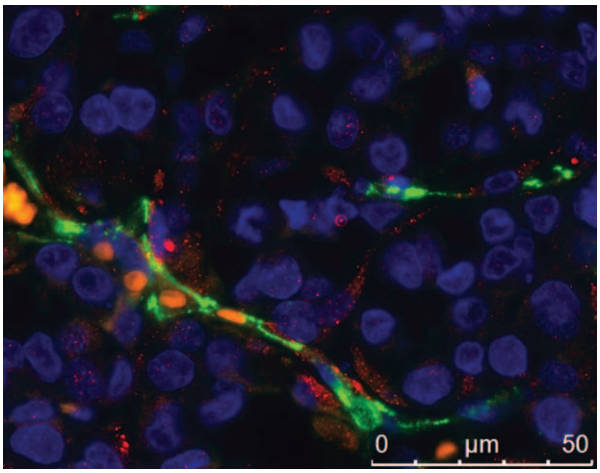

Fig. 4. Immunostaining of tumor tissue samples. Sample from an animal injected with MDA and AD-MSC, double-stained for CD31 and CD90. Single CD90$^+$ cells (*red*) were found in proximity to CD31$^+$ cells (*green*).

study also establishes the effects of varying concentrations of adipose-derived mesenchymal stromal cells on breast cancer cells both in vitro and in vivo. A high adipose-derived mesenchymal stromal cell–to–breast cancer cell ratio revealed a significant increase in cancer cell viability for MDA in vitro. This demonstrates that increasing the adipose-derived mesenchymal stromal cell dose may stimulate even minimal numbers of residual breast cancer cells. These data underscore the potential risk of adipose-derived mesenchymal stromal cell–based strategies in postoncologic breast reconstruction, especially because microscopic tumor cell pockets may remain after the most thorough tumor resection. Self-limiting cancer cell death was turned off in MDA cells by a suggestive paracrine influence of adipose-derived mesenchymal stromal cells in our indirect co-culture system.

Adipose-derived mesenchymal stromal cells influenced tumor progression and metastasis formation, depending on the breast cancer cell type. Adipose-derived mesenchymal stromal cells not only activated the breast cancer cells but also stimulated a more aggressive and proliferative phenotype. Indeed, a phenotypic switch was observed in vivo in both MDA and MCF cell lines, resulting in increased tumor progression and metastatic spread. The MCF cell line switched from a nonmetastasizing to a metastasizing phenotype with similar expression profiles for HER2/neu, follistatin, and osteopontin as for the MDA cell type. The latter two markers are predictive of malignant potential and are associated with poor prognosis and higher rates of metastatic spread and recurrence in patients. Our results confirm previous reports of murine CD34$^+$ cells and adipose-derived mesenchymal stromal cells enhancing growth and progression of different murine and human breast cancer cell lines.[17,28] Earlier, other authors used CD34$^+$ progenitors from adipose tissue,[29] mesenchymal stem cells from other sources,[30] or nonhuman cells.[17,28] Recently, Rowan et al. found significantly increased multiorgan spreading of MDA tumors and partial epithelial-to-mesenchymal transition after local addition of human adipose-derived mesenchymal stromal cells; however, in contrast to earlier reports, they found no increase of breast cancer cell growth.[16] In our study, we could demonstrate dose-dependent effects, which may have a high impact on the clinical scenario.

Adipose-derived mesenchymal stromal cells did not alter HER2/neu expression and down-regulation of follistatin and osteopontin in MDA cells, as these were already expressed or down-regulated in the more aggressive MDA cell line. In agreement with our findings, others have reported NCOA4, FOS oncogene up-regulation, and IGF-1R– and BCL2-related antiapoptotic effects.[31]

However, adipose-derived mesenchymal stromal cells in co-cultures with breast cancer cells led to increased levels of TNF-α, RANTES, and eotaxin expression, which can be correlated to higher migratory and metastatic potential. This was verified in vivo by microdialysis. Ahmad et al. described the importance of the microenvironmental influence of adipose-derived mesenchymal stromal cells, including nuclear factor kappa β regulation of angiogenesis and invasion,[32] whereas Chaturvedi et al. could show a hypoxia inducible factor-1α–dependent potential of mesenchymal stem cells to promote breast cancer progression and metastasis[33] as reflected by SDF-1/CXCR4 regulation.[17,34]

Although adipose-derived mesenchymal stromal cells have been shown to have high regenerative potential and express high levels of vascular growth factors, we observed down-regulation of PDGF and Tie-2 in cancer tissue samples. Even though solid tumors and metastases developed (which depends on sufficient vascularity), interestingly, these vascular factors were not supportively up-regulated. That mesenchymal stem cells may also have anti-angiogenic effects on cancer has been shown previously.[35,36] Surprisingly, our results for PDGF-D are contrary to the current literature, because PDGF-D expression of breast cancer cells has been associated with aggressive cancer biology.[22,32] We believe that this may be attributable to the large tumor mass, and PDGF might be expressed to a higher

extent in the transition zone, where cancer angiogenesis would be most needed to feed the growing mass. Another group detected antiangiogenic effects of mesenchymal stem cells in high local concentration, which resulted in tumor cell apoptosis and abrogated tumor progression.[36] These results would be in line with our antivascular regulation as found for Tie-2 and PDGF.

Our study is the first to take advantage of primary human adipose-derived mesenchymal stromal cells. Others have used $CD34^+$ progenitors from adipose tissue,[29] mesenchymal stem cells from other sources,[30] or nonhuman cells previously. Furthermore, our approach is unique in the way that it shows results being dependent on the adipose-derived mesenchymal stromal cell–to–breast cancer cell ratio. Mesenchymal stem cells have been investigated previously demonstrating promotion of breast cancer growth and cell viability in vivo and in vitro[37] in relation to the cytokine and growth factor expression modulating the cancer microenvironment as reported by Karnoub et al. and Kidd et al.[38,39]

Our findings are worrisome, as the rationale behind our study was to investigate the early events surrounding the cellular, cytokine, oncogene, or growth factor mediators that trigger or promote growth, dedifferentiation, or spread of breast cancer cells under the influence of different adipose-derived mesenchymal stromal cell ratios. Our experimental data may also support the significant clinical findings by Petit et al. for higher recurrence in intraepithelial breast cancer patients after autologous fat grafting following breast-conserving surgery.[40]

Alharbi et al. reported a case of aggressive breast cancer recurrence 2 years after autologous fat grafting, with eventual spread to lungs and brain.[41] However, the causal link between the events remains speculative.

Limitations of our study include the use of a humanized murine model, which might not depict the human tumor microenvironment closely enough. Furthermore, primary human breast cancer cells and adipose-derived mesenchymal stromal cells from the same patient would better represent the clinical scenario. The promoting effects found here are not necessarily mesenchymal stem cell–specific and could potentially extend to additional cell types (e.g., fibroblasts). Donor biology can also influence the results in a relevant manner.[16]

CONCLUSIONS

Our data suggest that adipose-derived mesenchymal stromal cells bear potential to promote tumor progression in breast cancer cell lines. This was demonstrated by increased breast cancer cell viability, tumor growth, and metastasis, in addition to oncogene up-regulation in the presence of adipose-derived mesenchymal stromal cells. However, our data also show decreased expression of the angiogenic factors Tie-2 and PDGF under the influence of adipose-derived mesenchymal stromal cells. Under the line, promotion of breast cancer might depend on the cancer cell type and their ratio to adipose-derived mesenchymal stromal cells. Reconstructive efforts with stem cell–enhanced fat grafting may therefore require strict guidelines to ensure a cancer cell-free environment. There may be a narrow physiologic window regarding factor concentration in the complex interplay between adipose-derived mesenchymal stromal cells and breast cancer cells. Further experimental and clinical research is required to determine the critical concentration of adipose-derived mesenchymal stromal cells in relation to the type and stage of cancer to prevent progression or recurrence of breast cancer.

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ACKNOWLEDGMENT

This study was supported with funding from the Swiss National Science Foundation (no. 310030_127577).

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