Cancer Research

Interactions between Adipocytes and Breast Cancer Cells Stimulate Cytokine Production and Drive Src/Sox2/miR-302b–Mediated Malignant Progression

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Abstract

Consequences of the obesity epidemic on cancer morbidity and mortality are not fully appreciated. Obesity is a risk factor for many cancers, but the mechanisms by which it contributes to cancer development and patient outcome have yet to be fully elucidated. Here, we examined the effects of coculturing human-derived adipocytes with established and primary breast cancer cells on tumorigenic potential. We found that the interaction between adipocytes and cancer cells increased the secretion of proinflammatory cytokines. Prolonged culture of cancer cells with adipocytes or cytokines increased the proportion of mammosphere-forming cells and of cells expressing stem-like markers *in vitro*. Furthermore, contact with immature adipocytes increased the abundance of cancer cells with tumor-forming and metastatic potential *in vivo*. Mechanistic investigations demonstrated that cancer cells cultured with immature adipocytes or cytokines activated Src, thus promoting Sox2, c-Myc, and Nanog upregulation. Moreover, Sox2-dependent induction of miR-302b further stimulated *cMYC* and *SOX2* expression and potentiated the cytokine-induced cancer stem cell–like properties. Finally, we found that Src inhibitors decreased cytokine production after coculture, indicating that Src is not only activated by adipocyte or cytokine exposures, but is also required to sustain cytokine induction. These data support a model in which cancer cell invasion into local fat would establish feed-forward loops to activate Src, maintain proinflammatory cytokine production, and increase tumor-initiating cell abundance and metastatic progression. Collectively, our findings reveal new insights underlying increased breast cancer mortality in obese individuals and provide a novel preclinical rationale to test the efficacy of Src inhibitors for breast cancer treatment. *Cancer Res; 76(2); 491–504. ©2016 AACR.*

Introduction

Obesity is associated with a more aggressive course and greater mortality for several cancers (1–3). Breast cancers in obese individuals show increased inflammation, hypoxia, angiogenesis, and changes in endocrine factors, including insulin, IGF-1, leptin, adiponectin, and estrogen (4). Obese tissue adipocytes upregulate chemokines and leptin, which attract macrophages that produce proinflammatory proteins, including TNFα and IL1β, and proangiogenic factors, providing a microenvironment favorable for adipose tissue expansion, but also propitious for tumor progression (2).

Obese individuals frequently have elevated proinflammatory cytokine levels in serum (3, 5). High-serum cytokines are also associated with poor breast cancer patient outcome (6–8). Because autocrine and paracrine cytokine pathway activation can promote oncogenesis and tumor metastasis (9), we investigated how interactions between invading breast cancer and adipose cells could alter the potential for aggressive tumor behavior and metastasis.

The cancer stem cell (CSC) model posits that tumors are generated and maintained by a subpopulation of cells that self-renew and yield heterogeneous progeny with reduced proliferative potential (10). Increasing evidence indicates that

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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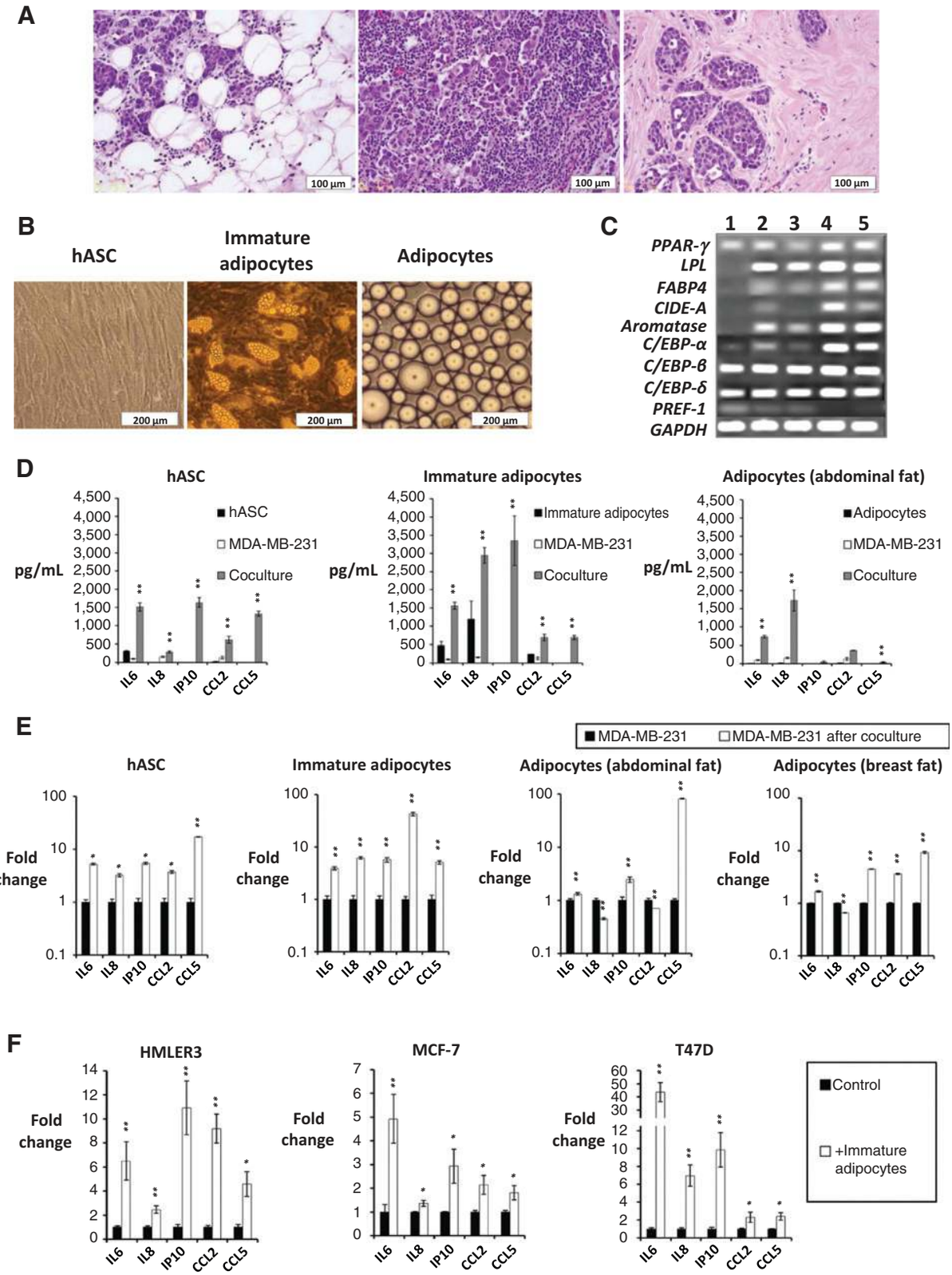


CSC underlie drug resistance tumor recurrence and metastasis (10). Deregulation of pathways that control normal embryonic stem cells (ES) may contribute to breast cancer–initiating cell expansion (11, 12). ES-transcription factors (ES-TF) Oct4, Sox2, Klf4, Nanog, and Myc are master regulators of ES self-renewal whose enforced expression in somatic cells yields induced pluripotent stem cells (13). ES-TFs govern expression of short non-coding miRNAs that modulate gene expression programs (14, 15). miR-302 drives ES self-renewal and its loss predicates differentiation (16). miR-302b overexpression on its own can induce pluripotency in somatic cells (17, 18). Mechanisms governing ES-self-renewal are often subverted by cancer cells. Src kinase is not only critical for maintaining ES pluripotency and proliferation, but is also a key mediator of oncogenesis (11, 19). Src is activated by proinflammatory cytokines and can mediate malignant transformation (9).

Although tissue niche influences ES self-renewal (11), little is known of how cancer cell interactions with the tissues they invade promote cancer progression. When breast cancer cells transgress the mammary duct basement membrane, they encounter fibroblasts, inflammatory cells, and adipocytes. Activated fibroblasts and tumor-associated macrophages (TAM) are known to drive breast cancer invasion (20, 21). This work indicates that the interaction between breast cancer cells and adipocytes, which occurs early in breast cancer invasion, markedly upregulates proinflammatory cytokines. These increase the abundance of stem cell–like cells that initiate highly metastatic breast cancers. Furthermore, these cytokines activate Src to upregulate Sox2 and induce miR-302b, generating feed-forward loops that contribute to mammary CSC self-renewal and drive metastatic tumor progression.

Materials and Methods

Cell culture

MDA-MB-231, MCF-7, and T47D were mycoplasma free, authenticated by short tandem repeat profiling, and passaged for <6 months. HMLER3 and BPLER3 were cultured as in ref. 22. Dissociated tumor cells, DT28, and carcinoma-associated fibroblasts (CAF23) were isolated from primary triple-negative breast cancers (23) and used at early passage. Human mammary fibroblasts (HMF; ScienCell) were cultured as per the manufacturer's instructions. Human adipocyte stem cells (hASC) and mature adipocytes were isolated from human abdominal fat from cytoreduction surgery and breast fat obtained at mastectomy (three samples each), collected with informed consent and Institutional Review Board approval (see Supplementary Methods). Immature adipocytes were differentiated from hASC or MIAMI cells (bone marrow derived, human mesenchymal stem cells) using adipogenic differentiation media for 4 weeks as in refs. 24 and 25. hASC, *in vitro* differentiated (immature), and mature adipocytes were cocultured with breast cancer cells over 7 days (details in Supplementary Methods). For conditioned medium assays, MDA-MB-231 was cultured in immature adipocyte conditioned media over 1 week. Cytokines were used at 10 ng/mL (100 ng/mL for IL8; R&D Systems) for 7 days, unless otherwise indicated, and media renewed every 2 days.

Cytokine arrays

After 7 days of monoculture or coculture, media were collected and cytokine concentrations assayed by an xMAPBio-Plex cytokine array (Bio-Rad Life Sciences) using a Luminex 200 plate reader (Biosource, Invitrogen; ref. 26).

Xenograft assays

For orthotopic limiting dilution assays, MDA-MB-231-luc-GFP cells were flow sorted from immature adipocytes after 7 days coculture, and 10, 100, or 1,000 viable GFP^+ cells were suspended in 100 μL Matrigel and injected into the mammary fat pad of 4-week-old Balb/c nude mice ($n = 8$–10/group).

To analyze metastasis from primary tumors, tumors formed from 10^4 MDA-MB-231-luc-GFP cells were excised at 1 cm and metastatic tumor bioluminescence monitored by Xenogen IVIS weekly (27).

For tail vein injections, 2×10^5 MDA-MB-231-luc-GFP cells were injected and monitored by IVIS weekly (27). Lungs were photographed and surface tumors counted.

Primary tumors, metastatic lymph nodes, or lung tumors were excised and pooled for each experimental group and single-cell suspensions seeded for sphere assays as in ref. 27. All animal research was University of Miami Animal Care Committee approved.

Circulating tumor cell analysis

Circulating tumor cells (CTC) were recovered and quantitated as in ref. 28.

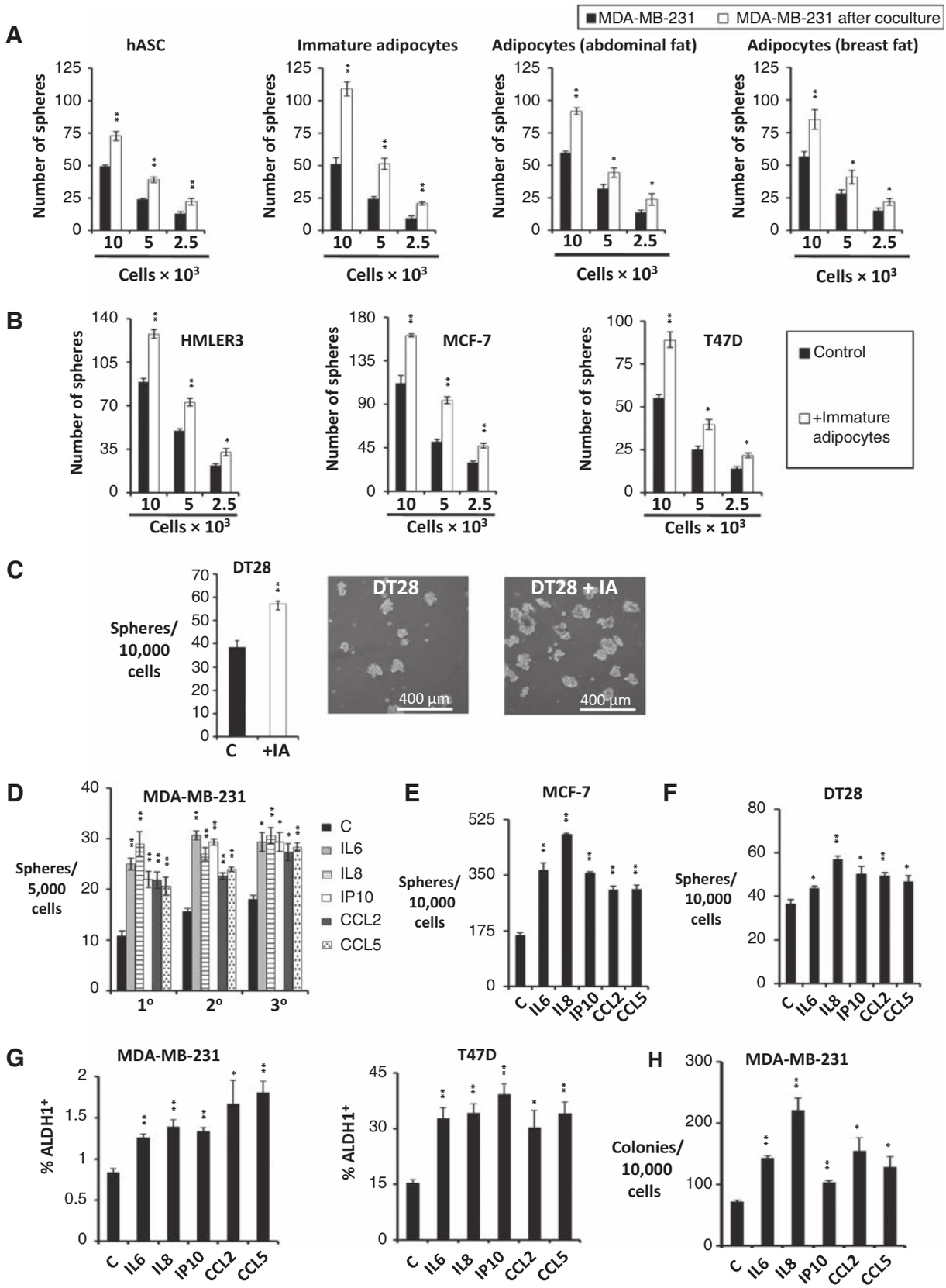
Src inhibition and SOX2 knockdown

Src inhibitors, 4-amino-5-(4-methylphenyl)-7-(*t*butyl) pyrazolo[3,4-*d*]pyrimidine (PP1, Sigma) or saracatinib/AZD0530 (from Astra Zeneca), each at 1 μmol/L, were added for 48 hours after 5 days of cytokine or adipocyte exposure, then RNA was extracted or spheres were seeded. For Western blot analyses, see Supplementary Methods. Src inhibitors were added 20 minutes before cytokine treatment, and proteins extracted at intervals for Western blot analyses.

At least three different siRNA oligonucleotides to *SOX2* and scrambled controls (Cell Signaling Technology) were used per the manufacturer's instructions. SOX2 knockdown was confirmed by Western blot analysis 48 to 72 hours after transfection.

Figure 1.

Adipocyte–breast cancer cell coculture increases proinflammatory cytokines. A, hematoxylin and eosin–stained sections of a single human invasive ductal carcinoma showing tumor cell contact with adipocytes (left), inflammatory cells (middle), and fibrous desmoplasia (right). B, photomicrographs of cultured hASC, immature adipocytes, and adipocytes. C, gel electrophoresis of PCR for adipogenesis markers in hASC (1); immature adipocytes differentiated from MIAMI cells (2); immature adipocytes differentiated from hASC (3); abdominal fat adipocytes (4); and breast fat adipocytes (5). D, cytokine concentrations in media conditioned by 7-day monocultures of hASC, immature adipocyte, adipocyte, or MDA-MB-231, or adipose–cancer cell cocultures graphed as mean pg/mL ± SEM ($n = 3$; *, $P < 0.05$; **, $P < 0.01$ vs. Σ monocultures). E, qPCR of cytokine expression in MDA-MB-231 cultured alone or with hASC, immature adipocytes, or adipocytes from abdominal or breast fat for 7 days. F, qPCR of cytokine expression in HMLER3, MCF-7, and T47D cells cultured alone or cocultured with immature adipocytes for 7 days. Data were normalized to 1 for monocultures using *GAPDH* as internal control and graphed as mean ± SEM ($n \geq 3$; *, $P < 0.05$; **, $P < 0.01$). See also Supplementary Fig. S2.



miRNA profiling

RNA was isolated from MDA-MB-231 with or without exposure to 10 ng/mL IL6, CCL5, or IP10 for 7 days. RNA integrity number was 9.3 to 10. miRNA array analysis assayed 742 miRNA targets and 6 reference genes (mirBase 13) using Human microRNA Ready-to-Use PCR Panels I and II. Details are in Supplementary Methods.

miR-302b overexpression and knockdown

Cells were transduced with lentiviral hsa-miR-302b construct, anti–miR-302b construct, or GFP control vectors (System Biosciences) as described in Supplementary Methods.

Statistical analysis

All graphs present mean ± SEM from ≥3 assays. The Student *t* test was used for experiments with two groups. Comparisons of more than two groups used one-way ANOVA followed by Dunnett or Tukey *post hoc* analysis. Some experiments used two-way ANOVA followed by Tukey *post hoc* tests. Tumor-initiating cell frequency was calculated by L-Calc Limiting Dilution Software (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx) from STEMCELL Technologies Inc., as given in ref. 29. Statistical differences between tumor growth curves (orthotopic or after tail vein) used the "Compare Growth Curves" function statmod software package (http://bioinf.wehi.edu.au/software/compareCurves/).

Results

Adipocyte–breast cancer cell coculture increases proinflammatory cytokine secretion

Primary human breast cancers invade surrounding fat and contact adipocytes, inflammatory infiltrate, and fibrous stroma (Fig. 1A). To investigate consequences of breast cancer/adipocyte interactions, human adipocyte stem cells (hASC) or mature adipocytes were isolated from human abdominal or breast fat. Adipocytes were differentiated from either hASC or marrow-isolated adult multilineage inducible (MIAMI) cells (Fig. 1B). Fat-derived hASC were cultured for more than three passages and did not contain contaminating immune cells (see Supplementary Fig. S1A for hASC characterization; refs. 24, 25). *In vitro* differentiated adipocytes formed adherent cultures containing abundant lipid droplets (see Fig. 1B), and expressed fat lineage markers that were undetectable in hASC. *In vitro* differentiated adipocytes showed intermediate maturation, expressing preadipocyte factor 1 (*PREF-1*), which was absent in mature adipocytes, and lower levels of aromatase, PPARγ, and other markers than mature adipocytes (Fig. 1C). These are referred to hereafter as immature adipocytes.

hASC, immature adipocytes, and mature adipocytes were cocultured with estrogen receptor–negative (ER^-) MDA-MB-231 breast cancer cells. Over 200 secreted cytokines and growth factors were assayed by Luminex 200 in media from monocultures or after 1 week of adipose cell–cancer cell coculture. Of these, concentrations of proinflammatory cytokines IL6, IL8, IFNγ-inducible protein-10 (IP10, also called CXCL10), CCL2 (MCP1), and CCL5 (RANTES) were each synergistically increased after breast cancer cell coculture with hASC or immature or mature adipocytes (Fig. 1D). GFP-tagged MDA-MB-231 cells were sorted from hASC, immature adipocyte, or adipocyte cocultures and cancer cell cytokine expression assayed by qPCR (Fig. 1E). Although all adipose cells significantly upregulated cytokine expression by MDA-MB-231, these five cytokines increased most consistently and strongly after immature adipocyte coculture (Fig. 1E).

In obese fat, the immature adipocyte:mature adipocyte ratio is increased and immature adipocytes are a major source of proinflammatory cytokines (4, 30, 31). Thus, subsequent investigation focused on immature adipocyte–cancer cell interaction. Comparison of relative cytokine expression before and after coculture showed immature adipocytes were the major producer of proinflammatory cytokines (Supplementary Fig. S2A). Coculture of immature adipocytes separated by a Transwell also increased MDA-MB-231 cytokine expression but less than after direct coculture (Supplementary Fig. S2B). Exposure to immature adipocyte conditioned medium modestly increased IL6 and CCL2 (Supplementary Fig. S2C).

To test the generalizability of these findings, immature adipocytes were also cocultured with oncogene-transformed ER^- human mammary epithelial HMLER3 (22), and with ER^+ MCF-7 and T47D. All cell lines showed a significant increase in expression (Fig. 1F) and in secretion of IL6, IL8, IP10, CCL2, and CCL5 after 1 week of coculture (Supplementary Fig. S2D).

Immature adipocyte and cytokine exposures increase the abundance of cells with stem cell properties *in vitro*

GFP-tagged MDA-MB-231 were flow sorted after 7 days coculture with hASC, immature adipocytes, or mature adipocytes from abdominal or breast fat, and seeded into limiting-dilution mammosphere assays as in ref. 32. Exposure to all adipose cell types significantly increased MDA-MB-231 sphere formation, but this was most notable with immature adipocytes (Fig. 2A). Immature adipocyte coculture also increased sphere formation by HMLER3, MCF-7, and T47D (Fig. 2B and Supplementary Fig. S3A). Results were validated using an early passage, primary dissociated breast cancer culture, DT28, whose tumor marker and gene expression profiles reflect that of its primary cancer (23). Immature adipocyte coculture significantly increased sphere formation (Fig. 2C).

Because secretion of these five cytokines, among 200 cytokines assayed, was synergistically increased following cancer cell–fat cell coculture (Fig. 1D), these were further investigated. A 7-day exposure to IL6, IL8, IP10, CCL2, or CCL5 significantly increased sphere-generating cells in breast cancer lines and DT28 cultures (Fig. 2D–F and Supplementary Fig. S3A–S3C). Notably, all

Figure 2.

Immature adipocyte or cytokine exposures increase sphere forming cells and ALDH1 activity. Mammosphere-formation after 7-day adipose cell or cytokine exposures. A and B, MDA-MB-231 cells alone or with indicated adipose cell types (A), and HMLER3, MCF-7 and T47D cells alone or with immature adipocytes (B). C, mammospheres from DT28 alone or with immature adipocytes. Representative photomicrographs (right). D, MDA-MB-231 cells were cultured ± indicated cytokines for 7 days, then seeded in sphere assays ±cytokines for further 7 to 10 days. Spheres were harvested and reseeded for secondary and tertiary sphere formation without further cytokine. MCF-7 (E) and DT28 (F) were cultured ± indicated cytokines and then seeded in sphere assays. G, percentage of $ALDH1^+$ cells in MDA-MB-231 and T47D cells cultured ± cytokines for 7 days. H, soft agar colonies from MDA-MB-231 cells seeded ± indicated cytokines. Results graphed as mean ± SEM ($n \geq 3$; *, $P < 0.05$; **, $P < 0.01$). See also Supplementary Fig. S3.

cytokines caused a sustained increase in sphere-forming cells when primary MDA-MB-231 spheres were dissociated and reseeded without cytokine over two serial sphere assays (Fig. 2D). Increased sphere formation was not a global mitogenic effect. These cytokines did not increase short-term population growth or stimulate cell cycle (representative data, Supplementary Fig. S3D and S3E).

Prolonged cytokine exposure also increased ALDH1$^+$ cells (shown for MDA-MB-231 and T47D, Fig. 2G) and clonogenicity in soft agar (Fig. 2H).

Normal and cancer-associated mammary fibroblasts express lower cytokine levels and yield less mammosphere formation after coculture

Breast cancer cells invading local fat also encounter fibroblasts. Thus, effects of breast cancer cell coculture with normal HMFs and cancer-associated fibroblasts (CAF) were compared with those of immature adipocytes. Both HMF and CAF coculture upregulated cancer cell cytokine expression, but generally to lower levels than with immature adipocytes (Fig. 3A). Baseline cytokine expression in HMFs and CAFs was lower than in adipocytes and, after coculture, increased to levels at least a log lower in magnitude than observed in cocultured immature adipocytes (Fig. 3B). Notably, HMF and CAF only modestly upregulate CCL2 after coculture, and neither increased CCL2 in cocultured MDA-MB-231. The increase in sphere formation by MDA-MB-231 after coculture with HMF or CAF was also less than after immature adipocyte coculture (Fig. 3C).

Immature adipocyte coculture increases tumor-initiating cells and metastasis

Effects of immature adipocyte coculture on tumor-initiating cell abundance were assayed *in vivo*. GFP+ MDA-MB-231-luc was grown with or without immature adipocytes for 1 week, then 10, 100, or 1,000 sorted cancer cells were injected orthotopically. All 1,000 cell injections yielded tumors. Tumor initiating cell frequency increased 3-fold and tumor latency decreased after prior adipocyte exposure (Fig. 4A and B, $P < 0.0278$). Despite the more rapid tumor growth from fat exposed cells (data for 10^4 injected cells, Fig. 4C), Ki-67 staining was similar ($n = 7$–10 tumors/group; Fig. 4D). Thus, immature adipocyte exposure did not increase global tumor proliferation, further supporting a stem cell effect.

Increasing data suggest that CSC may initiate metastasis (10). Prior MDA-MB-231 exposure to immature adipocytes or cytokines for 1 week increased subsequent Matrigel invasion *in vitro* (Supplementary Fig. S4A and S4B). To test whether prior immature adipocyte exposure increased metastasis, orthotopic tumors were excised at 1 cm and subsequent metastasis documented by IVIS. Notably, immature adipocyte-exposed cells generated more deeply invasive tumors, and 2 of 7 mice died at excision. Metastases arose in all remaining animals. Only 3 of 7 control mice developed metastasis. Although metastasis-bearing animal numbers did not differ significantly, adipocyte-exposed cells generated a greater volume of metastases than controls (Fig. 4E). Dissociated primary tumors and nodal metastasis generated by adipocyte-exposed cells yielded significantly more spheres than controls (Fig. 4F). Thus, exposure to immature adipocytes appears to cause a stable increase in the stem cell population, sustained during primary tumor formation and subsequent metastasis.

Immature adipocyte and cytokine exposures increase experimental lung metastasis

To further investigate effects on metastasis, GFP +MDA-MB-231-luc cells were exposed for 1 week to immature adipocytes or cytokines before tail vein injection. Cells exposed *ex vivo* yielded a significant increase in lung tumor bioluminescence and numbers compared with controls (Fig. 4G–I and Supplementary Fig. S4C) and more circulating tumor cells (28) at 6 weeks (Fig. 4J). Notably tail vein injection of cytokine-exposed cells led to the formation of distant nodal and myocardial metastasis in 40% to 60% of animals, not seen in controls (representative data, Supplementary Fig. S4D).

Immature adipocytes and cytokines activate Src to upregulate embryonic stem cell transcription factors

Several ES-TFs maintain ES self-renewal (13) and may regulate CSC self-renewal. Cytokine exposures increased *c-MYC*, *SOX2*, and *NANOG* expression in both ER$^-$ and ER$^+$ breast cancer lines (Fig. 5A). Because Src promotes ES self-renewal (13, 15), we tested its role in CSC upregulation by immature adipocyte exposure. All cytokines increased activated pSrc within 1 hour, with no change in kinase level (Fig. 5B, top). Sox2, c-Myc, Nanog, Klf4, and Oct4 increased 12 to 18 hours after Src activation and this was abolished by Src family kinase (SFK) inhibitors (PP1, 1 μmol/L) or saracatinib (AZD0530, 1 μmol/L; PP1 in Fig. 5B and Supplementary Fig. S5A). Immature adipocyte coculture for 7 days also caused an Src-dependent increase in *c-MYC*, *SOX2*, and *NANOG* expression in ER$^-$ and ER$^+$ lines (Fig. 5C).

SFK inhibition also impaired adipocyte- and cytokine-stimulated increases in mammosphere and soft agar colony formation (Fig. 5D and E and Supplementary Fig. S5B), without anti-proliferative effects (Supplementary Fig. S5C).

Src promotes cytokine upregulation following immature adipocyte–breast cancer cell coculture

Src is not only activated by immature adipocyte and cytokine exposures, but also appears to promote/sustain cytokine induction following coculture. SFK inhibition during the last 48 hours of immature adipocyte–MDA-MB-231 coculture dramatically decreased all cytokine levels (Fig. 5F). Thus, Src is not only activated by cytokine signaling, but Src and/or SFK members appear to drive further cytokine upregulation, creating a feed-forward loop to promote malignant stem cell expansion.

Immature adipocytes and cytokines modulate miRNAs that govern ES self-renewal

Certain miRNAs govern ES self-renewal (15). miRNA array analysis after 7-day cytokine treatments showed miR-302b was increased by IL6 and IP10, whereas Let7d*, miR34c-3p, and miR34b levels decreased (Fig. 6A). miR-302b is a Sox2 target (16). Since this potent ES self-renewal mediator was so strongly upregulated, its role was pursued further. qPCR showed immature adipocyte coculture also significantly increased miR-302b in MDA-MB-231, HMLER3, MCF-7, and T47D (Fig. 6B).

miR-302b activates *c-MYC* and *SOX2* expression to drive breast CSC self-renewal

MDA-MB-231 and MCF-7 treatment with Src inhibitors or *SOX2* silencing (Supplementary Fig. S6A) over 48 hours before plating of spheres decreased miR-302b compared with controls, and decreased miR-302b upregulation by IP10 (Fig. 6C).

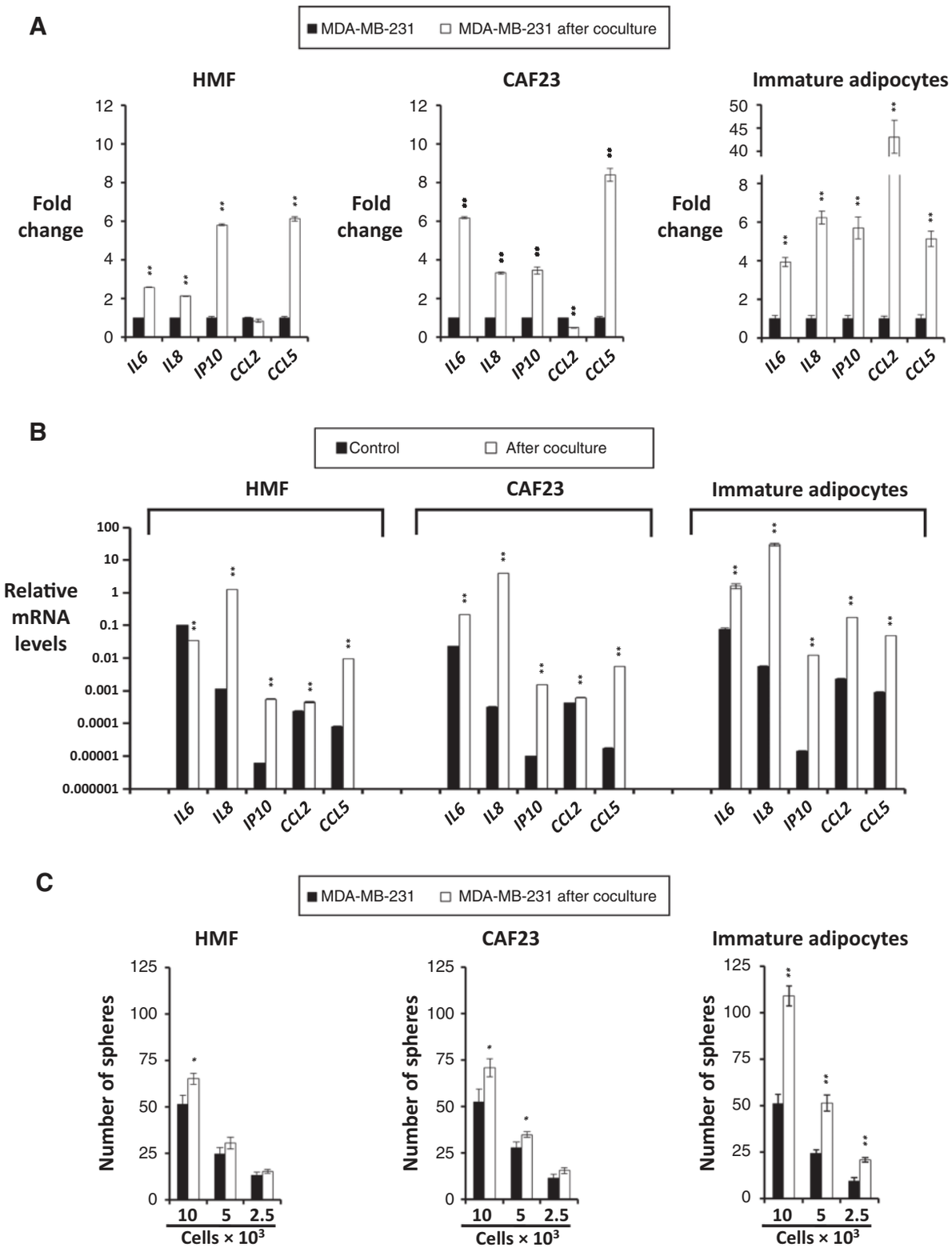
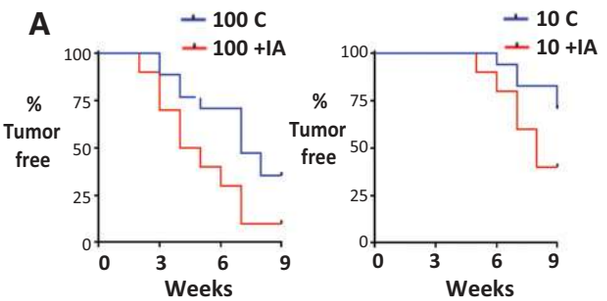
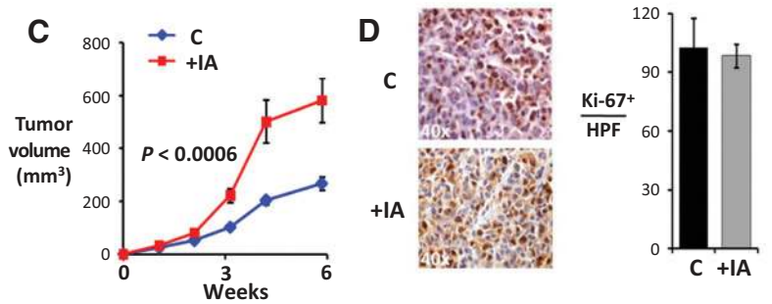


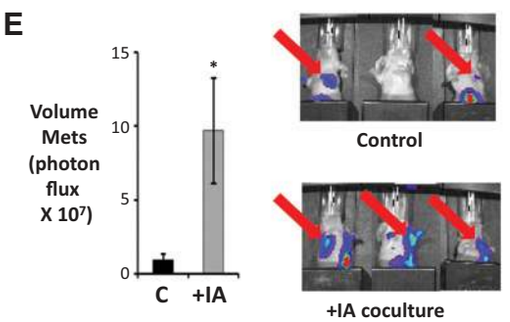
Figure 3.
Coculture with normal and cancer-associated mammary fibroblasts yields less breast cancer cell cytokine induction and mammosphere formation than with immature adipocytes. A, qPCR of cytokine expression in MDA-MB-231 cultured alone or with HMFs, CAF23, or immature adipocytes for 7 days. Data normalized to 1 for monocultures using *GAPDH* as internal control. B, relative cytokine expression in HMFs, CAF23, and immature adipocytes cultured alone or after 7-day coculture with MDA-MB-231. Data normalized using *GAPDH* expression as value 1 for each group. A and B, qPCR data graphed as mean ± SEM ($n = 3$; **, $P < 0.01$ vs. monoculture). C, sphere formation by MDA-MB-231 after 7-day monoculture or coculture with HMFs, CAF23, or immature adipocytes, graphed as mean ± SEM ($n = 3$; *, $P < 0.05$; **, $P < 0.01$).

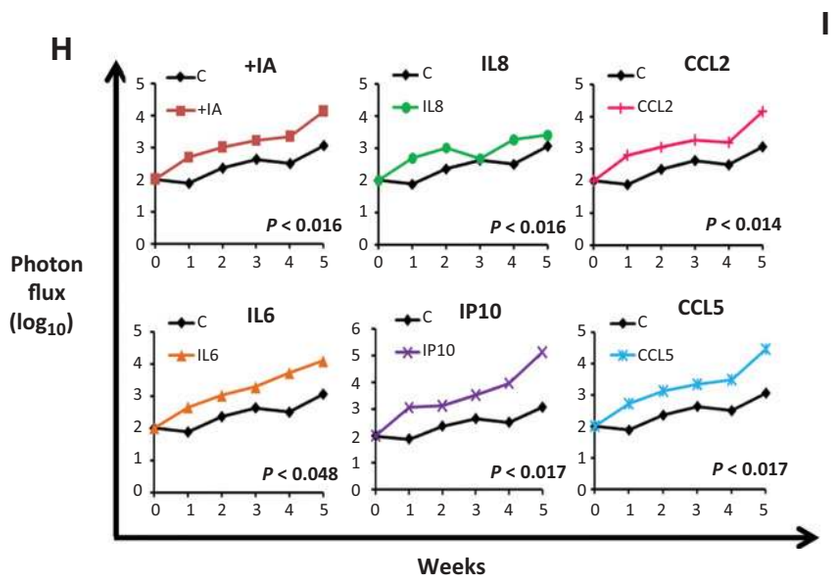


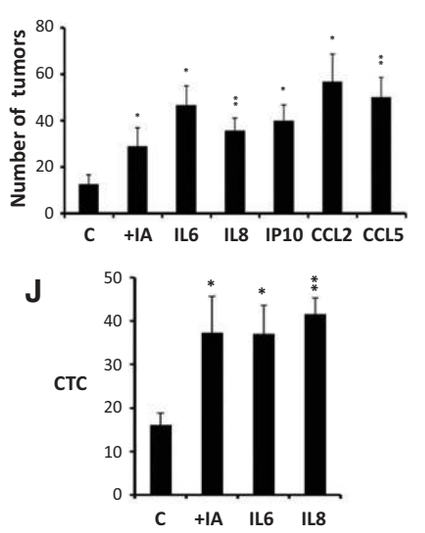
B

Group	Limiting dilution tumors/mice injected		CSC frequency (1 in/...) $P = 0.0278$
	100 cells	10 cells	
C	11/17	5/17	73
+IA	9/10	6/10	25









Stable miR-302b overexpression in both MDA-MB-231 and MCF-7 (Supplementary Fig. S6B) increased sphere formation over parental ($P < 0.0004$ and $P < 0.0005$, respectively) and made both lines resistant to loss of sphere formation by SFK inhibitors or *SOX2* knockdown (Fig. 6D). SFK inhibition and *SOX2* knockdown abolished the IP10-driven increase in sphere formation in parental cells, but not in cells overexpressing miR-302b (Fig. 6E and Supplementary Fig. S6C). Notably, *SOX2* and *c-MYC* were upregulated by miR-302b overexpression and decreased by miR-302b knockdown (Fig. 6F and Supplementary Fig. S6D). Thus, SFK activation and SOX2 both drive cytokine-mediated miR-302b induction, which further stimulates *c-MYC* and *SOX2* expression to feed forward and sustain self-renewal.

Discussion

Obesity affected over 600 million worldwide in 2014 and numbers are rising (33). Obesity is associated with greater morbidity and mortality for many cancers (2). Obese women have a higher risk of postmenopausal breast cancer and worse disease outcome at any age (1, 3), but mechanisms thereof remain obscure. This study informs the more aggressive course of breast cancer in obese individuals.

Obese adipose tissue is a mediator of chronic inflammation (5, 30). In obesity, adipocyte maturation and adiponectin production are both reduced. Leptin is elevated and promotes differentiation of macrophages that produce TNFα, FGF, EGF, and cytokines that stimulate immature adipocyte proliferation and vasculogenesis. High local TNFα and IL1β oppose adipocyte maturation, increasing immature adipocytes, which, in turn, produce further proinflammatory cytokines and chemokines to yield a chronic inflammatory milieu (4, 30, 31). IL6 and CCL2 attract monocytes and CCL5 reduces anti-tumor immunity (4, 34). Thus, tumor-adjacent fat, particularly in obese individuals, is rich in factors that promote tumor progression.

Present data indicate that close proximity or contact between breast cancer cells and hASC, immature adipocytes, and the mature adipocytes present in breast or central fat can increase cytokines that stimulate mammosphere formation in breast cancer models. Of over 200 peptide growth factors and cytokines assayed, secretion of IL6, IL8, IP10, CCL2, and CCL5 was synergistically increased upon breast cancer cell–fat cell coculture. Because immature adipocytes are increased and act as proinflammatory mediators in obese adipose tissue, their effects were assayed in both ER^+ and ER^- breast cancer models. Immature adipocytes express higher basal cytokine levels and these were increased by coculture to considerably higher levels than in cancer cells. While cytokine upregulation did not require direct contact between the two cell types, it was significantly less upon coculture across a Transwell membrane or using immature adipocyte–conditioned medium, suggesting a concentration effect that would be greatest with the two populations in close contact. Adipocyte or cytokine exposures increased ALDH1 activity and mammosphere formation in both breast cancer lines and in an early passage primary dissociated breast cancer culture. Prolonged immature adipocyte exposure also significantly increased tumor-initiating cell abundance and the volume of metastasis generated from orthotopic primary cancers. That tumor growth increased without stimulation of global proliferation is compatible with increased cancer stem cell abundance. Experimental lung metastases were also increased, and metastatic tumors contained more sphere-forming cells and yielded more circulating tumor cells than controls, suggesting a stable increase in these properties.

The relevance of these cytokines to tumor progression is supported by clinical observations. Increased IL8, IL6, CCL2, and CCL5 in primary breast cancers are each correlated with advanced stage, reduced differentiation, and poor patient outcome, and increased circulating cytokines are associated with worse prognosis (6–8). Although obese women also have higher circulating cytokines (4), the possibility that increased circulating cytokines may in part drive poor cancer outcome in obese patients has not been fully appreciated.

These cytokines are known mediators of tumorigenesis and metastasis. IL6 and IL8 are vasculogenic in adipose tissue and angiogenic in cancers (4, 34). Tumor-derived CCL2 drives tumor-associated macrophage (TAM) recruitment and metastasis in breast cancer models (35), while CCL5, produced by invading mesenchymal stem cells (MSC; ref. 26), also increases breast cancer xenograft invasion and metastasis (8). Ras upregulates IP10 and decreases the growth-inhibitory IP10 receptor, CXCR3-B (36), and IL6 and IL8 are both required for Ras-transformation (37). Notably, IL6 induction by cancer-associated adipocytes promotes tumor invasion (38) and metastasis (39). Although these cytokines have been shown individually to promote xenograft growth and metastasis (8, 35, 39), present data reveal that their simultaneous upregulation upon cancer cell–adipose cell/stromal tissue interaction that generates tumor-driving loops that promote CSC expansion and metastasis. That some of these cytokines were also upregulated upon cancer cell coculture with

Figure 4.

Prior preadipocyte coculture increases orthotopic tumor initiation and metastasis. A, Kaplan–Meier curves showing the percentage of tumor-free mice injected by limiting dilution assay with MDA-MB-231-luc-GFP with (+IA) or without (control, C) prior immature adipocyte coculture for 7 days. GFP^+ cells were flow sorted and animals injected with 100 or 10 cells. B, tumor formation and CSC frequency calculated (see Materials and Methods). C, mean tumor volume over time following injection of 10^4 cells from monoculture (control, C) or after prior 7-day immature adipocyte coculture (+IA), graphed as mean ± SEM ($n = 8$). D, Ki-67 staining of tumors formed from cells without (control, C) or with (+IA) prior immature adipocyte coculture (representative photomicrographs, left) and graphed as mean $Ki\text{-}67^+$ cells/high power field (HPF) ± SEM from three high power fields (right). E, tumor metastasis volume, measured as mean photon flux ± SEM (C $n = 3$; +IA $n = 5$; *, $P < 0.05$) from primary tumors formed from cells with (+IA) or without (control, C) prior immature adipocyte exposure (left). Representative IVIS images, with arrows indicating metastasis (right). F, primary tumors and lymph nodes from both experimental groups were dissociated, seeded into sphere assays, and spheres counted at day 10. Data graphed as mean ± SEM ($n = 3$; *$P < 0.05$). G–J, prior immature adipocyte or cytokine exposure effects on tumors formed after tail vein injection. G and H, MDA-MB-231-luc-GFP cells were grown without (control, C) or with immature adipocytes (+IA), or with the indicated cytokines as discussed in Materials and Methods for 7 days before tail vein injection into nude mice, followed by weekly IVIS. Statistical difference of curves calculated using the "Compare Growth Curves" Statmod software. Representative lung bioluminescence 1 and 6 weeks after injection (G) and normalized mean photon flux over 6 weeks ($n = 5$; H). I, lung tumor numbers from each group, graphed as mean ± SEM ($n = 5$), *, $P < 0.05$; **, $P < 0.01$. J, circulating tumor cells graphed as mean ± SEM ($n = 5$). See also Supplementary Fig. S4.

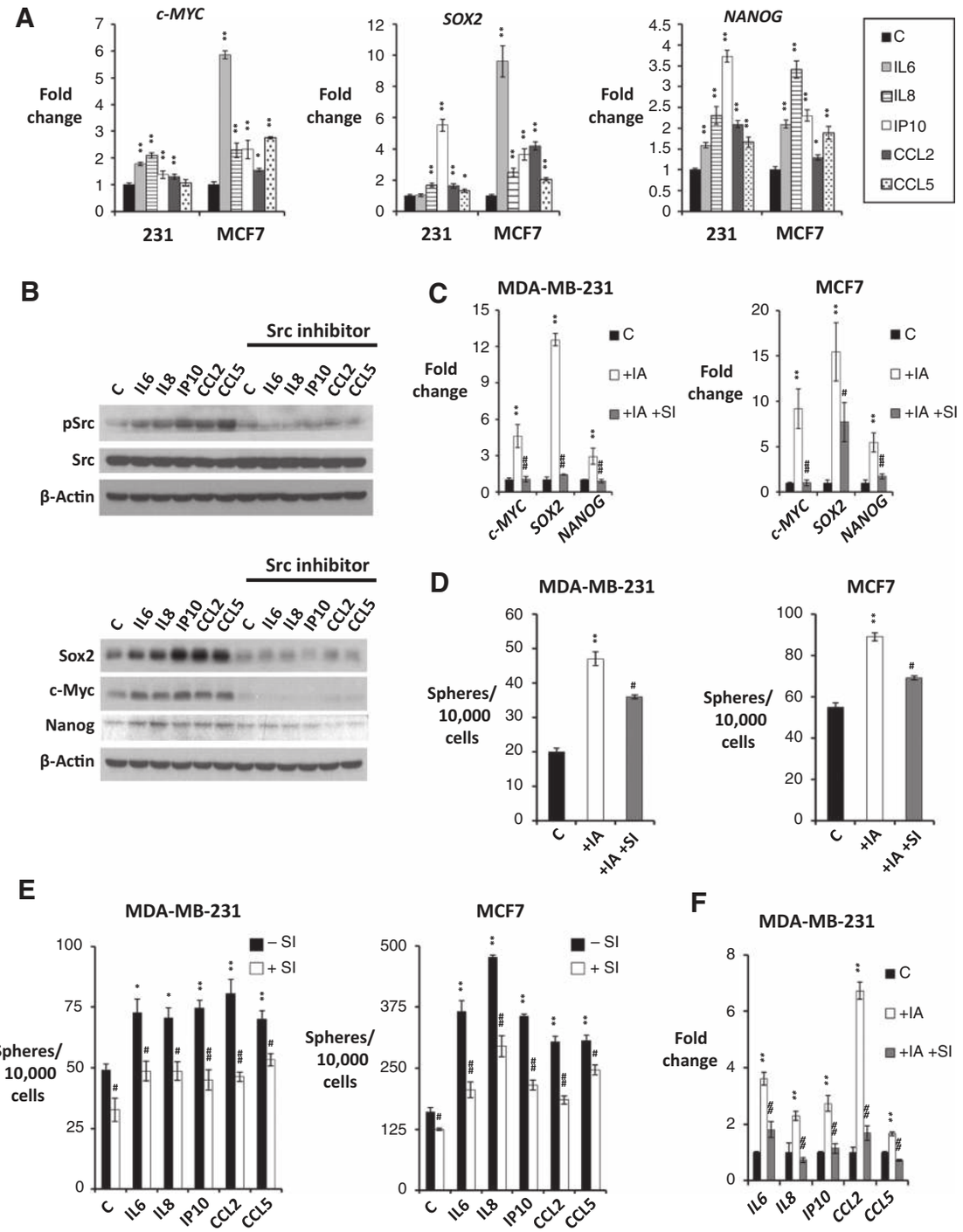


Figure 5.

Src mediates cytokine effects by upregulating embryonic stem cell transcription factors. A, fold change over untreated cells (control, C) in *c-MYC*, *SOX2* and *NANOG* expression assayed by qPCR after 7 days ±cytokine exposure, graphed as mean ± SEM ($n = 3$); *, $P < 0.05$; **, $P < 0.01$. B, Western blot analyses show effect of cytokines ± 2 hours Src inhibitor pretreatment (1 μmol/L PP1) on total or phosphorylated Src at 2 hours (top) and on ES-TF expression at 12 to 18 hours (bottom). C and D, MDA-MB-231 and MCF-7 were cultured alone or with immature adipocytes for 7 days, ± Src inhibitor for the last 48 hours (±IA, ±SI); **, $P < 0.01$ vs. control; #, $P < 0.05$; ##, $P < 0.01$ vs. +IA. C, qPCR of *c-MYC*, *SOX2*, and *NANOG* expression levels in flow sorted breast cancer cells (data normalized to 1 for monocultures). D, mammospheres formed at day 10. E, cells were grown for 7 days ± cytokines, and ± Src inhibitor (SI) for the last 48 hours before seeding into sphere assays. Spheres were counted at 7 to 10 days (*, $P < 0.05$; **, $P < 0.01$ for cytokine treated vs. control without Src inhibitor; #, $P < 0.05$; ##, $P < 0.01$ control or cytokine treated with vs. without SI). F, qPCR showing relative cytokine expression levels in sorted MDA-MB-231 after monoculture (control, C), coculture with immature adipocytes (+IA) for 7 days with or without 1 μmol/L AZD0530 (Src inhibitor, SI) added in the last 48 hours (*, $P < 0.05$; **, $P < 0.01$ +IA vs. C; #, $P < 0.05$; ##, $P < 0.01$ +IA +SI vs. +IA). Data are graphed as mean ± SEM. See also Supplementary Fig. S5.

A

	IL6 vs. control		IP10 vs. control		CCL5 vs. control	
Upregulated	Fold change	*P* value	Fold change	*P* value	Fold change	*P* value
miR-302b	**21.7**	**0.0004**	**12.4**	**0.002**	2.4	0.1
miR-19a	**2.4**	**0.0003**	**2.2**	**0.001**	0.9	0.4
miR-191	**2.3**	**0.006**	**2.8**	**0.002**	**3.4**	**0.0005**
Down-regulated						
Let7d	0.8	0.2	**0.05**	**10^{-5}**	**0.08**	**10^{-5}**
miR-34c	1.1	0.6	**0.2**	**10^{-5}**	**0.3**	**0.0005**
miR-34b	**0.2**	**0.0005**	**0.4**	**0.01**	**0.4**	**0.006**

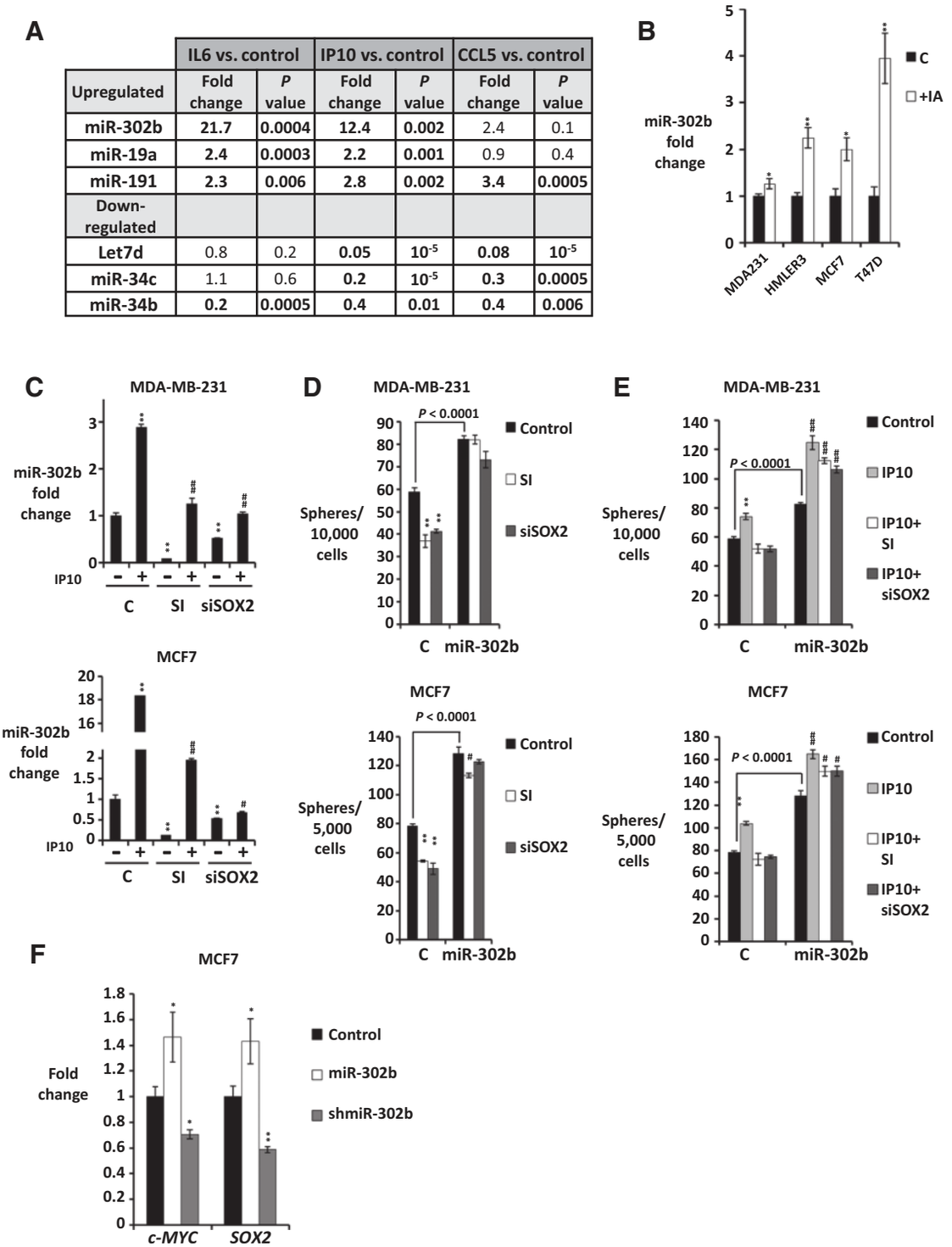


Figure 6.

Immature adipocytes and cytokines upregulate miR-302b via Src activation and SOX2 expression to govern breast cancer cell self-renewal. A, miRNA changes induced in MDA-MB-231 cells by cytokine exposure. B, miR-302b expression by qPCR in indicated cells cultured alone (Control, C) or with immature adipocytes (+IA) for 7 days. Data normalized to 1 for monocultures and presented as mean ± SEM ($n = 3$; *, $P < 0.05$; **, $P < 0.01$). C, cells were grown ± IP10 for 7 days and Src inhibitor (SI) added in the last 48 hours. For *SOX2* knockdown (*siSOX2*), cells were transfected with *siSOX2* oligo or controls 48 hours before the start of IP10 treatment. miR-302b expression was assayed after 7 days ± IP10 exposure. Fold change over untreated cells. (control, C) was graphed as mean ± SEM ($n = 3$; *, $P < 0.05$; **, $P < 0.01$ for values compared to control without IP10; #, $P < 0.05$; ##, $P < 0.01$ for values compared with control +IP10). D and E, indicated cells were exposed to IP10 for 7 days and Src inhibition (SI) and *SOX2* knockdown (*siSOX2*) were as in C above. Sphere numbers were quantitated from controls (C) or from miR-302b overexpressing cells (miR-302b) ± SI or *siSOX2*. Data are graphed as mean ± SEM ($n = 3$; **, $P < 0.01$ for effects of SI or *siSOX2* in parental cells; #, $P < 0.05$ for effects of SI or *siSOX2* in miR-302b-overexpressing cells). F, effect of miR-302b overexpression/knockdown on *c-MYC* and *SOX2* expression determined by qPCR. Fold change over parental controls (C) is graphed as mean ± SEM ($n = 3$; *, $P < 0.05$; **, $P < 0.01$). See also Supplementary Fig. S6.

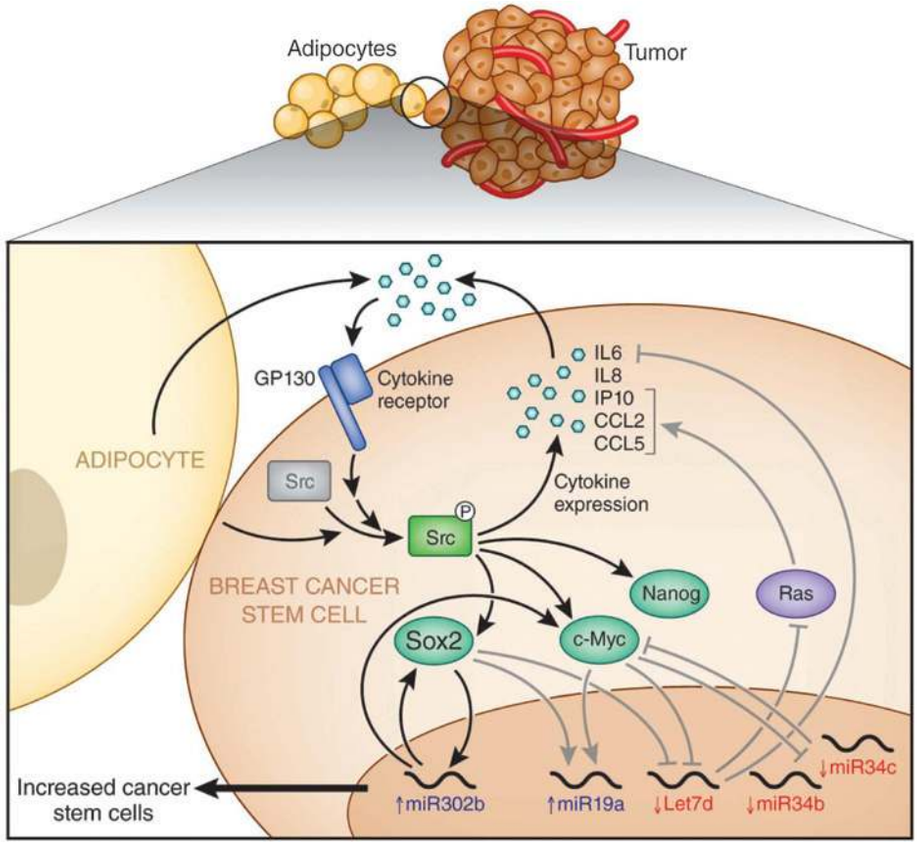


Figure 7.
Schematic model of cytokine-activated feed-forward loops. Present (black arrows) and prior work (gray arrows) in cancer and ES cells support a model in which adipocyte–cancer cell contact–induced cytokine signaling activates Src to drive cytokines and ES-TF expression. Cytokines modulate miRNA expression to establish feed-forward loops that upregulate tumor-initiating cells and stimulate further cytokine production. Src upregulates *SOX2*, which induces miR-302b, and miR-302b, in turn, maintains *c-MYC* and *SOX2* expression, likely by targeting a repressor of both genes.

mammary fibroblasts and CAFs raises the possibility that cytokine induction may be stimulated by aberrant heterotypic cell–cell interactions in many ectopic tissue locations during metastasis.

Several cytokines have been implicated in CSC self-renewal. IL8 increases ALDH1, sphere, and breast cancer xenograft formation, and IL8 blockade increased chemotherapy response (29). IL6 stimulates Notch3-dependent mammosphere formation (40) and MSCs drive breast CSC self-renewal via IL6 and CXCL7 (41, 42). Src was shown to activate NF-κB via pSTAT3 to induce *IL6* and oppose Let7-mediated *IL6* repression (19). Stromal CCL2 and CCL5 were recently implicated in CSC self-renewal (43, 44). This study adds IP10 to the cytokine network that drives mammary CSC self-renewal and reveals that adipocyte–cancer cell interaction engenders feed-forward mechanisms in which CCL2, CCL5, IL6, IL8, and IP10 all activate Src. SFK action, in turn, sustains cytokine induction and promotes Sox2-dependent miR-302b upregulation, to further induce *MYC* and *SOX2* expression and increase CSC (see model, Fig. 7). Oncogenic Src activation, frequently observed in human breast cancers (45), would prime this process. Although we posit that adipocyte exposure enhances CSC self-renewal, methods utilized cannot exclude the possibility that adipocytes may also induce a "dedifferentiation" of more differentiated cancer cells.

Immature adipocyte or cytokine exposures increased subsequent serial mammosphere and xenograft tumor formation over months, long after stimulus withdrawal. Feed-forward mechanisms established by Src-driven *SOX2* and miR-302b induction would sustain subsequent cytokine production after initial exposures. *SOX2* is amplified in certain cancers and can drive clonogenic tumor growth (46). Moreover, miR-302b expression in breast cancers was recently associated with expression of stem cell markers, nodal metastasis, and poor patient outcome (47). In ES, Sox2 transactivates *OCT4*, *NANOG*, *MYC*, and *KLF4* and these, in turn, transactivate *SOX2* (13). Sox2 maintains the undifferentiated state in ES, in part by directly inducing miR302 and miR17-92 clusters (16). miR-302b powerfully mediates ES self-renewal and can efficiently reprogram somatic cells to iPSC (17, 48). Present data reveal a role for miR-302b in the effects of an adipocyte niche on breast CSC. miR-302b was induced in both ER^+ and ER^- breast lines after adipocyte coculture, and IP10 and IL6 both upregulate miR-302b and miR19a (part of the miR17-92 cluster). miR-302b overexpression significantly increased sphere formation and its upregulation by IP10 required both SFK action and Sox2. Notably, miR-302b overexpression did not make cells fully resistant to either *SOX2* knockdown or SFK inhibition, suggesting that miR-302b-independent mechanisms also drive cytokine-activated CSC self-renewal. miR-302b also upregulated both *SOX2* and *c-MYC* expression; thus, cytokine-driven *SOX2* induction may feed forward to maintain high ES-TF levels in part via miR-302b.

Present and prior observations suggest two additional feed-forward mechanisms that would sustain cytokine production and tumor progression. IP10 and CCL5 both downregulated Let7d*. The Let7 family governs CSC and oncogenesis by targeting *RAS* and *HMG2a* (49, 50). Ras drives cytokine secretion (36, 37, 51);

thus, Let7 repression would feed forward to upregulate Ras and drive further IL6, IL8, and IP10 expression (19). IP10 upregulates CCL2 (51) and CCL2, in turn, stimulates CCL5 production by breast cancer cells (8); thus, coordinated upregulation of IP10, IL6, IL8, CCL2, and CCL5 following cancer cell invasion into fat would generate a self-perpetuating mechanism to support CSC expansion and metastasis even after cancer cell egress to other sites. Finally, coculture and cytokine exposures all upregulated c-Myc, potentially due to IL6-, IP10-, and CCL5-dependent suppression of miRs 34c-3p and/or 34-b, which target *c-MYC*, and also due to miR-302b-dependent *c-MYC* induction. c-Myc transactivates *SOX2*, providing yet another feed-forward loop to sustain self-renewal (13, 15).

Epithelial–stromal and inflammatory cell interactions contribute importantly to breast cancer progression (20, 21) but tumor cell–adipocyte interactions are less well understood. This study provides mechanistic insight into the aggressive behavior of breast cancer in obesity and supports a model in which cancer cell invasion into local fat would stimulate secretion of IL6, IL8, CCL2, CCL5, and IP10 by stromal cells, including mammary fibroblasts, which become CAFs, adipose cells, and cancer cells, leading to Src activation. It is tempting to speculate that the higher cytokine secretion by immature adipocytes in obese fat may initiate CAF activation. Src, in turn, would drive further cytokine induction, upregulate Sox2 and other ES-TFs to induce miR-302b, creating feed-forward loops to sustain cytokine and ES-TF induction, causing a sustained increase in tumor-initiating cell abundance and competence for metastasis. The increased inflammatory milieu generated as cancer cells migrate though mammary fat and upregulate circulating cytokines may also act systemically to promote metastasis. This mechanism may have relevance to the tropism of many malignancies to highly fatty bone marrow and may also increase metastatic potential of colon and ovarian cancers that directly infiltrate mesenteric fat. Up to 20% of cancer deaths in the United States may be attributable to obesity (52). Although the chronic inflammation of obesity is known to promote metabolic syndrome and heart disease (5), this study reveals novel feedforward mechanisms whereby tumor invasion into fat would drive inflammation and tumor progression. The obesity epidemic in the developed world may be a harbinger of an unprecedented increase in morbidity and mortality from cancer in decades to come.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Chan DS, Vieira AR, Aune D, Bandera EV, Greenwood DC, McTiernan A, et al. Body mass index and survival in women with breast cancer-systematic literature review and meta-analysis of 82 follow-up studies. Ann Oncol 2014;25:1901–14.
2. Gilbert CA, Slingerland JM. Cytokines, obesity, and cancer: new insights on mechanisms linking obesity to cancer risk and progression. Annu Rev Med 2013;64:45–57.
3. Carmichael AR. Obesity as a risk factor for development and poor prognosis of breast cancer. BJOG 2006;113:1160–6.
4. Vona-Davis L, Rose DP. Angiogenesis, adipokines and breast cancer. Cytokine Growth Factor Rev 2009;20:193–201.
5. Gregor MF, Hotamisligil GKS. Inflammatory mechanisms in obesity. Annu Rev Immunol 2011;29:415–45.
6. Nicolini A, Carpi A, Rossi G. Cytokines in breast cancer. Cytokine Growth Factor Rev 2006;17:325–37.
7. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. Clin Cancer Res 2008;14:6735–41.
8. Soria G, Ben-Baruch A. The inflammatory chemokines CCL2 and CCL5 in breast cancer. Cancer Lett 2008;267:271–85.
9. Bromberg J, Wang TC. Inflammation and cancer: IL-6 and STAT3 complete the link. Cancer Cell 2009;15:79–80.
10. Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. Cell Stem Cell 2012;10:717–28.
11. He S, Nakada D, Morrison SJ. Mechanisms of stem cell self-renewal. Annu Rev Cell Dev Biol 2009;25:377–406.
12. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 2005;7:86–95.
13. Li YQ. Master stem cell transcription factors and signaling regulation. Cell Reprogram 2010;12:3–13.
14. Martinez NJ, Gregory RI. MicroRNA gene regulatory pathways in the establishment and maintenance of ESC identity. Cell Stem Cell 2010; 7:31–5.
15. Gunaratne PH. Embryonic stem cell microRNAs: defining factors in induced pluripotent (iPS) and cancer (CSC) stem cells? Curr Stem Cell Res Ther 2009;4:168–77.
16. Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell 2008;134:521–33.
17. Anokye-Danso F, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, et al. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell 2011;8:376–88.
18. Subramanyam D, Lamouille S, Judson RL, Liu JY, Bucay N, Derynck R, et al. Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. Nat Biotechnol 2011;29:443–8.

19. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell 2009;139:693–706.
20. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell 2010;141:39–51.
21. Goruppi S, Dotto GP. Mesenchymal stroma: primary determinant and therapeutic target for epithelial cancer. Trends Cell Biol 2013;23: 593–602.
22. Ince TA, Richardson AL, Bell GW, Saitoh M, Godar S, Karnoub AE, et al. Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. Cancer Cell 2007;12:160–70.
23. Drews-Elger K, Brinkman JA, Miller P, Shah SH, Harrell JC, da Silva TG, et al. Primary breast tumor-derived cellular models: characterization of tumorigenic, metastatic, and cancer-associated fibroblasts in dissociated tumor (DT) cultures. Breast Cancer Res Treat 2014;144:503–17.
24. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci 2004;117:2971–81.
25. Marchal JA, Picon M, Peran M, Bueno C, Jimenez-Navarro M, Carrillo E, et al. Purification and long-term expansion of multipotent endothelial-like cells with potential cardiovascular regeneration. Stem Cells Dev 2012;21: 562–74.
26. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007;449:557–63.
27. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007; 1:555–67.
28. Lin HK, Zheng S, Williams AJ, Balic M, Groshen S, Scher HI, et al. Portable filter-based microdevice for detection and characterization of circulating tumor cells. Clin Cancer Res 2010;16:5011–8.
29. Ginestier C, Liu S, Diebel ME, Korkaya H, Luo M, Brown M, et al. CXCR1 blockade selectively targets human breast cancer stem cells *in vitro* and in xenografts. J Clin Invest 2010;120:485–97.
30. Osborn O, Olefsky JM. The cellular and signaling networks linking the immune system and metabolism in disease. Nat Med 2012;18:363–74.
31. Simons PJ, van den Pangaart PS, van Roomen CP, Aerts JM, Boon L. Cytokine-mediated modulation of leptin and adiponectin secretion during *in vitro* adipogenesis: evidence that tumor necrosis factor-alpha- and interleukin-1beta-treated human preadipocytes are potent leptin producers. Cytokine 2005;32:94–103.
32. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253–70.
33. World Health Organization: Obesity and Overwight - Fact Sheet #311 (Updated January 2015).
34. Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med 2008;14:109–19.
35. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 2011;475:222–5.
36. Datta D, Flaxenburg JA, Laxmanan S, Geehan C, Grimm M, Waaga-Gasser AM, et al. Ras-induced modulation of CXCL10 and its receptor splice variant CXCR3-B in MDA-MB-435 and MCF-7 cells: relevance for the development of human breast cancer. Cancer Res 2006;66:9509–18.
37. Ancrile B, Lim KH, Counter CM. Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. Genes Dev 2007;21:1714–9.
38. Walter M, Liang S, Ghosh S, Hornsby PJ, Li R. Interleukin 6 secreted from adipose stromal cells promotes migration and invasion of breast cancer cells. Oncogene 2009;28:2745–55.
39. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. Cancer Res 2011;71:2455–65.
40. Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M, et al. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J Clin Invest 2007; 117:3988–4002.
41. Liu S, Ginestier C, Ou SJ, Clouthier SG, Patel SH, Monville F, et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. Cancer Res 2011;71:614–24.
42. Korkaya H, Kim Gi, Davis A, Malik F, Henry N, Ithimakin S, et al. Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2+ breast cancer by expanding the cancer stem cell population. Mol Cell 2012;47:570–84.
43. Velasco-Velazquez M, Jiao X, De La Fuente M, Pestell TG, Ertel A, Lisanti MP, et al. CCR5 antagonist blocks metastasis of basal breast cancer cells. Cancer Res 2012;72:3839–50.
44. Tsuyada A, Chow A, Wu J, Somlo G, Chu P, Loera S, et al. CCL2 mediates crosstalk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. Cancer Res 2012;72:2768–79.
45. Chu I, Sun J, Arnaout A, Kahn H, Hanna W, Narod S, et al. p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2. Cell 2007;128: 281–94.
46. Chen Y, Shi L, Zhang L, Li R, Liang J, Yu W, et al. The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. J Biol Chem 2008;283:17969–78.
47. Volinia S, Nuovo G, Drusco A, Costinean S, Abujarour R, Desponts C, et al. Pluripotent stem cell miRNAs and metastasis in invasive breast cancer. J Natl Cancer Inst 2014;106.
48. Lipchina I, Studer L, Betel D. The expanding role of miR-302-367 in pluripotency and reprogramming. Cell Cycle 2012;11:1517–23.
49. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. Cell 2005;120:635–47.
50. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 2007;131: 1109–23.
51. Aronica SM, Fanti P, Kaminskaya K, Gibbs K, Raiber L, Nazareth M, et al. Estrogen disrupts chemokine-mediated chemokine release from mammary cells: implications for the interplay between estrogen and IP-10 in the regulation of mammary tumor formation. Breast Cancer Res Treat 2004; 84:235–45.
52. van Kruijsdijk RC, van der Wall E, Visseren FL. Obesity and cancer: the role of dysfunctional adipose tissue. Cancer Epidemiol Biomarkers Prev 2009; 18:2569–78.