Chronic Social Isolation Is Associated with Metabolic Gene Expression Changes Specific to Mammary Adipose Tissue

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Abstract

Chronic social isolation is linked to increased mammary tumor growth in rodent models of breast cancer. In the C3(1)/SV40 T-antigen FVB/N (TAg) mouse model of “triple-negative” breast cancer, the heightened stress response elicited by social isolation has been associated with increased expression of metabolic genes in the mammary gland before invasive tumors develop (i.e., during the in situ carcinoma stage). To further understand the mechanisms underlying how accelerated mammary tumor growth is associated with social isolation, we separated the mammary gland adipose tissue from adjacent ductal epithelial cells and analyzed individual cell types for changes in metabolic gene expression. Specifically, increased expression of the key metabolic genes Acaca, Hk2, and Acly was found in the adipocyte, rather than the epithelial fraction. Surprisingly, metabolic gene expression was not significantly increased in visceral adipose depots of socially isolated female mice. As expected, increased metabolic gene expression in the mammary adipocytes of socially isolated mice coincided with increased glucose metabolism, lipid synthesis, and leptin secretion from this adipose depot. Furthermore, application of media that had been cultured with isolated mouse mammary adipose tissue (conditioned media) resulted in increased proliferation of mammary cancer cells relative to group-housed–conditioned media. These results suggest that exposure to a chronic stressor (social isolation) results in specific metabolic reprogramming in mammary gland adipocytes that in turn contributes to increased proliferation of adjacent preinvasive malignant epithelial cells. Metabolites and/or tumor growth-promoting proteins secreted from adipose tissue could identify biomarkers and/or targets for preventive intervention in breast cancer. Cancer Prev Res; 1–12. ©2013 AACR.

Introduction

Human epidemiologic studies have revealed that social isolation is associated with an increased risk of both all-cause mortality and metabolic diseases such as diabetes (1). Although association studies examining social isolation and human cancer risk have shown mixed results (2), the conclusions of these studies are likely inconsistent because human populations have immense genetic and environmental variation as well as heterogeneous breast cancer subtypes (3, 4). These issues make identifying the mechanisms connecting social stressors to breast cancer biology challenging. They also underscore the importance of developing well-defined preclinical models for identifying the specific biologic mechanisms linking an individual’s response to social stressors to specific cancer subtypes.

Recent models of breast cancer examining the effects of imposed social isolation, a well-defined chronic stressor for female rodents, have found an association with increased mammary tumor growth. For example, in SV40 T-antigen FVB/N (TAg) mice (5) and Sprague–Dawley rats (6), social isolation was associated with larger mammary gland tumor burden and increased tumor invasiveness independently of changes in circulating estrogen and progesterone levels. Furthermore, our laboratories discovered that genes encoding key enzymes regulating lipid metabolism were differentially upregulated in the mammary glands of socially isolated versus group-housed mice, even before differences in tumor development (5). These results suggested that changes in lipid metabolism (in the premalignant epithelial cells and/or the adjacent adipocytes and stromal cells) could be driving the relatively aggressive mammary tumor growth of the social isolates. In mouse models, social stressors have been linked to obesity (7), disruption of metabolism (8, 9), and diabetes (10), supporting an association between exposure to social stressors, the physiologic stress response, and metabolic disorders. However, the mechanisms through which disrupted metabolism promote mammary tumorigenesis are
still unclear. A link between metabolic syndrome/obesity and breast cancer has been suggested (11). Obesity is associated with increased local production of estrogen in mammary gland fat likely contributing to estrogen receptor positive (ER+) breast cancer progression (12). However, emerging data also link metabolic diseases to ER negative (ER−) breast cancer, suggesting that factors other than estrogen are involved (13). Indeed, there is increasing evidence that in addition to estrogenic factors, mammary gland tumorigenesis can be influenced by both local and systemic metabolic signaling molecules, including insulin and leptin (14–16). Adding complexity, the stromal compartment and its reciprocal communication with the mammary epithelium is likely an important factor influencing breast cancer (17). Many cell types, including fibroblasts, adipocytes, and immune and endothelial cells compose the mammary stroma. Mammary gland adipocytes are arguably the least well-understood component. The relative lack of studies on mammary adipocytes in breast cancer biology is surprising considering the abundance of mammary gland adipose tissue and the well-established role of fat as an endocrine/paracrine tissue (18).

Endocrine action by adipose tissue includes the release of growth factors, hormones, and cytokines as well as adipocyte-specific factors (adipokines), many of which have been implicated in cancer progression (19). For example, leptin, an adipokine that was first identified as a gene product influencing satiety and body mass (20), has since been shown to affect the differentiation and proliferation of other cell types, including breast cancer cells (21). Adding to the complexity of adipocyte endocrine action is the fact that the metabolic activity and the profile of secreted substances in adipose tissue varies depending on its location (e.g., visceral vs. subcutaneous depots; ref. 22). Properties specific to mammary adipose tissue and the mammary microenvironment that influence breast cancer biology remain largely unexplored. Therefore, whether abnormal function in mammary adipocytes and the ensuing effects on local metabolism contribute to ER-independent breast cancer biology has not been established.

Using a global gene expression approach, we previously identified key metabolic genes including hexokinase 2 (Hk2), ATP citrate lyase (Achy), and acetyl-CoA carboxylase alpha (Acaca) as significantly upregulated in the mammary glands of isolated versus group-housed TAg mice. Interestingly, increased expression of these gene products is associated with the hallmark metabolic changes observed in cancer cells (23). However, in our previous experiments, RNA was obtained from whole mammary gland tissue so we could not determine the specific cell type(s) that were contributing to increased metabolic gene expression. Because it has become increasingly clear that mammary epithelial cell proliferation is influenced by adjacent nonepithelial stromal cells (17), we sought to establish the specific cell types contributing to mammary gland metabolic gene expression changes. Our new findings reveal an association between social isolation, the ensuing stress response, and increased mammary gland adipose tissue lipid metabolism, without a measurable concomitant effect on systemic metabolism. While previous studies have implicated increased mammary fat estrogen production in ER+ breast cancers, our results implicate mammary adipocyte function and its secretome as an important modulator in a model of ER-negative breast cancer growth.

Materials and Methods

C3(1)/SV40 TAg FVB/N transgenic and CD1-outbred mice

FVB/N mice homozygous for the SV40 TAg transgene (The FVB-Tg(C3-1-TAg)cJeg/N mouse strain, originally provided as hemizygous TAg mice by Jeff Green of the National Cancer Institute’s Mouse Models of Cancer Consortium), nontransgenic FVB/N mice (Charles River), and Swiss CD1 mice (Charles River) were weaned at 3 weeks of age and transferred to differential housing as described in the Supplementary Methods section. TAg homozygous animals were maintained and bred to generate TAg homozygous study populations. Female TAg mice were no longer bred following birth of a litter. To minimize confounding influences from estrous cycle hormones on experimental results, all study animals were sacrificed in estrus phase, as determined by vaginal cytology (24). NI H and University of Chicago Animal Care Guidelines were followed for all studies. A detailed outline of experiments and setup before animal sacrifice are described in Supplementary Methods.

Measurement of circulating factors and food/caloric consumption

Blood glucose was measured via tail bleed using a Bayer contour glucose meter. Tail blood was collected for plasma isolation using heparinized capillary microvets (Andwin Scientific) and was diluted for corticosterone measurements 1:50 in buffer provided with a corticosterone ELISA kit (Enzo Life Sciences). Immediately at sacrifice, cardiac puncture was carried out to collect blood, and serum was isolated and stored at −80°C. Serum insulin and leptin were measured by ELISA (ALPCO Diagnostics, Crystal Chem; respectively). Serum-free fatty acids were measured by enzymatic assay (Wako Diagnostics). Food consumed was calculated weekly as initial food mass minus the final mass at end of the week. Calories consumed were calculated by multiplying the consumed food mass by the diet’s caloric density (Teklad #8904, 3.0 kcal/g). Statistical analyses are provided in Supplementary Methods.

Adipose tissue harvest, collagenase digestion, and centrifugal separation of mammary adipocytes

Mice were sacrificed at 15 weeks of age and mammary fat pads with palpable tumors were excluded from experiments. Gonadal fat pads were excised from their adjacent fallopian tubes and snap-frozen. Excised mammary fat pads were immediately placed in 2 ml microcentrifuge tubes containing 700 μL Dulbecco’s modified Eagle medium (DMEM) with 10% FBS. Details of adipose tissue harvest and adipocyte isolation are provided in Supplementary Methods and are a modification of the procedure reported previously (25).
Quantitative RT-PCR

mRNA (1 μg) was reverse-transcribed using the qScript cDNA synthesis kit (Quanta Biosciences). Quantitative real-time PCR was conducted with PerfeCta SYBR Green FastMix (Quanta Biosciences). All reactions were conducted in a Bio-Rad iCycler iQ real-time PCR system. Details of the statistical analysis are provided in Supplementary Methods section.

Adipocyte glucose consumption and lipogenesis

For each 15-week-old animal, mammary fat pads were minced in 1 mL of DMEM with 1% bovine serum albumin (BSA). Following mincing, 4 mL of 1.0% BSA and 2 mg/mL collagenase (Type II, Worthington) were added. Tubes were agitated at 37°C for 60 minutes and pipette-mixed every 15 minutes. Cells were filtered through a 100 μm nylon mesh and then spun at 100 × g for 60 seconds. Floating adipocytes were transferred to microcentrifuge tubes and repeat centrifugation (30 seconds, 100 × g) followed by media removal with syringe and needle were conducted to obtain isolated packed adipocytes.

To compare adipocyte glucose consumption, adipocytes (10 μL) from individual animals (group-housed n = 5; socially isolated n = 3) were placed into 96-well plates containing 90 μL of DMEM (1 g/L glucose) and 1% BSA. Cells were stimulated with 50 nmol/L insulin or vehicle control, incubated for 4 hours, and medium was then collected and glucose consumption measured as loss of glucose from the media between 0 and 4 hours (26).

To assess lipogenesis, 10 μL of adipocytes from individual animals (n = 3, each condition) were placed into scintillation vials containing 400 μL of DMEM (1 g/L glucose) and 1% BSA. Cells were treated with vehicle or 10 nmol/L insulin and 100 μL of 14C-labeled glucose (0.001 μCi/ml, 1 μCi/Rxn) was immediately added to each vial. The cells were incubated at 37°C for 60 minutes and 4 mL of Beta-fluor was added, followed by vortexing. The following day, 3.2 mL of the upper (lipid-containing) fraction was transferred to a new scintillation vial and radioactivity measured using a scintillation counter. Statistical analyses are provided in Supplementary Methods.

Leptin measurements

Mammary adipocytes were sonicated in 1× PBS containing protease inhibitors, and spun at 4°C and 13,400 × g for 15 minutes. The infranatant between the top lipid layer and pelletted nuclei that contained soluble proteins was transferred to a new tube. For Western blot analysis, 20 μL of mammary adipocyte protein lysate from individual animals (n = 5 per housing condition) was boiled with 4× Laemmli buffer for 10 minutes. Samples were resolved on a 15% SDS gel, transferred to PVDF (Millipore), and immunoblotted with antileptin antibody (A-20, Santa Cruz Biotechnology), whereas anticyclopheilin-B antibody was used as a loading control (PAI-027, Affinity Bio-Reagents). Leptin ELISA (Crystal Chem Inc.) assays were conducted on protein lysates (group housed, n = 7; socially isolated, n = 5), following BCA-based protein normalization (Thermo Scientific). Leptin receptor immunoblotting was conducted with Abcam 5593 (Supplementary Fig. S3B). Statistical analyses are provided in Supplementary Methods.

For secreted leptin measurements, fat pads from 4 group-housed or 4 socially isolated animals were pooled, miniced and spun at 100 × g for 1 minute. The floating tissue was weighed and 1 g incubated with 10 mL of media (SH30240.01, Hyclone), containing 1% BSA and 1% penicillin/streptomycin (P/S), at 37°C for 24 hours. Medium was harvested and sterile-filtered through a 0.22 μm syringe filter, aliquoted, and stored at −80°C. Media (40 μL) was used for ELISA leptin measurements (Crystal Chem Inc.). Statistical analyses are provided in Supplementary Methods.

Premalignant SV40-TAg-mammary epithelial cell proliferation

The SV40-Tag M27H4 mammary cell line (a kind gift by Dr. Cheryl Jurcyk, Boise State University) was originally isolated from a hyperplastic, noninvasive lesion in a 2-month-old C3(1)/Tag mouse (27). The cells were authenticated by microscopic morphology and were routinely tested via growth curve comparison to the original series of cell lines (27). Cells (5,000/well, ~50% confluence after 16 hours) were seeded in 96-well plates containing DMEM, 10% FBS, and 1% P/S. After 16 hours, medium was removed and cells were washed with PBS and then cultured with either 100 μL of mammary adipose tissue culture media from grouped or isolated animals (described above), or in control media (serum free, 1% BSA, 1% P/S). Replicate wells from each condition were washed with PBS and fixed with 10% trichloroacetic acid at 24-hour intervals. On the final day, total protein from each well, representing relative cell number, was measured via the SRB method (28). The SRB assay was also used to measure proliferation of M27H4 cells in response to recombinant leptin (Life Technologies). Statistical analyses are provided in Supplementary Methods.

Results

Social isolation versus group housing is associated with increased vigilance followed by accelerated mammary tumor growth

Hemizygous FVB/N TAg female mice typically progress to palpable carcinomas at approximately 16 weeks of age (29), whereas homozygous mice can develop palpable mammary tumors as early as 10 weeks of age (30). Our previous work has shown that socially isolated homozygous TAg mice have a larger invasive tumor burden (the total palpable tumor volume per mouse) compared with group-housed mice (5). These results needed to be confirmed to investigate the metabolic and molecular changes underlying the increased tumor burden.

In agreement with our previous work (5), among the mice that had palpable tumors by 18 weeks of age (Supplementary Fig. S1A), isolated mice developed a significantly larger average tumor burden than group-housed mice (P = 0.013, Supplementary Fig. S1B). Also, we again observed that socially isolated mice had become more vigilant by 16
weeks of age, as shown by a longer time to leave their home base and explore a novel environment \( (P < 0.0001; \) log-rank test, Supplementary Fig. S1C). These results confirm that exposure to social isolation is associated with increased vigilance and increased mammary tumor growth \( (5) \).

**Social isolation is associated with metabolic gene expression changes in mammary adipocytes**

Next we sought to determine whether the increased tumor burden of socially isolated mice was associated with local and/or systemic changes in lipid metabolism. Our previous studies in the TAg model found that social isolation results in increased mRNA steady-state levels of key genes encoding glucose metabolism and lipid synthesis enzymes (\( Hk2, Acly, \) and \( Acaca \)) in whole-mammary gland extracts from 15-week-old mice, before differences in tumor formation \( (5) \). Increased glucose metabolism and de novo lipid synthesis are associated with the hallmark metabolic changes observed in cancer cells. However, de novo synthesis of lipids from glucose is also a primary function of adipocytes, and therefore one of or both of these cell populations could account for the observed overall change in mammary gland metabolic gene expression. To separate the cell types, we used a collagenase digestion and centrifugation protocol \( (25) \) and isolated the floating adipocytes from other cells in minced mammary glands of 15-week-old mice. Representative images of fractionated cells are shown in Fig. 1A. mRNA was isolated from each fraction and gene expression was analyzed by qRT-PCR.

A comparison of the relative gene expression in the adipocyte versus stromal/epithelial fraction (regardless of housing condition) revealed 20- to 60-fold higher overall metabolic gene expression in the adipocytes (Fig. 1B). Adipocytes from socially isolated TAg mouse mammary glands expressed significantly higher steady-state \( Hk2 \) \( \left( 2^{-\Delta\Delta Ct} = 1.84, *P = 0.012 \right) \), \( Acly \) \( \left( 2^{-\Delta\Delta Ct} = 2.93, ***P = 0.0001 \right) \), and \( Acaca \) \( \left( 2^{-\Delta\Delta Ct} = 2.94, ***P = 0.0001 \right) \) mRNA levels compared with mRNA from group-housed mammary gland adipocytes (Fig. 1C). In cells of the nonadipocyte fraction, expression of these genes was not statistically different between isolated and group-housed animals \( (P > 0.12 \) for all 3 genes, Fig. 1D, Supplementary Table S1). These results indicate that these metabolic gene expression changes in the mammary gland following social isolation occur primarily in the adipocyte tissue fraction.

To determine whether the elevated metabolic gene expression was specific to the mammary gland fat depot or similarly found in other adipose tissue depots, we also harvested gonadal fat attached to the uterus and fallopian tubes. Unlike mammary adipose tissue, there were no
significant differences in the gonadal fat metabolic gene expression from the isolated versus grouped mice (Fig. 1E, Supplementary Table S1, \( P > 0.43 \) for all 3 genes), suggesting that the mammary adipocytes were more sensitive to stress-induced upregulation of metabolic gene expression.

**Social isolation is not associated with detectable systemic metabolic changes**

Our gene expression data suggested the intriguing possibility that social isolation in female rodents is associated with mammary adipose-specific metabolic changes; therefore, we conducted additional analyses of systemic metabolism to examine the local versus systemic effects of social isolation on metabolism. We measured food consumption and weight in isolated and grouped cohorts. Animal weights did not differ between isolated and group-housed mice before palpable tumor formation (age 10 weeks, Table 1) or after tumor formation (age 17 weeks, Table 1). However, isolated mice consumed significantly more kilocalories per day compared with group-housed mice both before palpable tumor formation (age 8–10 weeks; \( P = 0.03 \), Table 1) and after (age 11–17 weeks; \( P = 0.0016 \), Table 1), suggesting a possible effect of social condition on eating behavior and/or systemic energy metabolism.

To test this possibility, parallel cohorts of chronically isolated and group-housed female TAg mice were placed in individual metabolic cages. This was also a test of the enduring effects of living in groups, as all mice had to be isolated during the metabolic cage studies because grouped metabolic cages are not available. When animals were isolated during the metabolic cage studies because grouped and isolated cohorts (Supplementary Fig. S2 and Supplementary Table S2), suggesting that the superimposed stress of social isolation in the metabolic cages affected the eating behavior of the grouped mice.

In addition to food consumption, animal weight, and metabolic cage analyses, we measured several circulating markers of systemic metabolism while the animals were in their assigned social conditions. As shown in Table 1, at 15 weeks of age, we did not observe significant differences in systemic circulating blood glucose, serum insulin, serum free-fatty acids, or serum leptin, even though profound changes were seen in mammary adipose gene expression at this age. Thus, circulating metabolic factor levels did not suggest a significant effect of social isolation on systemic metabolism.

### Upregulation of metabolic genes in the mammary gland upon social isolation occurs independently of the SV40 TAg transgene and is not limited to the FVB/N mouse strain

The TAg transgenic mouse breast cancer model used in this study is on the FVB/N background strain. In addition, previous studies have shown that different mouse breast cancer models (e.g., MMTV-neu vs. MMTV-pymt) on the FVB/N background strain can have differential effects on metabolic phenotypes including obesity (31). To rule out potential FVB/N-specific or SV40-TAg–associated effects of social isolation on mammary fat gene expression or systemic metabolism, we repeated the isolation versus group-housed studies using nontransgenic FVB/N (WT) and outbred CD1 female mice.

We measured whole mammary gland gene expression from chronically isolated and group-housed 15-week-old female WT and CD1 mice. The results recapitulated the upregulation of \( Hk2 \) (WT, \( \ast P = 0.02 \); CD1, \( P = 0.09 \)), \( Acly \) (WT, \( \ast\ast P < 0.001 \); CD1, \( \ast\ast P = 0.007 \)), and \( Acaca \) (WT, \( \ast P = 0.03 \); CD1, \( \ast P = 0.01 \)) steady-state mRNA that we observed in mammary glands from isolated 15-week-old TAg mice (Fig. 2A and B; Supplementary Table S3). Moreover, as we had observed in the TAg mice, gene expression in gonadal fat was not significantly different between isolated versus

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**Table 1. Measurements of circulating metabolic parameters, food consumption, and weights in grouped versus isolated TAg female mice**

<table>
<thead>
<tr>
<th></th>
<th>Grouped</th>
<th>Isolated</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>113.4 ± 9.85</td>
<td>110.4 ± 3.54</td>
<td>0.79</td>
</tr>
<tr>
<td>Serum Insulin (pg/mL)</td>
<td>361.5 ± 78.8</td>
<td>349.3 ± 82.8</td>
<td>0.92</td>
</tr>
<tr>
<td>NEFA (mEq/L)</td>
<td>0.93 ± 0.10</td>
<td>0.79 ± 0.07</td>
<td>0.24</td>
</tr>
<tr>
<td>Serum Leptin (ng/mL)</td>
<td>1.36 ± 0.39</td>
<td>1.31 ± 0.55</td>
<td>0.94</td>
</tr>
<tr>
<td>Serum Cort. (ng/mL)</td>
<td>76.8 ± 37.0</td>
<td>67.9 ± 39.3</td>
<td>0.66</td>
</tr>
<tr>
<td>Food consumption: 8–10 weeks (kcal/day)</td>
<td>9.33 ± 0.36</td>
<td>10.60 ± 0.26</td>
<td>0.03</td>
</tr>
<tr>
<td>Food consumption: 11–17 weeks (kcal/day)</td>
<td>10.47 ± 0.35</td>
<td>12.58 ± 0.35</td>
<td>0.002</td>
</tr>
<tr>
<td>Weight: age 10 weeks (g)</td>
<td>18.9 ± 0.48</td>
<td>18.8 ± 0.23</td>
<td>0.85</td>
</tr>
<tr>
<td>Weight: age 17 weeks (g)</td>
<td>21.2 ± 0.47</td>
<td>21.4 ± 0.54</td>
<td>0.81</td>
</tr>
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</table>

**NOTE:** Circulating markers were obtained from 15-week-old animals (9 group housed, 8 isolated). Food consumption and weights (16 group housed, 15 isolated) are divided into pre- and posttumor time periods to investigate the effects of tumor burden on metabolism. Data indicate means ± SD, with \( P \) value obtained using a Student \( t \) test.
group-housed female CD1 mice (Fig. 2C, \( P > 0.59 \) for all genes), and only one of the 3 metabolic genes was significantly upregulated in the WT visceral fat (\( \text{Acaca} \) \( *P = 0.04, \text{Hk2} \) \( P = 0.47, \text{Acly} \) \( P = 0.10 \); Fig. 2D).

As was observed in TAg mice, CD1 mice metabolic cage measures (Supplementary Fig. S2; Supplementary Table S2) and other markers of systemic metabolism (Supplementary Table S4) were not significantly different between grouped and isolated CD1 mice. Together, these results support the hypothesis that depot-specific upregulation of metabolic gene expression in mammary fat is a broader characteristic of mammary fat from chronically isolated mice, rather than dependent on mammary tumor formation.

**Upregulation of metabolic genes in mammary adipocytes results in their elevated glucose consumption and lipid synthesis**

Changes to cellular metabolism can be achieved through the increased expression of glucose metabolism and lipid synthesis gene products, such as the proteins encoded by \( \text{Hk2} \), \( \text{Acly} \), and \( \text{Acaca} \). Phosphorylation of glucose by hexokinase 2 (encoded by \( \text{Hk2} \)) effectively traps glucose within cells for subsequent metabolic processes, including lipid synthesis (lipogenesis). \( \text{Acly} \) and \( \text{Acaca} \) gene products also play essential roles in regulating lipid synthesis pathways (Fig. 3A). Therefore, we sought to determine whether the upregulation of \( \text{Hk2} \), \( \text{Acly} \), and \( \text{Acaca} \) in the mammary adipocytes of socially isolated animals was associated with the predicted functional increases in glucose metabolism and/or lipid synthesis.

Following 15 weeks of either group housing or social isolation, we purified mammary adipocytes from individual TAg mouse mammary glands and measured their relative cellular glucose uptake from culture media in the presence (stimulated) or absence (basal) of insulin. Under basal and stimulated conditions, mammary adipocytes from socially isolated animals consumed roughly twice the amount of glucose compared with adipocytes from group-housed animals (Fig. 3B; \( *P = 0.025, \) Wilcoxon rank sum test).

In a parallel experiment, we assessed the relative amount of radio-labeled glucose incorporated into lipid (a measurement of lipogenesis) in mammary adipocytes from the same set of animals. Under basal conditions, lipogenesis was nearly twice as high in mammary adipocytes from socially isolated mice (Fig. 3C; \( *P = 0.05 \)). Following insulin stimulation, the level of de novo lipid synthesis in isolated animals’ mammary adipocytes further increased to roughly 3-fold higher than group-housed animals’ adipocytes (Fig. 3C; \( *P = 0.05, \) Wilcoxon rank sum test). Taken together,
these results show that increased HK2, Acly, and Acaca mRNA expression in mammary adipocytes of socially isolated animals is indeed associated with a concomitant relative increase in both glucose consumption and glucose incorporation into newly synthesized lipids.

**Leptin expression and secretion is increased in adipocytes from socially isolated animals.**

Both adipocyte glucose metabolism and lipid synthesis are suspected to be important regulators of the production and secretion of the adipokine and leptin (32, 33). Furthermore, previous *in vitro* and *in vivo* studies have implicated leptin in cancer cell proliferation and tumor growth (34–37). Although there were no significant differences in circulating leptin between isolated and group-housed animals (Table 1), the metabolic changes we saw in the mammary adipose tissue of socially isolated mice suggested that adipocytes may increase leptin secretion within the local mammary microenvironment. Therefore, we measured the leptin content of mammary adipocytes from socially isolated versus group-housed mice.

Using both Western blot and ELISA analyses, we observed approximately 60% to 70% more leptin in the mammary adipocytes of social isolates (Fig. A and B; ELISA, \( P = 0.01 \); Western blot, \( ** P = 0.009 \)). To determine whether the elevation in intracellular leptin correlated with increased leptin secretion, we cultured mammary adipose tissue from isolated and group-housed animals for 24 hours under serum-free conditions. The media was then harvested and its leptin content was assessed. As observed in Fig. 4C, leptin levels were elevated in the mammary adipose tissue conditioned media from the socially isolated animals relative to media from group-housed mouse mammary adipose tissue (\( P = 0.02 \)). Thus, social isolation and the ensuing stress response seem to result in increased mammary adipocyte leptin protein expression and secretion. In contrast, systemic leptin levels were not significantly affected (Table 1), again indicating an effect of social isolation and associated neuroendocrine responses to stressors on gene expression specifically in the mammary adipose tissue microenvironment, as opposed to a generalized effect on all fat depots.

**Mammary adipose tissue conditioned media potentiates the proliferation of SV40-TAg mammary epithelial cancer cells *in vitro***

Adipose tissue depots are considered to be endocrine organs, secreting numerous factors including leptin. Because we observed elevated leptin secretion from the mammary fat of socially isolated animals, we hypothesized that differential secretion of leptin and/or other adipokine factors could contribute to the larger mammary tumor burden associated with social isolation. We next evaluated the possibility that secreted factors from mammary fat contribute to cancer cell proliferation, using conditioned media from the leptin secretion experiments (Fig. 4C).

Conditioned media was applied to an SV40 TAg transgenic mammary epithelial cell line (M27H4) derived from an *in situ* hyperplastic lesion (27). Media derived from culturing mammary adipose tissue, regardless of animal housing, was sufficient to drive cancer cell growth without serum (Fig. 4D), indicating that secreted factors and/or metabolites from mammary adipose tissue can support cancer cell proliferation. Interestingly, media derived from culturing mammary adipose tissue from socially isolated
animals resulted in significantly more M27H4 cell proliferation than media from group-housed mouse mammary adipose tissue \((/C3/C3/C3 P < 0.0001)\). However, addition of exogenous recombinant purified leptin (final concentration 0.1 ng/mL–10 ug/mL) did not affect cell proliferation (Supplementary Fig. S3A), suggesting that factor(s) other than leptin drive proliferation of the epithelial cells in culture and may also contribute to the increased tumor burden observed in socially isolated animals. Thus, isolated animals’ mammary adipose tissue seems to be enriched in the production of proliferative factors. Furthermore, differences in the secretome components likely contribute to the larger tumor growth seen in socially isolated versus group-housed mice.

Discussion

Although association studies examining social stressors and human cancer risk have shown mixed results, several rodent models suggest that the social stress response can contribute to cancer progression \((5, 6, 38–40)\). Because the biology of the stress response involves complex changes in physiology, identifying the precise aspects of the stress response that influence cancer biology is challenging. Here, we identify a relationship between exposure to a chronic social stressor, altered mammary adipose tissue metabolism, and breast cancer progression. Our results revealing metabolic alterations within the adipocytes of the mammary gland microenvironment expand the importance of maintaining metabolic homeostasis in cancer prevention.

It is well-established that exposure to unmitigating low-level psychosocial stressors is correlated with an increased metabolic and cardiovascular disease risk \((41, 42)\). Social isolation, an established psychosocial stressor for female rodents, is associated with an increased corticosterone responsiveness to a mild acute stressor (e.g., restraint for 30 minutes) and with increased mammary tumor growth in both the TAg mouse \((5)\) and Sprague–Dawley rat \((6)\) models of breast cancer. Additional studies have also reported effects of psychosocial stressors in rodent models of mammary tumorigenesis \((39, 40)\). Using a carcinogen-induced mouse breast cancer model, Boyd and colleagues observed increased expression of ER-\(\alpha\) and promotion of mammary tumorigenesis in adulthood when neonates were exposed to chronic, moderate psychosocial stress \((39)\). In a more general genetic mouse model of human cancer \((p53^{+/–}\) mice), Hasen and colleagues reported an initial higher mortality rate in isolated female mice. Among the various types of cancers that arose in this model \((p53^{+/–}\) mice), mammary cancer incidence was actually lower in the surviving social isolates, although other cancers appeared earlier and
were higher in incidence in the isolates (40). Taken together, these studies support a psychosocial influence on breast cancer biology.

Adipose tissue secretes numerous hormones, growth factors and adipokines, some of which have been linked to both inflammation (43, 44), and cancer progression (45–48). Of note, we observed elevated leptin secretion from the mammary fat of socially isolated animals. Despite this observation, the mouse mammary epithelial cancer cell line used in this study did not proliferate more when exposed to recombinant mouse leptin (Supplementary Fig. S3A). This is likely because the mouse cell line does not express detectable levels of the leptin receptor long isoform (Supplementary Fig. S3B) required for full activity (49). However, expression of the leptin receptor long isoform was observed in tumor samples from TAg mice (Supplementary Fig. S3B). Thus, we cannot rule out an increase in the local mammary leptin concentration playing an important role in the increased tumor burden observed in vivo in socially isolated animals. Indeed, several studies support a role for increased leptin in breast cancer risk (14, 36).

The M27H4 cell line we used in this study was chosen because of its derivation from a TAg mouse mammary carcinoma in situ. However, mouse immortalized cells in culture inevitably undergo genotypic and phenotypic changes over time. We are currently attempting to identify the additional factor(s) in the conditioned media from adipose tissue that contribute to the differential effects on epithelial cell proliferation, and in the future will test the

Figure 5. Working model showing interactions between the social environment (exposure to a chronic stressor), the individual’s ensuing stress response, and the neuroendocrine axis with mammary tumorigenesis. The HPA axis and sympathetic nervous system mediate the physiologic response to chronic stressors such as social isolation. Altered neuroendocrine signaling in the mammary microenvironment results in metabolic changes within mammary adipocytes. These include changes in the mammary adipocyte secretome (e.g., increased leptin production) which can promote cancer cell proliferation. Neuroendocrine signaling (e.g., via glucocorticoids) could also act directly on the mammary epithelium and initiate oncogenic pathways.
requirement for these factors on a variety of mammary epithelial cell lines and for their presence in vivo.

The finding that changes in metabolic gene expression are more prominent in mammary versus visceral fat depots was completely unanticipated. However, given the critical role of lactation in reproductive fitness, heightened transcriptional responsiveness of the mammary fat to stress hormones may allow preservation of milk production during times of chronic environmental stress exposure (50). In fact, the depot-specificity of adipose tissue adipokine secretion and hormone responsiveness is increasingly appreciated (51). Our observations that mammary adipose tissue displays a unique physiologic association with chronic environmental stress exposure is in line with the current appreciation for “depot-specificity” in adipose tissue biology (51). Furthermore, interactions between adipocytes and cancer epithelium have been increasingly reported in the literature (52); however, mammary fat as an independent depot and its specific influences on breast cancer biology remain largely uncharacterized. Our data raise important questions about local crosstalk between mammary adipocytes and epithelium in the in situ stages of breast cancer, when preventive intervention may be most relevant. For example, do metabolic changes in mammary adipocytes, independent of obesity, contribute to tumor incidence and progression? Can mammary adipocyte biology be favorably altered through a dietary intervention (e.g., increased omega-3 fatty acids) or with small molecules (e.g., metformin) to reduce secretion of pro-tumorigenic factors and cancer progression? Answers to these and other questions will require in-depth studies of mammary fat biology and biochemistry.

The exact neuroendocrine mechanisms by which the response to chronic social stressors triggers progonocytic changes in mammary adipocyte physiology remain to be detailed. Notably, social isolation of female rodents is also associated with heightened systemic glucocorticoid responsiveness to superimposed stressors. Human adipose tissue responds to either chronically high or pulsed glucocorticoid exposure, which can in turn result in adipose depot-specific effects including fat redistribution, decreased insulin sensitivity and increased fatty acid efflux (53). In vivo, glucocorticoids may promote tumor growth and previous work suggests a predominant role for glucocorticoids and GR activation in inhibiting epithelial cell apoptosis (54). Based on our findings, we propose a model wherein social isolation and its ensuing neuroendocrine effects potentiate tumor growth by altering mammary adipocyte metabolism and associated metabolite/adipokine secretion, as well as by directly affecting mammary cancer cell apoptosis and possibly proliferation (Fig. 5). Although much emphasis has been placed on the role of excess adiposity and its contribution to ER+ breast cancer (12), our findings suggest that local mammary adipocyte biology also influences ER-negative breast cancer.

Understanding the mammary adipocyte physiology associated with in situ and invasive tumor formation may aid in identifying new biomarkers and/or targets for breast cancer prevention and treatment. Furthermore, employing behavioral interventions and bolstering the social support of at-risk individuals, in addition to the obvious quality of life benefits, may have important physiologic consequences relevant to cancer prevention. Our findings suggest that lifestyle and pharmacologic interventions (e.g., diet, exercise, and possibly, metformin) targeting metabolic abnormalities in adipose tissue may be effective preventive measures because of their ability to alter the local microenvironment that supports breast cancer development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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