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Cancer Research

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Cancer-Associated Adipocytes Exhibit an Activated Phenotype and Contribute to Breast Cancer Invasion

Béatrice Dirat[1,2,3], Ludivine Bochet[1,2,3], Marta Dabek[1,2], Danièle Daviaud[1,3], Stéphanie Dauvillier[1,2], Bilal Majed[6], Yuan Yuan Wang[1,2,3], Aline Meulle[1,2,3], Bernard Salles[1,2], Sophie Le Gonidec[1,3], Ignacio Garrido[4], Ghislaine Escourrou[5], Philippe Valet[1,3], and Catherine Muller[1,2]

Abstract

Early local tumor invasion in breast cancer results in a likely encounter between cancer cells and mature adipocytes, but the role of these fat cells in tumor progression remains unclear. We show that murine and human tumor cells cocultivated with mature adipocytes exhibit increased invasive capacities *in vitro* and *in vivo*, using an original two-dimensional coculture system. Likewise, adipocytes cultivated with cancer cells also exhibit an altered phenotype in terms of delipidation and decreased adipocyte markers associated with the occurrence of an activated state characterized by overexpression of proteases, including matrix metalloproteinase-11, and proinflammatory cytokines [interleukin (IL)-6, IL-1β]. In the case of IL-6, we show that it plays a key role in the acquired proinvasive effect by tumor cells. Equally important, we confirm the presence of these modified adipocytes in human breast tumors by immunohistochemistry and quantitative PCR. Interestingly, the tumors of larger size and/or with lymph nodes involvement exhibit the higher levels of IL-6 in tumor surrounding adipocytes. Collectively, all our data provide *in vitro* and *in vivo* evidence that (i) invasive cancer cells dramatically impact surrounding adipocytes; (ii) peritumoral adipocytes exhibit a modified phenotype and specific biological features sufficient to be named cancer-associated adipocytes (CAA); and (iii) CAAs modify the cancer cell characteristics/phenotype leading to a more aggressive behavior. Our results strongly support the innovative concept that adipocytes participate in a highly complex vicious cycle orchestrated by cancer cells to promote tumor progression that might be amplified in obese patients. *Cancer Res; 71(7); 2455–65. ©2011 AACR.*

Introduction

Cells that compose the tumor stroma are associated, if not obligate, partners in tumor progression (1–3). In breast cancer, stromal cells include resident adipocytes and fibroblasts, a wide range of recruited hematopoietic cells, and newly formed blood vessels with their associated cells (3). Dynamic and reciprocal communication between epithelial and stromal compartments occurs during breast cancer progression (1, 2). Cancer cells usually go about generating a supportive microenvironment by activating the wound-healing response of the host (4). Conversely, the stromal cells, such as cancer-associated fibroblasts or tumor-associated macrophages, promote tumor progression by secreting growth factors, chemokines, and promigratory extracellular matrix components (2). To date, most of the studies focused on cancer cell–mesenchymal cell interactions have emphasized the contribution of fibroblasts, endothelial, and inflammatory cells (1). Little attention has been given to the mature adipocytes despite the fact that in breast cancers, early local tumor invasion results in immediate proximity of cancer cells to adipocytes (3). Until recently, adipocytes were mainly considered as an energy storage depot, but there are now clear evidences pointing adipocytes as endocrine cells producing hormones, growth factors, cytokines, and other molecules named adipokines (5). Therefore, mature adipocytes represent excellent candidates to influence tumor behavior through heterotypic signaling processes.

Adipokines, such as leptin, adiponectin (APN), hepatocyte growth factor, or collagen VI, stimulate the growth and survival of breast tumor cells even in estrogen-negative cells (6, 7). Moreover, it has been shown that metalloproteinase (MMP)-11/stromelysin-3 is induced in adipose tissue by cancer cells as they invade their surrounding environment (8).

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Obesity, wherein the normal balance of adipose tissue secretory proteins is disturbed, has been recently identified as a negative prognosis factor for breast cancer (9, 10). This poor prognosis seems to be independent of menopausal status, tumor stage, and tumor hormone–binding characteristics (for review, see refs. 11–13). Several studies pointed out that obese women exhibit at diagnosis an increase in lymph nodes involvement and a higher propensity to distant metastasis (10, 11, 14–17). Furthermore, obesity is also associated with the occurrence of tumors of high grade with increased local and distant invasion in prostate cancer (18). Taken together, these compelling observations suggest that excess adiposity may favor tumor invasiveness by yet uncharacterized mechanism(s). We show here that tumor-surrounding adipocytes exhibit profound phenotypic changes *in vitro* and in human tumors, and these modified adipocytes promote tumor cell invasion and metastasis.

Materials and Methods

Cell culture

The murine 3T3-F442A preadipocyte cell line was differentiated as described previously (19). To estimate the adipocyte phenotype, triglyceride (TG) content was quantified using a colorimetric kit (Wako Chemicals), and red oil staining was carried out as previously described (20). The human breast cancer cell line ZR 75.1 (provided by Prof. K. Bystricky, Laboratoire de Biologie Moléculaire Eucaryote, Toulouse, France) and the murine 67NR and 4T1 breast cancer cell lines (ref. 21; provided by Dr. S. Vagner, INSERM U563, Toulouse, France) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (ZR 75.1 and 67NR) and 5% (4T1) fetal calf serum (FCS). The human breast cancer cell line SUM159PT (provided by Dr. J Piette, IGMM, Montpellier, France) was grown in Ham F12 (50:50) medium complemented with 5% FCS, 1 μg/mL hydrocortisone (Sigma), and 0.2 UI/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. For ZR 75.1 and SUM159PT cell lines, the expression status of estrogen receptor (ER) α, E-cadherin, N-cadherin, and vimentin was confirmed by immunoblotting before they were used in the experiments (22). For all cell lines, their invasive behavior was confirmed using Boyden chamber assays before they were used in the experiments. Cell lines 67NR and 4T1 were also tested for their ability to form (4T1) or not (67NR) metastatic tumors when injected in syngenic BALB/c mice (21).

Human samples

Breast adipose tissue samples were collected from reduction mammoplasty (RM) or tumorectomy (TU), according to the guidelines of the Ethical Committee of Toulouse-Rangueil. All subjects gave their informed consent to participate in the study, and investigations were conducted in accordance with the Declaration of Helsinki as revised in 2000. In cases of TU, the fat tissue samples obtained were immediately close to the tumor mass and were devoid of fibrosis, as assessed macroscopically (data not shown). Adipose tissue pieces were immediately used for collagenase digestion as previously described (23) and centrifuged (20 minutes, room temperature, 1,000 rpm) to separate adipocytes from the stroma vascular fraction (pellet, SVF).

Coculture and invasion assays

Tumor cells and adipocytes were cocultured using a Transwell culture system (0.4-μm pore size; Millipore). A total of 5×10^4 (67NR), 1.5×10^5 (SUM159PT, 4T1), or 3×10^5 (ZR 75.1) cells were seeded in the top chamber of the Transwell system in the culture medium of adipocytes and cocultivated with or without mature adipocytes in the bottom chamber for the indicated times. Adipocytes or tumor cells cultivated alone in similar conditions served as controls. To evaluate the effect of adipocyte-conditioned medium (Ad-CM) on tumor cell invasion, mature adipocytes were cultivated overnight with serum-free medium containing 1% of delipidated bovine serum albumin (Sigma) and medium was collected. CM was also collected from adipocytes previously cocultivated during 3 days with tumor cells [cancer-associated adipocytes conditioned medium (CAA-CM)]. After 3 days of coculture in the presence of mature adipocytes, Ad-CM, or CAA-CM (complemented with 10% FCS), tumor cells were used to conduct Matrigel invasion assays as previously described (24). A total of 35 to 40 fields per filter were counted using an optical microscope. Invasion assays were also conducted with tumor cells previously cultivated for 3 days with recombinant human IL-6 (PeproTech).

Western blot analysis

Whole cell extracts and immunoblots were prepared as previously described (25). All the antibodies used in our study are presented in Supplementary Information.

Tail vein metastasis assays

A total of 1×10^6 4T1 cells, previously cocultivated either with or without mature adipocytes for 4 days, were injected intravenously in BALB/c mice (6 animals per condition). Mice were sacrificed after 14 days, and lungs were taken for analyses. Nodules were scored from 1 to 5 according to their likelihood to represent metastasis to lung (0, normal lung tissue; 1, tissue with 1–5 nodules; 2, 6–10 nodules; 3, 11–20 nodules; 4, 21–30 nodules; and 5, >31 nodules). Lungs were then fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and nodules were observed. The mean number of nodules per lung area and the mean nodule area as a function of lung area (that defined the nodule area index) were measured by ImageJ software program. The local Institutional Animal Care and Use Committee approved the experimental protocols described in the study.

Immunofluorescence, immunohistochemistry, and determination of adipokines secretion

Immunofluorescence and immunohistochemistry were carried out as previously described (8, 24). Concentration of

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secreted factors in supernatants was determined using the xMAP Technology (Luminex), with a Milliplex kit (Millipore).

RNA extraction and quantitative PCR

Gene expression was analyzed using real-time PCR as described previously (23). The sequences of the primers are presented in Supplementary Information.

Statistical methods

Quantitative expression of biological markers measured by quantitative PCR (qPCR) in isolated human adipocytes was assessed using estimations of means and SDs. Hence, a normal distribution of these measures was assumed. However, the size of subpopulations was limited in most cases. Thus, we tested differences about the expression of biological markers between subpopulations of interest, using nonparametric tests (i.e., Wilcoxon's nonparametric rank test). Quartiles were also computed; box plots permitted to appreciate the presence of outliers and to illustrate differences in the expression of biological markers. The threshold of statistical significance was fixed at alpha risk at 5%. SPlus statistical software was used for the data analysis (26). For the *in vitro* experiments, the statistical significance of differences between the means (at least 3 independent experiments) was evaluated using Student's *t* test, done using Prism (GraphPad Inc.). The values of $*P \geq 0.05$, $**P < 0.01$, and $***P < 0.001$ were deemed as significant whereas "NS" stands for not significant.

Results and Discussion

Tumor cells cocultivated with mature adipocytes exhibit an enhanced invasive phenotype

Human cancer cells with low (ZR 75.1) or high (SUM159PT) invasive capacities (27) were grown for 3 days on Transwells, allowing the diffusion of soluble factors, in the presence or absence of murine mature adipocytes (28). After 3 days, tumor cells were trypsinized and Matrigel invasion assays were conducted in a medium containing either 0% or 10% FCS. As shown in Figure 1A, the invasive capacity of ZR 75.1 tumor cells in 10% FCS containing medium was increased by 3-fold in cells previously cocultivated for 3 days with adipocytes as compared with tumor cells grown alone ($P < 0.01$). No additional increase in the invasive capacity of ZR 75.1 was observed when the time of coculture with adipocytes was increased up to 5 days (data not shown). This effect was not observed when tumor cells were cocultivated with preadipocytes (Supplementary Fig. S1A). Similar results were obtained with the SUM159PT cell line, although to a lesser extent (Fig. 1A, $P < 0.05$). Experiments were also carried out with the mouse mammary cell line 4T1 and its nonmetastatic sibling cell line 67NR (21). Coculture with adipocytes increases both 67NR and 4T1 invasive capacities by 2-fold (Fig. 1B, $P < 0.05$ and 0.01, respectively). Therefore, mature adipocytes are able to stimulate the invasive capacities of murine and human breast cancer cell lines that are either positive (ZR 75.1) or negative (SUM159PT, 67NR, 4T1) for the ER. In addition, the proinvasive effect was independent from the tumor growth–promoting effect because coculture with adipocytes has no effect on ZR 75.1 or murine tumor cell lines proliferation in contrast to SUM159PT cells (Supplementary Fig. S1B). When tumor cells were grown in the presence of CM obtained from adipocytes, which have never been cocultivated with tumor cells (Ad-CM), invasive capacities were not significantly increased for any of cell lines (Fig. 1C). When CM was prepared from adipocytes previously cocultivated with tumor cells (CAA-CM), a significant stimulation of tumor invasive capacities was observed in all the tested models as compared with cells grown in control medium ($P < 0.05$ for the 67NR cells, $P < 0.01$ for the ZR 75.1, SUM159PT, and 4T1 cells). These results highlight that a cross-talk between the 2 cell populations is necessary to obtain this effect (Fig. 1C).

Noncocultivated ZR 75.1 cells displayed a characteristic epithelial morphology and form compacted colonies. Yet, when these cells were cocultivated with adipocytes from 3 to 5 days, morphologic changes became apparent. Cells formed less compacted colonies and exhibited a more scattered aspect. In addition, these cells displayed a downregulation of membrane E-cadherin expression, in association with β-catenin redistribution, resulting into a disorganized state in the cytoplasm (Fig. 2A). Decreased E-cadherin expression was observed both at mRNA (Fig. 2B) and protein levels (Fig. 2C) without an increased expression of mesenchymal markers (Fig. 2B and C), suggesting the occurrence of an incomplete epithelial-mesenchymal transition (EMT). For the SUM159PT cell line, which has already undergone a complete EMT, morphologic changes were also observed along with the reorganization and polarization of vimentin (Fig. 2A) without a change in its expression level (Fig. 2C).

Our coculture system shows that adipocytes stimulate the invasiveness of tumor cells *in vitro*. To test the hypothesis that "priming" tumor cells with adipocytes regulate tumor cell distal organ seeding and growth, we conducted tail vein metastasis assays, using the 4T1 cell line. 4T1 tumor cells were cultured *in vitro* for 4 days in the presence or absence of adipocytes, harvested, and injected intravenously into BALB/c mice. As shown in Figure 3A, the number of lung metastases was enhanced in mice injected with 4T1 cells previously cocultivated with adipocytes as compared with mice injected with 4T1 grown alone ($P < 0.05$). Histologic analysis (Fig. 3B) confirmed that both the number and size of the nodules were significantly increased in mice injected with adipocytes-cocultivated 4T1 compared with 4T1 cells grown alone (Fig. 3C and D, $P < 0.05$ for the 2 parameters). Therefore, our results clearly show that adipocytes promote the invasive phenotype of breast tumor cells both *in vitro* and *in vivo*.

Cocultivated adipocytes exhibit extensive phenotypic changes

Adipocytes have been shown to promote tumor survival and growth *in vitro* and *in vivo* (6, 29), but their contribution to tumor cell invasion is an emerging concept. Because our results showed that a cross-talk between the 2 cell populations is necessary to observe the proinvasive effect (Fig. 1C), we next examined the phenotype of cocultivated adipocytes. As shown in Figure 4A (left), mature adipocytes cocultivated with ZR 75.1 tumor cells exhibited a dramatic reduction in

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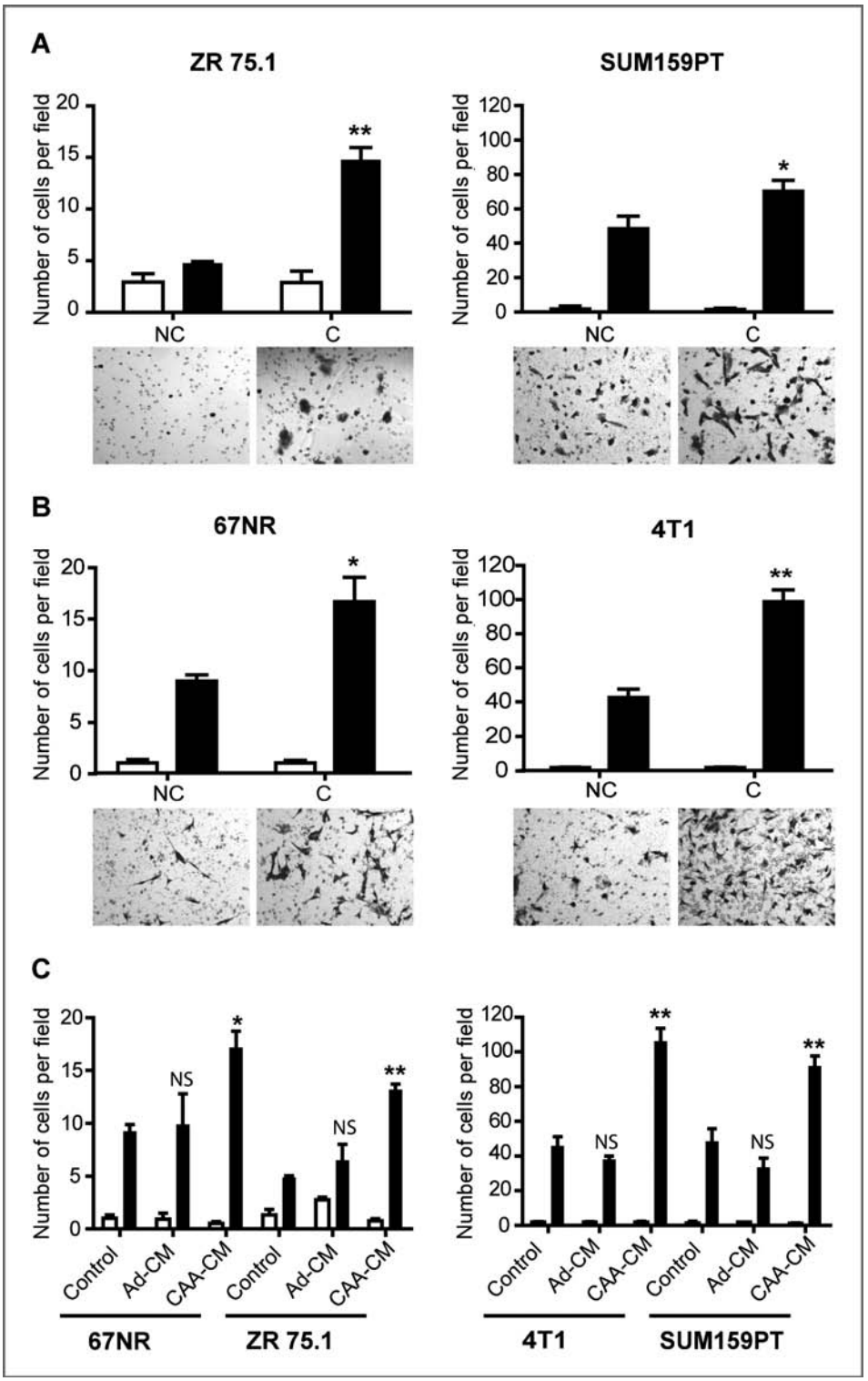


Figure 1. Coculture of breast tumor cells with mature adipocytes stimulates their invasive capacities. A, ZR 75.1 or SUM159PT cells were cocultivated in the presence (C) or absence (NC) of mature adipocytes. After 3 days, tumor cells were trypsinized and used for Matrigel invasion assay in a medium containing either 0% (white bars) or 10% FCS (black bars). B, similar experiments were done with the 67NR and 4T1 murine cancer cells. A and B, representative images of Matrigel invasion assay conducted in a medium containing 10% FCS are shown below each graph. C, indicated cells were cultivated either in normal medium (control), in CM obtained from adipocytes (Ad-CM), or in CM obtained from adipocytes previously grown in the presence of tumor cells (CAA-CM). After 3 days, tumor cells were trypsinized and used for Matrigel invasion assays in a medium containing either 0% (white bars) or 10% FCS (black bars).

the number and size of lipid droplets that correlated with a significant decrease in lipid accumulation (Fig. 4A, right, $P < 0.05$). These adipocytes lose several terminal differentiation markers exemplified by the strong decrease in HSL (hormone-sensitive lipase), APN, resistin, and aP2 mRNA (ref. 5; Fig. 4B, $P < 0.05$ for all the markers), this loss was also confirmed at protein levels for APN and resistin (Fig. 4C, $P < 0.05$). In cocultivated adipocytes, the level of C/EBPα was dramatically reduced ($P < 0.001$) whereas that of PPARγ was not significantly decreased (Fig. 4B). As previously stated,

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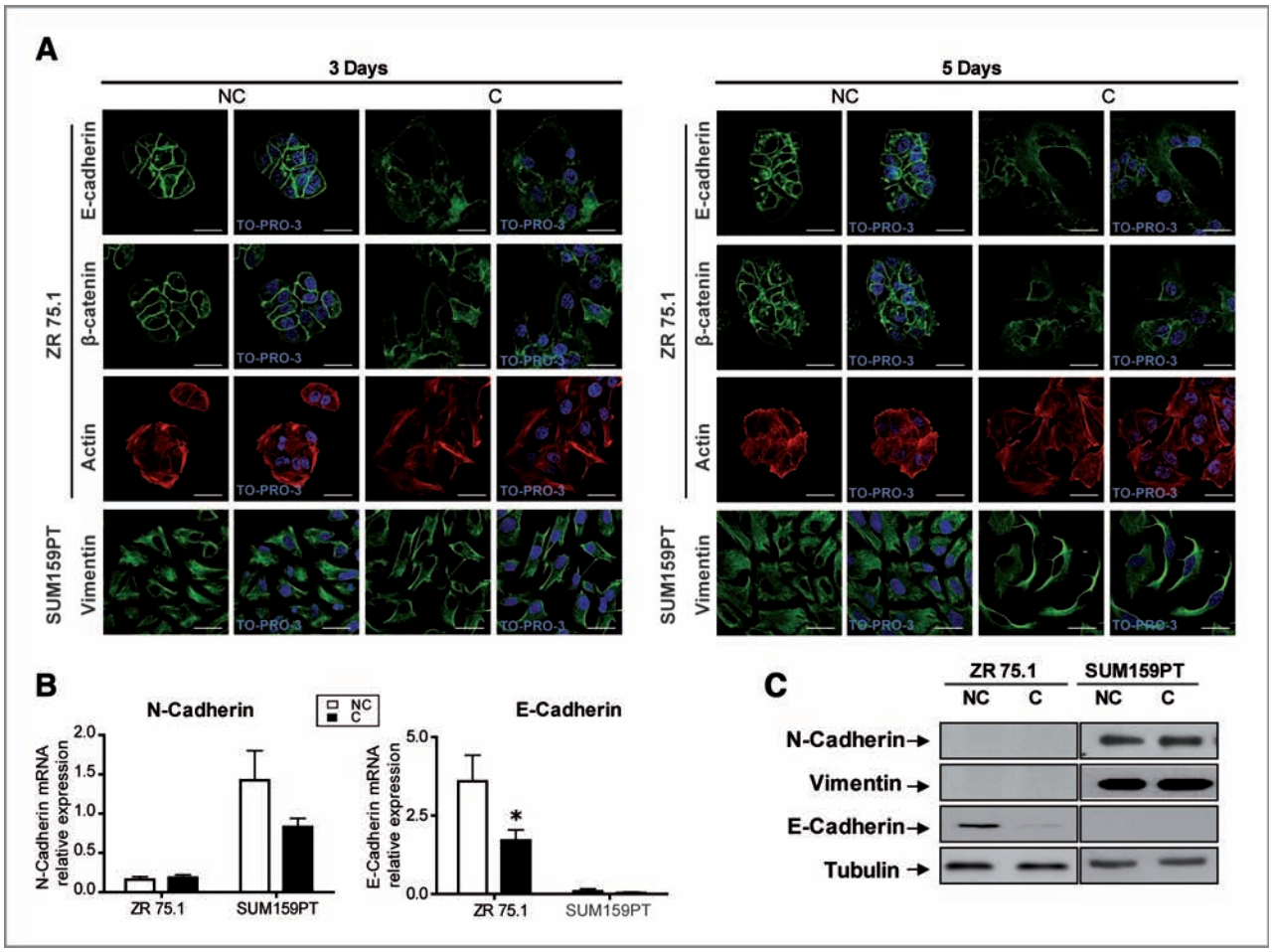


Figure 2. Cancer cells exhibit morphologic changes and incomplete EMT on coculture with adipocytes. A, ZR 75.1 or SUM159PT were grown on coverslips in inserts, the lower wells cocultivated in the presence (C) or absence (NC) of mature adipocytes. After 3 or 5 days, cells were fixed and stained with the indicated antibodies. Nuclei were labeled with TO-PRO-3; scale bars, 40 μmol/L. B, relative mRNA expression of N-cadherin and E-cadherin in indicated tumor cell lines cocultivated in the presence (C) or absence (NC) of murine adipocytes for 5 days evaluated by qPCR. C, analysis of protein expression of the indicated markers done by Western blots with extracts obtained from tumor cells cocultivated in the presence (C) or absence (NC) of adipocytes (5 days).

MMP-11/stromelysin-3 is induced *in vivo* in adipose tissue by cancer cells as they invade their surrounding environment (8). Similarly, an upregulation for this protease was noted in the cocultivated adipocytes, highlighting the *in vivo* relevance of this coculture model (Fig. 4B, $P < 0.001$). In contrast, the levels of MMP-9 (Fig. 4B) and MMP-2 (not shown) remain unchanged whereas a significant increase (10-fold) of plasminogen activator inhibitor-1 (PAI-1) expression was found (Fig. 4B, $P < 0.01$). In cocultivated adipocytes, a 5-fold increase in levels of IL-6, IL-1β, and TNF-α was shown at mRNA levels (Fig. 4B, $P < 0.001$ for all cytokines). At protein levels, however, only IL-6 and IL-1β were found to be significantly higher in the supernatant of cocultivated cells (Fig. 4C, $P < 0.05$ for both cytokines) whereas the levels of TNF-α remained undetectable in both conditions (data not shown). Of note, these extensive adipocyte phenotypic changes were obtained with other tumor cells lines (Supplementary Fig. S2). Furthermore, these results were confirmed with mammary adipocytes derived from *ex vivo* differentiation of progenitors purified from mammary adipose tissue of healthy donors undergoing RM (6 independent donors; Supplementary Fig. S3). Therefore, we extensively described tumor-induced changes in adipocytes that are reproducibly displayed by both human and mouse cocultivated adipocytes. Collectively, our observations clearly show that cocultivated adipocytes generally exhibit a loss of lipid content, a decrease in late adipose markers, and overexpression of inflammatory cytokines and proteases. We named these cells "cancer associated adipocytes" (11).

CAAs are found in human tumors

We next investigate whether these cells are present in primary human tumors. To address this critical issue, we first conducted a histologic analysis of human breast tumors. As exemplified in Figure 5A, we consistently observed at the invasive front of human breast tumors the presence of adipocytes of smaller sizes with a dilated interstitial space, which

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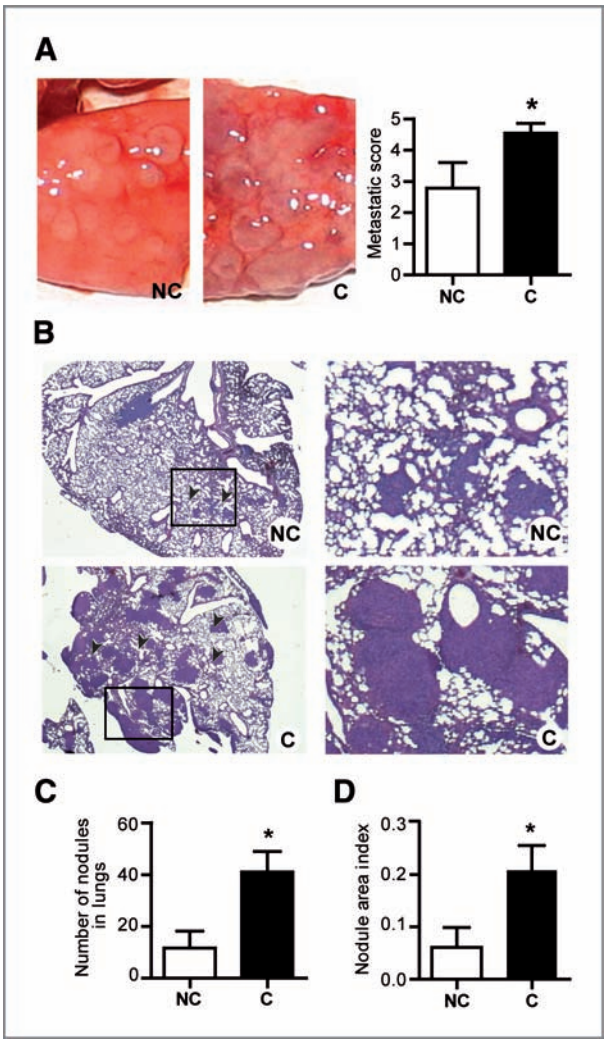


Figure 3. Coculture with adipocytes stimulates the metastatic potential of 4T1 cells *in vivo*. A, left, representative lungs harvested at necropsy after tail vein injection of 4T1 cocultivated in the presence (C) or absence (NC) of adipocytes during 4 days prior to injection. Right, metastatic score as determined by macroscopic examination. B, left, H&E stained transverse sections of host lungs from mice injected with 4T1 cells previously cocultivated in the presence (C) or absence (NC) of adipocytes during 4 days (original magnification × 40). Examples of invading lesions are shown by arrows. Right, magnified views of invading lesions. C and D, metastasis number obtained from mice injected with 4T1 cells, previously cocultivated in the presence (C) or absence (NC) of adipocytes during 4 days, is expressed either as the average number of metastasis sites per lung area (C) or as nodule area index (D) representing the average metastasis area normalized by total lung area.

might be related to extensive extracellular matrix occurring in tumor-surrounding adipocytes such as collagen VI overexpression (6). Further characterization of these CAAs was done by immunohistochemistry. As shown in Figure 5B (i and ii), we observed a decreased expression of the adipocyte marker APN in CAAs as compared with normal mammary adipose tissue. Although MMP-11 and IL-6 were not detected in mature adipocytes of RM (Fig. 5B, iii and v, respectively), these proteins were expressed within the rim of cytoplasm of the adipocytes located proximally to the invasive cancer cells (Fig. 5B, iv and vi, respectively). To further assess the expression of these markers by a quantitative approach, adipocytes were isolated from fat samples associated with TU or RM (23). A series of 28 samples, with 14 samples in each group (Supplementary Tables S1 and S2 for the clinical characteristics of the patients), was used comparable with respect to age (mean: 51.8 ± 7.3 vs. 60 ± 13) and BMI (26.2 ± 2.8 vs. 25.2 ± 2.3). Adipocytes isolated from tumor samples overexpressed MMP-11 ($P = 0.001$), PAI-1 ($P < 0.01$), and IL-6 ($P < 0.001$), as compared with adipocytes purified from normal mammary gland, whereas the mRNA levels of IL-1β and TNF-α were not statistically different (Fig. 5C). The adipose tissue also contains stromal cells (present in the SVF), such as fibroblasts, endothelial cells, and adipocyte precursors. The levels of expression of MMP-11, PAI-1, IL-6, IL-1β, and TNF-α remained unchanged between normal and tumor-surrounding adipose SVF, highlighting that the observed increase in proinflammatory molecules and proteases is an adipocyte-dedicated event (Fig. 5C). Furthermore, when the expression levels of IL-6 in adipocytes were analyzed as a function of tumor characteristics, we found that higher levels of this cytokine were associated with the tumors of larger size and with lymph nodes involvement (Fig. 5D). This correlation was not found either for PAI-1 or for MMP-11 (Supplementary Fig. S4). Taken together, these results show that CAAs are found in human tumors.

Our data strongly support the concept that an intimate cross-talk is established between cancer cells and mature adipocytes at the invasive front of the tumor. Invading tumor cells are able to modify adipocytes phenotype, which, in turn, stimulate cancer cells aggressive behavior (30). The mechanism(s) responsible for the transition from mature adipocytes to CAAs remain(s) unclear. Modification resembling to CAAs is also observed in adipose tissue of tumor-bearing mice far from the tumor site. In cachexia, adipocytes also exhibit morphologic modification, decreased in adipogenic transcription factors such as C/EBPα and late differentiation markers such as leptin (31). However, during cancer cachexia, adipocytes exhibit a decrease in resistin expression and upregulation of lipolytic enzymes such as HSL or ATGL (adipose triglyceride lipase) (31), events not found in CAAs (Fig. 4 and Supplementary Fig. S5), alluding to their unique phentotype. Several molecules expressed by tumor cells have been described as strong suppressors of adipogenesis such as IL-1β, TNF-α, or even IL-6 (32), representing one mechanism by which tumor cells might directly influence adipocyte behavior. An alternative mechanism might be adipocytes themselves as the source of autocrine signals that induce the CAAs phenotype. In favor of the latter hypothesis, we showed that tumor CM was not able to reproduce CAAs phenotype, suggesting that either adipocyte factors or inducible factors in tumor cells are necessary to observe this effect (Supplementary Fig. S6).

Adipocyte IL-6 plays a key role in mediating adipocyte-dependent invasive activity of tumor cells

Our results show that IL-6 is overexpressed in tumor-surrounding adipocytes both *in vitro* and *in vivo*. We showed

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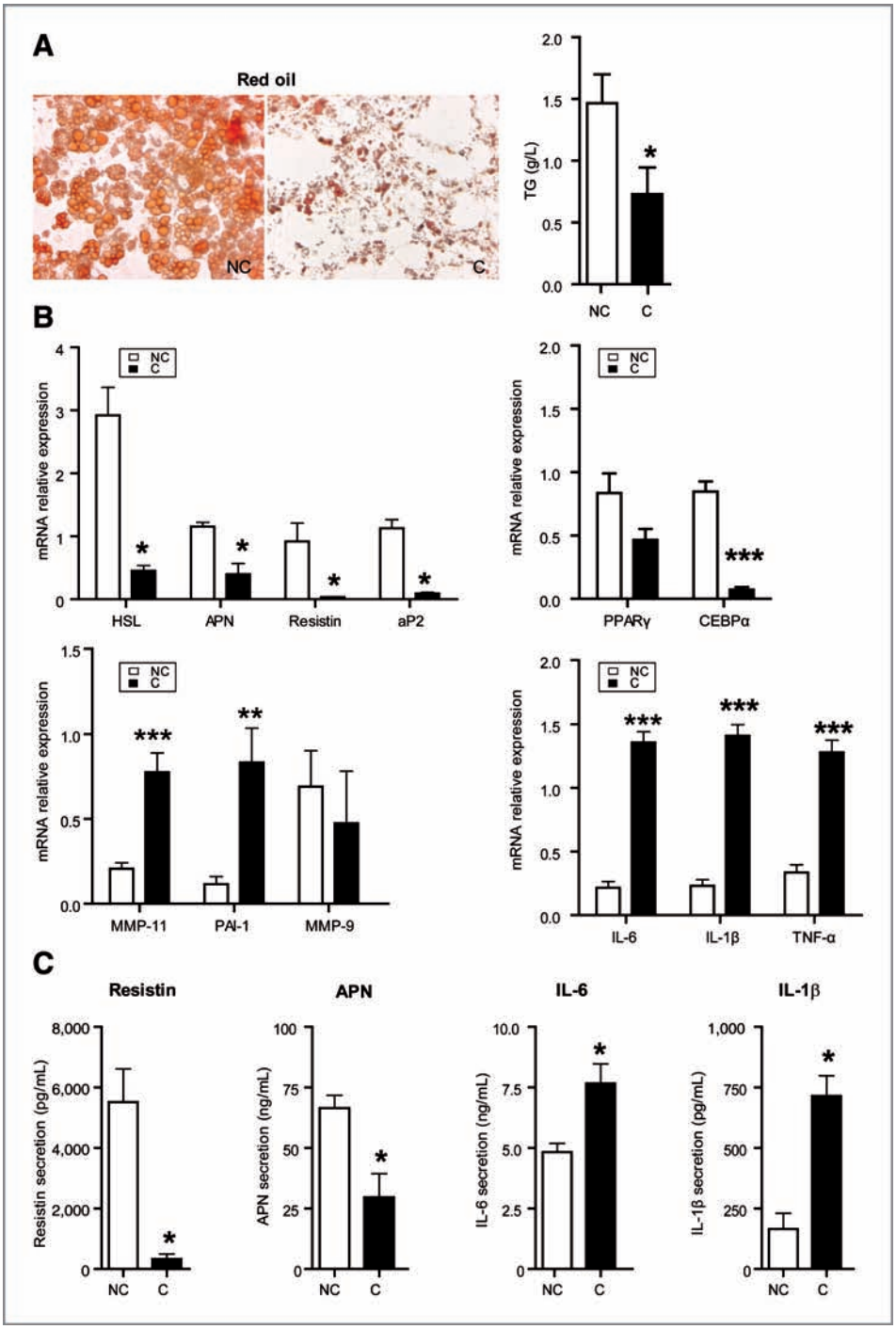


Figure 4. Adipocytes cocultivated with breast tumor cells undergo extensive phenotypic changes. A, left, mature murine adipocytes cocultivated in the presence (C) or absence (NC) of ZR 75.1 tumor cells were stained with red oil. Right, the TG content was dosed in adipocytes. B, adipocytes were cocultivated in the presence (C) or absence (NC) of ZR 75.1 tumor cells. After 3 days, mRNAs were extracted and expression of the indicated genes was analyzed by qPCR. C, dosage of the indicated murine secreted factors in the supernatants of adipocytes grown alone (NC) or in the presence of breast tumor cells (C).

that IL-6 (at 10 ng/mL, a dose equivalent to the levels secreted by CAAs, Fig. 4C) significantly increased the invasive capacities in ZR 75.1 and 67NR cells (Fig. 6A, $P < 0.01$ for the 2 cell lines). As compared with untreated cells, the epithelial-like ZR 75.1 cells exposed to IL-6 form less compacted colonies and exhibit a more scattered aspect (Fig. 6B). A downregulation of membrane E-cadherin expression resulting into a disorganized state in the cytoplasm, in association with β-catenin redistribution (Fig. 6B), was observed in treated cells in accordance with the literature (33–35). Strikingly, when ZR 75.1 cells were cocultivated with adipocytes in the presence of murine IL-6 blocking antibody, the adipocyte proinvasive effect was significantly decreased (Fig. 6C, $P < 0.01$). mIL-6 blocking antibody specifically

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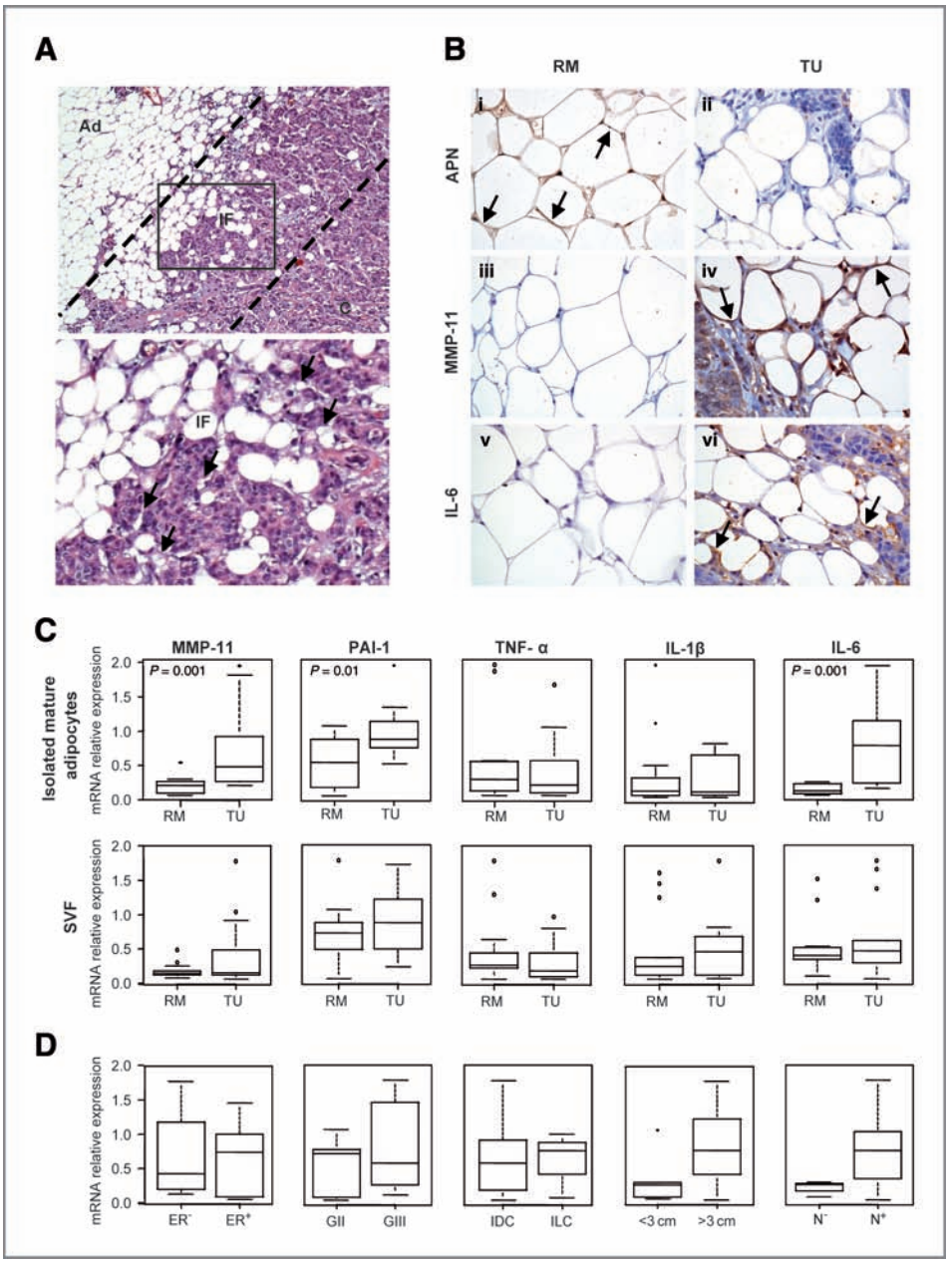


Figure 5. CAAs from human breast tumor exhibit extensive phenotypic changes in accordance with the *in vitro* coculture assays. A, top, histologic examination of an invasive breast tumor after H&E staining (original magnification × 200). Ad, adipose tissue; IF, invasive front; C, tumor center. Bottom, histologic magnification of the invasive front of the tumor. Note that the number and the size of adipocytes were reduced (adipocytes of smaller size are indicated by arrows). B, the expression of APN (i and ii), MMP-11 (iii and iv), and IL-6 (v and vi) was visualized (in brown) in adipocytes (lipids in white, nucleus in blue) located adjacent to invading cancer cells (right) as compared with normal adipose tissue (left). Arrows indicate the expression of proteins of interest within the rim of cytoplasm of adipocytes. C, top, the expression of the indicated genes was analyzed by qPCR in mature adipocytes obtained from RM or TU (14 independent samples per group). Bottom, similar experiments were done with the SVF of the samples. D, the expression of IL-6 was measured as a function of tumor characteristics. G, grade; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; N, lymph node involvement.

inhibits the proinvasive effect of CAA-CM in both 67NR and 4T1 models and had no effect on the invasive capacities of cells grown in control medium (Fig. 6D, $P < 0.01$ and $P < 0.05$, respectively). Altogether, these results indicate that IL-6 plays a key role in the CAAs proinvasive effects.

In breast cancer patients, the extent of the increase in serum IL-6 correlates with poor disease outcome and reduced prognosis (36). Recent evidence shows that fibroblast-derived IL-6 supports breast tumor growth and metastasis (37). Our results show that the stromal IL-6 is also produced by mature adipocytes surrounding the tumors. The absence of IL-6 overexpression in the SVF of human adipose tissue isolated from breast tumor biopsies (Fig. 5C) apparently contradicts a recent study conducted *ex vivo*, and not *in vivo*, using human adipose tissue–derived stromal cells cocultivated with tumor cells (38). Our results obtained in human tumors suggest that within the cellular complexity of the adipose tissue, the cross-talk with tumor cells is rather established with mature, than with precursors, adipose cells (Fig. 5C and D). In addition, because mature adipocytes are the most abundant cells in the breast tumor stroma (3), their cumulative secretion of IL-6 could therefore have profound impact on tumor progression.

Finally, this work paves the way for future clinical and cellular studies aimed at determining the impact of this

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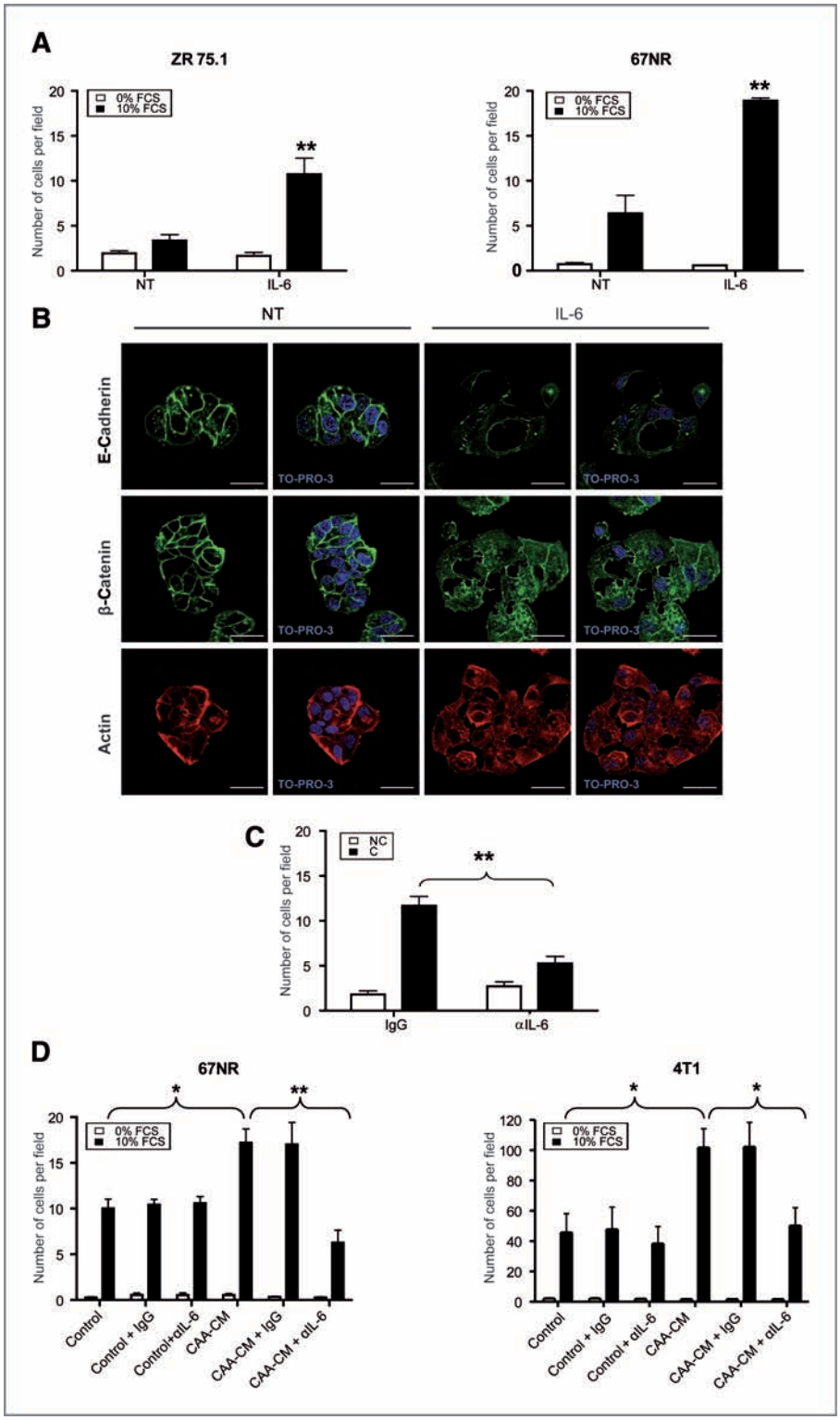


Figure 6. IL-6 is involved in the adipocyte-driven proinvasive effect. A, ZR 75.1 and 67NR cells were cultivated either alone (NT) or in the presence of IL-6, and after 3 days, they were harvested to conduct Matrigel assays in a medium containing either 0 or 10% serum. B, similarly treated ZR 75.1 cells were fixed and stained with the indicated antibodies. Nuclei were labeled with TO-PRO-3; scale bars, 40 μmol/L. C, ZR 75.1 were cocultivated for 3 days in the presence (C) or absence (NC) of murine adipocytes. As indicated, blocking antibody directed against murine IL-6 (αIL-6) or control antibody (IgG) was added in the culture medium. Tumor cells were then harvested to conduct Matrigel invasion assays in a medium containing 10% FCS. D, 67NR and 4T1 cells were cultivated in normal medium (control), normal medium containing control immunoglobulin (control + IgG), or blocking antibody directed against murine IL-6 (control + αIL-6) conditioned medium obtained from adipocytes previously grown in the presence of tumor cells (CAA-CM), CAA-CM containing control (CAA-CM + IgG), or IL-6 blocking antibody (CAA-CM + αIL-6). After 3 days, tumor cells were harvested to conduct Matrigel invasion assay in a medium containing either 0% or 10% FCS.

cross-talk in obesity conditions. Indeed, our results suggest that the cross-talk between the CAAs and tumor components might be amplified in obesity in which the normal balance of adipose tissue secretory proteins is disturbed. Obesity is related to a condition of low chronic inflammation characterized by the abnormal production of inflammatory cytokines involved in insulin resistance (5). Obesity and the now-defined profile of CAAs share inflammation as common

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traits, including overexpression of IL-6 with the noticeable absence of overexpression of TNF-α in CAAs (Fig. 5). Interestingly, the association of high levels of IL-6 in CAAs with high tumor size and enhanced local invasion reflects those traits present in obese patients (10, 16, 17). Therefore, CAAs within a context of obesity should be more prone to amplify the negative cross-talk with tumor cells that we have identified and characterized in this study. This is a hypothesis that remains to be shown in both murine and human models. 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.

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