Adipocyte-Derived Fibroblasts Promote Tumor Progression and Contribute to the Desmoplastic Reaction in Breast Cancer

Ludivine Bochet1,2,3, Camille Lehuède1,2,3, Stéphanie Dauvillier1,2, Yuan Yuan Wang1,2,3, Béatrice Dirat1,2,3, Victor Laurent1,2, Cédric Dray1,3, Romain Guiet1,2, Isabelle Maridonneau-Parini1,2, Sophie Le Gondiec1,3, Bettina Couderc4, Ghislaine Escourrou5, Philippe Valet1,3, and Catherine Muller1,2

Abstract

Cancer-associated fibroblasts (CAF) comprise the majority of stromal cells in breast cancers, yet their precise origins and relative functional contributions to malignant progression remain uncertain. Local invasion leads to the proximity of cancer cells and adipocytes, which respond by phenotypical changes to generate fibroblast-like cells termed as adipocyte-derived fibroblasts (ADF) here. These cells exhibit enhanced secretion of fibronectin and collagen I, increased migratory/invasive abilities, and increased expression of the CAF marker FSP-1 but not α-SMA. Generation of the ADF phenotype depends on reactivation of the Wnt/β-catenin pathway in response to Wnt3a secreted by tumor cells. Tumor cells cocultivated with ADFs in two-dimensional or spheroid culture display increased invasive capabilities. In clinical specimens of breast cancer, we confirmed the presence of this new stromal subpopulation. By defining a new stromal cell population, our results offer new opportunities for stroma-targeted therapies in breast cancer. Cancer Res; 73(18); 5657-68. ©2013 AACR.

Introduction

One of the features of breast cancer is the presence of a dense collagenous stroma, the so-called desmoplastic response, comprising of fibroblast-like cells known as cancer-associated fibroblasts (CAF; ref. 1). Accumulating experimental evidence has shown that CAFs play an active role in breast cancer progression through the release of growth factors and chemokines and by contributing to the remodeling of the extracellular matrix (for review see refs. 2–4). CAFs are “activated” fibroblasts and were first identified upon morphologic criteria and α-smooth muscle actin (α-SMA) expression, in the same manner as wound-healing myofibroblasts (5). However, this marker is not ubiquitous, highlighting that CAFs is a heterogeneous cell population (6). Indeed, CAFs are alternatively reported to stem from resident local fibroblasts, bone marrow–derived progenitor cells, or trans-differentiating epithelial/endothelial cells (for review, see refs. 4, 7). At present, neither the origin of these cells, nor the extent to which their origin determines their contribution to tumor progression can be unanimously agreed upon.

We have recently shown that invasive cancer cells have a dramatic impact on surrounding adipocytes. Both in vitro and in vivo, these adipocytes exhibit a decrease in lipid content, a decreased expression of adipocyte markers, and an activated state indicated predominantly by the overexpression of proinflammatory cytokines (8). These cells are present in vivo at the tumor invasive front and retain an adipocyte-like rounded morphology. We named them cancer-associated adipocytes (CAA; reviewed in refs. 8–10). CAs also display overexpression of extracellular matrix (ECM, such as collagen VI) and ECM-related molecules (11–13), events contributing to breast cancer progression. As the origin of CAFs is unclear, one attractive hypothesis is that during tumor evolution, peritumoral adipocytes undergo “dedifferentiation”, first into CAAs then into fibroblast-like cells that may account for a part of the CAFs population present in the desmoplastic reaction of breast cancers. Although suggested in several reviews (14, 15), this hypothesis has never been directly shown experimentally using combined in vitro and in vivo approaches. Independent of the effect of cancer cells, several recent studies have shown that mature adipocytes are in fact plastic cells capable of “dedifferentiation” towards fibroblast-like cells (16). Although limited data exists on the mechanisms that could potentially be involved in this effect, the role of soluble factors such as TNF-α, Wnt3a (17), or specific mitochondrial uncoupling (18) has been described. In vitro, recombinant MMP-11 is also able to “dedifferentiate” mature adipocytes (11). However, the existence of this phenomenon in human pathology remains elusive. In the present study, we use both in vitro and in vivo (including human...
tumors) approaches to show that breast cancer cells are able to force mature adipocytes towards fibroblast-like cells, named adipocyte-derived fibroblasts (ADF), in a Wnt-dependent manner. We also extensively describe the distinctive phenotype of these new stromal cells, expressing some CAFs markers like fibroblast-specific protein 1/S100A4, but not α-SMA, and underline their ability to promote the spread of breast cancer cells.

Materials and Methods

Antibodies, probes, and drugs
All the primary antibodies used in this study are presented in Supplementary Table S1. The Wnt/β-catenin inhibitor, ICG-001 (19) was from Enzo Life Sciences and was used at 2.5 and 5 μmol/L final concentrations. The bodipy lipid probe (used at 1 μg/mL) and TO-PRO-3 (used at 1 μmol/L final concentration) were obtained from Invitrogen.

Cell culture, cell lines, and isolation of primary adipocytes

The murine 3T3-F442A preadipocytes cell line was differentiated as previously described (20). To estimate the adipocyte phenotype, triglyceride content was quantified using a colorimetric kit (Wako Chemicals) and Red Oil staining was conducted as previously described (8). The murine 4T1 breast cancer cell line (provided by Dr. S. Vagner, INSERM U563, Toulouse, France) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum (FCS). The human breast cancer cell line SUM159PT (provided by Dr. J Piette, IGMM, Montpellier, France) was cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum (FCS), 1 μg/mL hydrocortisone (Sigma), and 0.2 U/mL insulin. All the cell lines were used within 2 months after resuscitation of frozen aliquots. Cell lines were authenticated on the basis of viability, recovery, growth, and morphology. The expression status of E-cadherin, N-cadherin, and vimentin using immunofluorescence approaches, as well as their invasive behavior, was confirmed before they were used in the experiments (8). Coculture of tumor cells and adipocyte was carried out using a Transwell culture system (0.4 μm pore size, Millipore) as previously described (8). In certain experiments, cells genetically modified by lentiviral vectors encoding either eGFP (Genethon, Evry; 3T3-F442A) or Hc-Red (vector core ICR; SUM159PT) were used. The isolation of mammary adipocytes either from tumor or reduction mammoplasty was carried out as previously described by our group (8).

In vivo GFP-subcutaneous fat pad formation and tumor injection

A total of 1 × 10^7 confluent GFP-3T3-F442A preadipocytes were injected into the flank of athymic nude mice, as described (21). Fat pad formation was allowed to proceed for 5 weeks, after which fat pads were injected with 4 × 10^4 4T1 breast cancer cells. Mice with fat pad alone or tumor cells alone were used as controls. Five mice were used in each group. After 10 days, mice were sacrificed and fat pad, tumors, and fat pads containing tumors were embedded in paraffin after 48 hours of fixation in buffered formalin. The local Institutional Animal Care and Use Committee approved the experimental protocol used in our study.

Immunofluorescence and immunohistochemistry

Adipocytes differentiated on coverslips grown alone, or cocultivated for the indicated times with breast cancer cells were processed for immunofluorescence as previously described (8). Fluorescence images were acquired with a confocal laser microscopy system (Leica SP2). The image resolution was 512 × 512 pixels and scan rate was 400 Hz. For each sample, more than 100 cells were examined in at least three independent experiments. Immunohistochemistry was conducted as previously described (11). Adjacent sections were stained with hematoxylin and eosin or Masson trichrome to visualize collagen fibers (murine fat pad experiments). Images were captured on a Leica DMRA2.

Western blot analysis
Whole-cell extracts and immunoblots were prepared as previously described (22). All the antibodies used in this study are presented in Supplementary Table S1.

RNA extraction and quantitative PCR
RNA extraction and quantitative PCR (qPCR) were conducted as previously described (23). All the primers used in this study are presented in the Supplementary Table S2.

Migration and invasion assays (two-dimensional)

After 3 or 8 days of coculture in the presence of SUM159PT cells, adipocytes were trypsinized and used to conduct migration or Matrigel invasion assays as previously described (23). Similar Matrigel invasion assays were conducted with tumor cells grown alone or cocultivated with ADFs or with mature adipocytes during 3 days (8). In these assays, FCS (10%) was used as a chemoattractant, and similar experiments were run in parallel in the absence of serum (no chemoattractant, negative control).

Spheroids formation and invasion in a three-dimensional collagen matrix

The hanging-drop method has been adapted to produce spheroids of similar diameter (24). Drops (20 μL) containing 1,000 tumor cells alone or mixed with 1,000 ADFs or preadipocytes were suspended on the lid of 2% agar-coated 24-well dishes containing culture media. After the 7 days required for cell aggregation, the spheroids were transferred to the bottom of a well containing culture medium. Multicellular spheroids were then allowed to grow for 14 days and the spheroids used in the invasion experiments had an average diameter of 500 μm. The spheroid invasion in collagen I matrix was conducted as previously described (25).

Glucose uptake experiments

Differentiated adipocytes cocultivated or not during 3 and 8 days with SUM159PT cells were used to measure the uptake of 2-deoxy[3H]glucose in the presence or not of insulin as previously described (26).
Statistical analysis

The statistical significance of differences (at least three independent experiments) was evaluated using Student t test or ANOVA using Prism (GraphPad Inc.). P values less than 0.05 (*), 0.01 (**), and 0.001 (***)) were deemed as significant while "NS" stands for not significant.

Results and Discussion

Breast tumor cells are able to cause mature adipocytes to revert to fibroblast-like cells in vitro and in vivo

To test our main hypothesis in vitro, we took advantage of the coculture model recently set up in our laboratory. As previously described, mature 3T3-F442A adipocytes cocultivated in the presence of breast cancer cells exhibited a decrease in the number and size of lipid droplets (8). We show here that this decrease is time-dependent and, after 8 days of coculture, these cells were almost devoid of lipid droplets (Fig. 1A, top). In contrast with CAAs (typically obtained after 3 days of coculture), which retain their rounded morphology (8), adipocytes that have been cocultivated for longer times in the presence of tumor cells took on a highly elongated, fibroblastic shape (Fig. 1A, middle). Preadipocyte factor 1 (Pref-1) expression, a preadipocyte transmembrane protein that inhibits adipogenesis (27), is progressively detected in cocultivated adipocytes (Fig. 1A, bottom). After 8 days of coculture, we also observed that mature adipocytes exhibit a loss in adipose markers expression that was more accentuated than in CAAs (8). An overall dramatic reduction of all the tested adipogenic markers including Adipisin, Adiponectin (APN), Ap2, HSL, as well as C/EBPα and PPARγ2 mRNA was observed by qPCR analysis (reduced by more than 70% for all tested genes, P < 0.001, Fig. 1B). These results were confirmed by Western blots for HSL, Adiponectin, PPARγ2, and C/EBPα expression, whose expression was no longer detected in cocultivated adipocytes (Supplementary Fig. S1A). Similar results were obtained by cocultivating adipocytes with the human ZR 75.1, HMT3522-T4.2 or mouse 4T1 breast cancer cell lines (Supplementary Fig. S1B), as well as when human mammary adipocytes derived from ex-vivo differentiation of progenitors, purified from mammary adipose tissue of healthy donors undergoing reduction mammoplasty, were used (Supplementary Fig. S1C). At 8 days, cocultivated
adipocytes exhibited a marked decrease in GLUT4 expression and a concomitant increase in GLUT1 expression (Fig. 1C) and became resistant to insulin (Fig. 1D). No gain of proliferative ability was observed in these cocultivated adipocytes exhibiting insulin resistance and a fibroblast-like shape. Indeed, the cell number, as well as the cell-cycle distribution, remained unchanged during the coculture period (see Supplementary Fig. S2).

We next investigated whether this process might be observed in vivo. To address this critical issue, we used GFP-tagged confluent 3T3-F442A preadipocytes implanted subcutaneously into athymic BALB/c nude mice, where they developed into typical fat pads after 5 weeks (21). Murine breast cancer cells 4T1 were then either injected into the newly formed fat pads or used alone as a control, whereas untreated fat pads also provided a separate control. Immunohistochemical staining for GFP was conducted in fat pads removed 10 days after the injection (Fig. 2A). Tagged-mature adipocytes were used to ensure that the cells with a fibroblast-like shape present in the tumors did in fact arise from local adipocytes and not from other sources. The fat pads derived from 3T3-F442A–confluent preadipocytes exhibited histologic features of adipose tissue. Adipocytes were positive for GFP whereas tumor cells were not (Fig. 2B, bottom). In the case of tumors injected into the fat pads, adipose tissue at the tumor periphery was composed of adipocytes of small size and elongated cells containing many small lipid droplets—all cells that are GFP positive (Fig. 2B, bottom, indicated by arrows). Within the tumor, GFP-positive cells with fibroblast-like shape appear to interdigitate with tumor cells (Fig. 2B, bottom, indicated by asterisks). Staining of the tissue sections with Masson trichrome revealed a significant amount of collagen fibrils in the tumor grown within the fat pad, whereas few collagen fibrils were seen in tumor grown alone (Fig. 2B, top). Taken together, these results are consistent with the fact that breast tumor cells induce phenotypic changes in mature adipocytes, leading to cells exhibiting low (if any) lipid content and profound morphologic changes associated with a marked increase in the expression of matrix proteins in the tumors. Therefore, our results compellingly suggest that cells with a fibroblast-like shape could be derived from mature adipocytes in a tumor-context both in vitro and in vivo.

**ADFs overexpress FSP-1, but not α-SMA, and exhibit an activated phenotype**

We then investigated the expression of major markers, previously used to identify CAFs in these cells (6). As shown in Fig. 3A, adipocytes cocultivated with tumor cells exhibit a gradual increase in FSP-1 expression, as opposed to α-SMA or FAP expression that remains very low. We confirmed that mature adipocytes cocultivated for 8 days with tumor cells overexpressed FSP-1 mRNA by 3-fold, compared with mature adipocytes grown alone (P < 0.01), whereas the difference in mRNA levels of α-SMA between these two conditions was not statistically significant (Fig. 3B). These results were confirmed with human mammary adipocytes (Supplementary Fig. S3). Functionally activated fibroblasts surrounding tumors are highly contractile and motile and also display increased ECM expression (3). As shown in Fig. 3C, increased expression of the ECM proteins, type 1 collagen and fibronectin, was also observed during adipocyte conversion. The fibrotic network produced by these cells in vitro is in accordance with the results obtained with Masson trichrome staining in vivo (see Fig. 2). As shown in Fig. 3D (top), coculture of adipocytes with tumor cells was associated with a profound reorganization of actin cytoskeleton, marked by the occurrence of stress fibers and the formation of punctuate spots of vinculin at the ends of actin bundles. Accordingly, cocultivated adipocytes, as they acquire a fibroblast-like phenotype, progressively exhibit increased migratory (Fig. 3D, bottom left) and invasive (Fig. 3D, bottom right) abilities. Therefore, our compelling results show that tumor cells can cause mature 3T3-F442A adipocytes, as well as human mammary adipocytes, to revert to an activated FSP-1 fibroblastic cell population. Because of the common characteristics of CAFs and these cells, highlighting that they represent a subpopulation of CAFs, we named them ADFs. In vitro, the CAA to ADF transition is time dependent (Figs. 1 and 3) and intermediate forms of adipocyte phenotypical changes (from small adipocytes to fibroblast-like cells that may or may not still contain small lipid droplets) could be observed in vitro and in vivo (Figs. 1 and 2). Once modified at the tumor invasive front, where the cross-talk between breast tumor and adipocytes is established (8), our data strongly suggests that ADFs are able to join the center of the tumor and participate in the desmoplastic reaction by exhibiting increased migratory and invasive abilities (Fig. 3), as shown in the murine fat pad experiments (Fig. 2).

**The Wnt/β-catenin pathway is reactivated in ADFs and contributes to the occurrence of the ADFs phenotype**

Among the many signaling networks involved in relaying information from extracellular signals during adipogenesis, the Wnt/β-catenin pathway plays a prominent role (28). This pathway negatively regulates precursors’ commitment and differentiation along the adipocyte lineage (28) and its activation has recently been shown to also induce a “dedifferentiated” state in mature adipocytes (17). As shown in Fig. 4A (top), adipocytes cocultivated with tumor cells exhibit a gradual increase in β-catenin expression, associated with its redistribution from the cell membrane to the cytoplasm. During activation of this pathway by Wnt ligands (the so-called canonical pathway), the β-catenin is hypophosphorylated and translocated into the nucleus where it contributes to the activation of Wnt target genes (28). A progressive increase in active β-catenin in the nucleus of cocultivated adipocytes was detected (Fig. 4A, bottom). In accordance, cocultivated adipocytes exhibit an upregulation of all Wnt target gene mRNAs analyzed as described for Dishevelled-1 (Dvl1), Wnt10b, Endothelin-1 (ET1), and metalloproteinase-7 (MMP7; Fig. 4B). Taken together, our results show that the canonical Wnt/β-catenin pathway is reactivated during coculture and is likely to contribute to the occurrence of the ADFs. Given our results, we reasoned that targeted inhibition of this signaling using ICG-001, a unique small molecule that selectively inhibits TCF/β-catenin transcription in a CBP-dependent fashion (19) might be able to mitigate the tumor-induced changes in adipocytes.
As shown in Fig. 4C, exposure to ICG-001 for 3 days significantly inhibits the increase in Wnt10b expression induced by coculture (about 3-fold decrease in 5 μmol/L ICG-001–treated cells compared with untreated cells, \( P < 0.05 \)) and partially restores lipid accumulation in cocultivated adipocytes in a dose-dependent manner. We next investigated the signal emanating from tumor cells that could have reactivated the canonical Wnt/β-catenin pathway. Soluble Wnt3a or TNF-α has been recently shown to induce the "dedifferentiation" of murine and human adipocytes through activation of the Wnt/β-catenin pathway (17). TNF-α was undetectable in the supernatant of all breast cancer cell lines that were cultivated with or without adipocytes. In contrast, Wnt3a was expressed and secreted by the breast cancer cells, as shown in Fig. 4D for the

Figure 2. The phenotypic changes of mature adipocytes towards fibroblast-like cells are observed in vivo. A, GFP-tagged confluent 3T3-F442A preadipocytes were implanted subcutaneously into athymic BALB/c nude mice, where they develop into fat pads after 5 weeks. Murine breast cancer cells 4T1 were then injected into the newly formed fat pads or assayed separately. Immunohistochemical staining for GFP was conducted in fat pads removed 10 d after the injection, tumor cells alone being also used as a control. B, representative Masson trichrome–stained sections from fat pads alone, tumors grown alone, or tumors grown into the fat pads with collagen stained in blue and nucleus in red (top). Representative images of the expression of GFP (brown) visualized in fat pads alone, tumors grown alone, or tumors grown into the fat pads (lipids, white; nucleus, blue; middle). Arrows indicate GFP-positive elongated cells with small lipid droplets present at the tumor periphery, whereas asterisks identify GFP-fibroblast-like cells within the tumor; scale bars, 50 μm. The bottom panel shows a two-fold magnified view of the framed areas.
SUM159PT cells. The levels of Wnt3a expression and secretion were not regulated by coculture. Similar results were obtained with the human breast cancer cell lines ZR 75.1 and MDA-MB231 (Supplementary Fig. S4). As shown in Fig. 4E (left), in the presence of Wnt3a blocking antibody, we observe a clear inhibition of β-catenin upregulation and redistribution induced by coculture, suggesting that Wnt3a is indeed upstream of the β-catenin stabilization. Of note, cells that have been treated with Wnt3a blocking antibody exhibit a significant decrease of Wnt10b mRNA expression (1.6-fold decrease in antibody-treated cells compared with untreated cells, \( P < 0.05 \)) and conversely, an increase in lipid content as compared with untreated cells (Fig. 4E, right). Therefore, the occurrence of ADFs is associated with activation of the Wnt/β-catenin pathway and blocking this pathway partially reversed the ADFs phenotype (Fig. 4C and E). Elevated levels of cytoplasmic and/or nuclear β-catenin are detectable by immunohistochemistry in the majority of breast tissue samples and high β-catenin activity significantly correlated with poor prognosis of the patients (29). This activation might rely on Wnt ligands because several studies have described overexpression of Wnt protein themselves, including Wnt3a, in breast cancer cells lines as well as breast tumors relative to normal tissue (30). Expression of these ligands is generally thought to activate the canonical Wnt/β-catenin pathway in tumor cells by an autocrine mechanism (31) to favor tumor growth, migration, inhibition of

Figure 3. ADFs overexpress FSP-1, but not α-SMA, and exhibit an activated phenotype. A, 3T3-F442A mature adipocytes were grown on coverslips and cultivated in the presence (C) or in the absence (NC) of tumor cells during 3, 5, and 8 days (D3, D5, and D8). After this period, cells were fixed and stained with the indicated antibodies. 3T3-F442A preadipocytes served as a control. Nuclei were labeled with TO-PRO-3; scale bars, 20 μm. B, α-SMA and FSP-1 gene expression was evaluated by qPCR in adipocytes cultivated in the presence (C) or in the absence (NC) of tumor cells during 8 days with tumor cells. Bars and errors flags represent the means ± SEM of at least three independent experiments; statistically significant by Student t test, \(^* \), \( P < 0.01 \) relative to NC cells. NS, non significant. C, fibronectin and collagen 1 expression were assessed by immunofluorescence in adipocytes cultivated in the presence (C) or in the absence (NC) of tumor cells during 3, 5, and 8 days (D3, D5, and D8). 3T3-F442A preadipocytes served as a control. Nuclei were labeled with TO-PRO-3; scale bars, 20 μm. D, top, 3T3-F442A mature adipocytes were grown on coverslips and cultivated in the presence (C) or in the absence (NC) of tumor cells during 3, 5, and 8 days (D3, D5, and D8). After this period, cells were fixed and stained with the indicated antibodies. 3T3-F442A preadipocytes served as a control. Nuclei were labeled with TO-PRO-3; scale bars, 20 μm. Bottom, adipocytes grown alone (NC) or in the presence of tumor cells (C) for 3 (D3) and 8 (D8) days. Adipocytes were then trypsinised and used for migration (left) or Matrigel invasion (right) assays towards a medium containing either 0% (white bars) or 10% FCS (black bars). Bars and errors flags represent the means ± SEM of three independent experiments; statistically significant by Student t test; \(^* \), \( P < 0.05 \); \(^{**} \), \( P < 0.01 \); \(^{***} \), \( P < 0.001 \) relative to NC cells.
Figure 4. The Wnt/β-catenin pathway is reactivated in ADFs and contributes to the occurrence of this new stromal cell population. A, adipocytes cultivated in the presence (C) or in the absence (NC) of tumor cells during 3, 5, and 8 days (D3, D5, D8) and 3T3-F442A preadipocytes were fixed and stained with the indicated antibodies. Nuclei were labeled with TO-PRO-3; scale bars, 20 μm. B, mRNA expression (qPCR) of the indicated Wnt/β-catenin targets was evaluated in adipocytes cultivated in the presence (C) or in the absence (NC) of tumor cells during 3, 5, and 8 days (D3, D5, D8). C, Wnt10b mRNA expression (right) and Bodipy accumulation or morphology observed through phase contrast microscopy (left; scale bars, 20 μm) were evaluated in adipocytes cultivated in the presence (C) or in the absence (NC) of tumor cells for 3 days with tumor cells in the presence of increasing concentrations of ICG-001. D, Wnt3a expression and secretion was measured in the SUM159PT cells cultivated in the presence (C) or in the absence (NC) of adipocytes. E, adipocytes were cultivated in the presence (C) or in the absence (NC) of breast tumor cells in the presence (+) or absence (-) of a Wnt3a blocking antibody. In these adipocytes, the expression of Wnt10b mRNA was measured by qPCR (right), and the β-catenin expression and lipid accumulation were evaluated by immunofluorescence. Nuclei were labeled with TO-PRO-3 (left); scale bars, 20 μm. Bars and errors flags represent the means ± SEM of three independent experiments; statistically significant by Student t test or ANOVA; *, P < 0.05; **, P < 0.01; ***, P < 0.001 relative to NC cells.
apoptosis, and potentially stem cell renewal in certain tissues (32). We propose here a new and paracrine effect of Wnt ligands in promoting the occurrence of a subpopulation of CAFs.

**ADFs stimulate tumor cells’ invasive phenotype in two-dimensional and three-dimensional coculture systems**

Mature adipocytes were cocultivated with breast cancer cells for 5 days to obtain ADFs, which were then used to carry out two-dimensional (2D) coculture experiments or to produce mixed spheroids with “naïve or unprimed” tumor cells. In 2D coculture experiments, ADFs strongly stimulated the invasive properties of SUM159PT cells (increase of 2.4-fold compared with tumor cells grown alone, \( P < 0.01 \); Fig. 5A), this effect being more pronounced in the presence of ADFs than in the presence of mature adipocytes (1.5-fold increase as compared with tumor cells grown alone, \( P < 0.05 \); 1.6-fold increase in cells cocultivated with ADFs as compared with mature adipocytes, \( P < 0.05 \)). In addition to the increased cell invasive abilities, we have previously shown that cocultivated SUM159PT exhibit morphologic changes along with the reorganization and polarization of vimentin (8). As shown in Fig. 5B, after 3 days of coculture, ADFs induce similar but more pronounced effects.

Figure 5. ADFs enhance tumor cells’ invasion in 2D and 3D coculture models. A, tumor cells cultivated in the presence (C) or in the absence (NC) of mature adipocytes or ADFs for 3 d were used for Matrigel invasion assay toward a medium containing either 0% (white bars) or 10% (black bars) FCS. Representative images of Matrigel invasion assay conducted towards a medium containing 10% FCS are shown below each graph; scale bar, 50 μm. Bars and errors flags represent the means ± SD of three independent experiments; statistically significant by Student’s t test; \( * \), \( P < 0.05 \); \( ** \), \( P < 0.01 \) relative to NC cells. B, immunofluorescence experiments using monoclonal antibody against vimentin or rhodamin phalloidin were conducted on tumor cells cultivated in the presence (C) or in the absence (NC) of mature adipocytes or ADFs for 3 days. Nuclei were labeled with TO-PRO-3; scale bar, 20 μm. C, a representative picture of spheroid invasion in a collagen matrix [left, scale bar, 250 μm and measurement of the invasion distance (in μm) 48 h after collagen embedding (right)]. Bars and errors flags represent the means ± SEM of three independent experiments, statistically significant by Student t test; \( ** \), \( P < 0.01 \) relative to spheroids composed by tumor cells alone (SUM), NS, nonsignificant. D, confocal microscopy analysis of spheroids invasion 48 hours after collagen inclusion, spheroids being composed by HcRed-SUM, by mixed HcRed-SUM + GFP-3T3-F442A, or by HcRed-SUM159PT + GFP-ADF; scale bar, 100 μm. SUM159PT, SUM.
on vimentin expression that was associated with profound actin reorganization, leading to the occurrence of stress fibers. As three-dimensional (3D) invasion models more closely follow the cell behavior observed in vivo (33), we confirmed our results using a spheroid invasion model within a collagen matrix (25; see Fig. 5C, left, for a representative experiment). We found that mixed ADFs/tumor cells spheroids exhibit higher invasive capacities compared with spheroids of tumor cell alone ($P < 0.01$), yet the presence of preadipocytes had no effect on 3D tumor cells' invasion (Fig. 5C, right), in accordance with the results previously obtained in 2D (8). HcRed-SUM159PT cells, GFP-preadipocytes, and GFP-ADFs were used to investigate the invasive behavior of each cell subpopulation. Interestingly, ADFs (and preadipocytes) remained in the center of the spheroids in contrast with tumor cells that invaded the collagen matrix (Fig. 5D). These combined results indicate that ADFs stimulate the invasive behavior of tumor cells.

Reorientation of mature adipocytes into ADFs is observed in human tumors

We next investigated whether ADFs are present in primary human tumors. To address this critical issue, we first conducted a histologic analysis of human breast tumors to investigate the presence of cells exhibiting ADF traits. As defined in vitro and in vivo in murine fat pads, ADFs originate from progressive changes of the morphology of adipocytes, giving rise to small adipocytes and of elongated cells containing many small lipid droplets. As shown in Fig. 6A, we observed a profound reorganization of the adipose tissue at the tumor invasive front (see left for the adipose tissue aspect at distance of the tumors) with numerous cells exhibiting the features of intermediate forms of ADFs. Of note, elongated cells with lipid droplets could be observed within the dense desmoplastic reaction surrounding the tumors (Fig. 6A, bottom, indicated by arrows).

Figure 6. ADFs are found in human tumors. A, histologic examination of human breast cancers showing distant adipose tissue (left) and tumor tissue containing reorientated adipocytes (middle) after H&E staining (original magnification, ×200; scale bar, 50 μm.). The right panels show a 2-fold magnification of the framed area. Results of representative experiments were obtained using tumors issued from 2 independent donors. B, histologic examination of adipose tissue (lipids, white; nucleus, blue) in a representative breast tumor after anti FSP-1 staining (brown). The results obtained in different areas from the adipose tissue away from the tumor to the tumor center are shown (scale bar, 50 μm). C, left, the expression of FSP-1 and αSMA was visualized (brown) in adipocytes (lipids, white; nucleus, blue) located adjacent to invading cancer cells (TU) as compared with normal adipose tissue (reduction mammoplasty (RM)). Right, the expression of the indicated genes was analyzed by qPCR in mature adipocytes obtained from RM or TU (8 independent samples per group), statistically significant by Student t test; $^*$, $P < 0.05$. NS, non-significant.
To further characterize these cells, we investigated FSP-1 expression in the breast-cancer surrounding adipocytes at variable distances from the tumor center. As shown in Fig. 6B, the mature adipocytes further from the tumor are negative for FSP-1 expression, whereas those closer to the tumor exhibit FSP-1 expression. FSP-1 positive fibroblasts were present in the center of the tumors as previously described (6). Therefore, as observed in vitro, we consistently observed a gradient of FSP-1 expression in mature adipocytes exhibiting first CAA, then ADF phenotype (Fig. 6B). Finally, we investigated the differential expression of both αSMA and FSP-1 in tumor-surrounding adipocytes by immunohistochemistry. In fact, one of the key findings of our study is that ADFs, at least in vitro, express FSP-1 but not αSMA (see Fig. 3A and B). Interestingly, using experimentally generated tumors in mice, Sugimoto and colleagues found unique FSP-1-expressing CAFs, which are clearly distinct from the αSMA-positive myofibroblasts (6). This subpopulation of CAFs is very important functionally, because FSP-1-positive fibroblasts promote tumor metastasis (34). Mammary carcinoma cells injected into FSP1−/− mice showed significant delay in tumor uptake and decreased tumor incidence (34). Coinjection of carcinoma cells with FSP1+/+ mouse embryonic fibroblasts partially restored the kinetics of tumor development and the ability to metastasize, underlying the role of this specific subset of stromal population in invasive processes (34). As shown in Fig. 6C (left), we found that α-SMA expression was not upregulated in adipocytes close to tumors when compared with normal breast adipose tissue (reduction mammoplasty) whereas in the same sample, an upregulation of FSP-1 expression was found. To further assess the expression of these markers by a quantitative approach, adipocytes were isolated from fat samples associated with tumors or reduction mammoplasty. A series of 16 samples, with 8 samples in each group (Supplementary Table S3 for the clinical characteristics of the patients), was used, matched with respect to age (mean: 54.5 ± 10.4 vs. 51.1 ± 8.3 years) and BMI (mean: 26.1 ± 3.5 vs. 24.9 ± 2.3 kg/m²). Adipocytes isolated from tumor samples overexpressed FSP-1 mRNA when compared with adipocytes isolated from normal mammary glands, whereas the mRNA levels of α-SMA were not statistically different between the two groups (Fig. 6C, right). Therefore, the phenotype described here both in vitro and in vivo for ADFs is clearly reminiscent of the one of FSP-1-positive CAFs, including the absence of α-SMA expression and the invasion-promoting effect. Because mature adipocytes represent the most abundant cellular type in breast cancer stroma, the process of adipocyte "dedifferentiation" is likely to provide a large part of this CAFs subpopulation. Interestingly, the impact of tumor cells on various components of adipose tissue might also participate in the heterogeneity of CAFs phenotype. In fact, it has been shown both in vitro and in vivo that breast cancer cells activate adipose stem cells towards a CAFs-like phenotype associated with α-SMA overexpression (for review see refs. 35, 10). Therefore, mature and precursor adipose cells could contribute to divergent CAFs subpopulation, whose relative contributions in cancer progression remain to be determined in lean and obese conditions.

Figure 7. Mechanism of ADFs occurrence and role in tumor progression. Schematic representation of the described bidirectional crosstalk established between breast cancer and adipose cells, underlying the presence of ADFs in the desmoplastic reaction of breast cancers.

To conclude, the proposed mechanism to explain the occurrence and the role of ADFs in breast cancer is summarized in Fig. 7. In our model, early local tumor invasion in breast cancer results in immediate proximity of tumor cells and adipocytes. Cancer cells secrete soluble factors, including Wnt3a, that are able to activate Wnt/β-catenin pathway in mature adipocytes, leading to adipocyte "dedifferentiation". Adipocytes undergo morphologic changes, first marked by a decrease in adipocyte size and lipid content (CAAs), then by acquiring a fibroblast-like morphology (ADF), yet still containing small lipid droplets. At the start of this process, tumor-primed adipocytes express FSP-1. ADFs exhibit increased migratory and invasive abilities and are able to join the center of the tumor, where they significantly promote tumor invasion. In the center of the tumor, these cells no longer express adipose markers nor exhibit lipid droplets, which render them indistinguishable from other CAFs cell population. Studying the process of occurrence of ADFs in breast cancer is of major clinical importance. In fact, the occurrence of specific stromal cells, such as ADFs, is critical to the aggressiveness of nearby tumor cells and they need to be identified to develop targeted therapeutic strategies inhibiting their development and/or tumor-promoting activity. Implicating adipose tissue in this crosstalk is also of major interest because several studies pointed out that obese women exhibit higher propensity for local invasion and distant metastasis (10). Obesity condition is also associated with an increase in adipose tissue fibrosis (15). Therefore, further studies should address whether ADFs are over-represented in the breast tumor stroma of obese patients.
in which they would provide an invasion-promoting population. Such studies will provide unique opportunities to set up specific strategies for the treatment of the subsets of patients exhibiting aggressive diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and Design: L. Bochet, B. Couture Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Bochet, S. Daviaud, C. Dray, Y. Wang, V. Laurent, S. Dauvillier, R. Guiet, I. Maridonneau-Parini, S. Le Gonidec, G. Escourrou Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Bochet, C. Lehuede, S. Daviaud, Y. Wang, B. Couture, V. Laurent, C. Dray, G. Escourrou, P. Valet Writing, review, and/or revision of the manuscript: L. Bochet, C. Lehuede, C. Muller

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Le Gonidec

Study supervision: C. Muller

References


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Ludivine Bochet, Camille Lehuédé, Stéphanie Dauvillier, et al.


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