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Award Number: W81XWH-11-1-0149

TITLE: How Do the Metabolic Effects of Chronic Stress Influence Breast Cancer Biology

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CONTRACTING ORGANIZATION: W. J. GIBBS & COMPANY, INC. [AS] [HI] [EI] [FA]

REPORT DATE: April 2013

TYPE OF REPORT: Annual summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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OMB No. 0704-0188

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1. REPORT DATE 01 APR 2013		2. REPORT TYPE Annual Report		3. DATES COVERED 1 April 2012 – 31 March 2013	
4. TITLE AND SUBTITLE How Do the Metabolic Effects of Chronic Stress Influence Breast Cancer Biology				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0149	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Paul Volden				5d. PROJECT NUMBER	
E-Mail: Voldenp@uchicago.edu				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Chicago Chicago IL 60637-5418				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT In the C3(1)/SV40 T-antigen (Tag) FVB/N mouse model of human estrogen and progesterone receptor-negative breast cancer, the stress response elicited by social isolation is associated with increased expression of metabolic genes in the mammary gland. To further understand accelerated tumor growth associated with social isolation, we separated mammary gland adipocytes from ductal epithelium and stroma and then analyzed individual fractions for changes in metabolic gene expression and function. The increased expression of the key metabolic genes Acaca, Hk2 and Acly was found to be significantly elevated in the adipocytes of the mammary gland, and surprisingly, was not significantly increased in visceral adipose depots of socially isolated female mice. Increased metabolic gene expression in the mammary gland of socially isolated mice coincided with increased glucose metabolism, lipid synthesis, and leptin expression. Furthermore, culture media from isolated versus group-housed mouse mammary adipose tissue resulted in relatively increased proliferation of mammary cancer cells. These results suggest that exposure to chronic social isolation results in metabolic changes in mammary gland adipocytes that contribute to increased growth of adjacent epithelial cell tumors. We propose a model in which environmental stress affects estrogen-independent mammary tumor growth, at least in part, through changes in mammary adipocyte biology.					
15. SUBJECT TERMS Breast cancer, adipocytes, social isolation, chronic stress, lysophosphatidylcholine, lysophosphatidic acid					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	58	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	2
Key Research Accomplishments.....	7
Reportable Outcomes.....	9
Conclusion.....	10
References.....	12
Appendices, section 1.....	13
Appendices, section 2.....	46

Introduction

We previously identified key lipid synthesis genes including acetyl-CoA carboxylase alpha (*Acaca*) and ATP citrate lyase (*Acly*) as significantly over-expressed in the mammary adipocytes from isolated vs. group-housed TAg mice. Increased expression of these metabolic genes was also associated with increased glucose consumption and increased lipid synthesis in mammary adipocytes. Furthermore, conditioned media from mammary adipose tissue of socially isolated animals potentiated the proliferation of an SV40 TAg mammary cancer cell line. These data identify an association between chronic social stress, altered mammary fat metabolism, and the secretion of tumor promoting factors from the mammary adipocytes of TAg mice. Ongoing studies have focused on the metabolic alterations within the mammary adipocytes of socially isolated mice. In particular, we have identified significant changes in mammary fat lipid species within the social isolates. Our current investigations test the hypothesis that pro-tumorigenic lipid species are synthesized in the mammary adipocytes of socially isolated mice and contribute to increased tumor burden.

Human epidemiological 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 had mixed results (2, 3), the conclusions of these studies are likely inconsistent because of the genetic and environmental variation inherent in human populations as well as the extensive heterogeneity of breast cancer subtypes (4). These issues make identifying underlying mechanisms connecting social stressors to breast cancer biology challenging. They also underscore the importance of developing well-defined preclinical models for identifying the variety of connections between social stressors and specific cancer subtypes.

In female rodents, imposed social isolation is a well-defined chronic stressor and has been associated with increased mammary tumor growth and malignancy within Sprague-Dawley rats and increased tumor burden in the SV40 T-antigen (TAg) FVB/N mouse breast cancer model (5, 6). Interestingly, increased tumor burden and increased tumor invasiveness occur independently of circulating estrogen and progesterone levels. Global gene expression data from our laboratory previously suggested a link between stress-induced mammary gland lipid synthesis gene expression and subsequently increased mammary tumor growth in the TAg model of ER-negative human breast cancer (6).

Our latest manuscript (appendix, section 2) reports that the increased metabolic gene expression is specific to the mammary adipocytes from socially isolated mice and is associated with a concomitant increase in mammary adipocyte glucose and lipid metabolism. Interestingly, the upregulation of *Acaca*, *Hk2*, and *Acly* steady-state mRNA that was involved in lipid synthesis, was not observed in the visceral fat depots of either FVB/N (both SV40-TAg and wild type) or CD1 female mice. This suggests that stress-induced changes in metabolic gene expression are mammary adipocyte-specific and not dependent on the animal strain. Furthermore, mammary adipocytes from socially isolated versus group-housed mice exhibited increased leptin production. When mammary fat was cultured, media from isolated animals' fat had significantly higher levels of secreted leptin and also stimulated SV40-TAg epithelial cell growth to a greater extent than media from group-housed adipose tissue. These results support the hypothesis that secreted factors from metabolically altered mammary adipose tissue contribute to larger tumor formation in socially isolated versus group-housed mice (appendix, section 1).

Our ongoing investigations have focused on the metabolic alterations within the mammary adipocytes of socially isolated mice. In particular, we have identified upregulation of the lipid synthesis-regulating transcription factor, ChREBP, in the mammary fat of socially isolated animals. Furthermore, lipidomics profiling of mammary lipids from isolated vs. grouped animals has revealed significant increases in specific

polar and neutral lipid species within the mammary fat of social isolates. Among these lipids, lysophosphatidylcholine (LPC), the major precursor to the important signaling lipid, lysophosphatidic acid (LPA), has been investigated as a lipid species that may contribute to the increased tumor burden in social isolates. Interestingly, whereas physiological LPC doses were toxic to TAg breast cancer cells and sub-physiological doses inhibited TAg cell proliferation, in the presence of mammary fat conditioned media, LPC protected TAg breast cancer cells from apoptosis and enhanced their proliferation. Furthermore, exogenous LPA protected TAg breast cancer cells from apoptosis and increased cell proliferations, suggesting that LPC acts as an intermediate metabolite with pro-tumorigenic effects upon conversion to LPA. These data identify LPC and LPA as potentially important lipid signaling species involved in the increased tumor burden observed in socially isolated female TAg mice.

The lipid synthesis-regulating ChREBP α/β genes are differentially regulated in the mammary fat of social isolates.

Upon a meta-review of microarray data comparing grouped and socially isolated animals' mammary fat pad gene expression, *MLXIPL* was identified as significantly increased in social isolates and also encoding for a transcription factor (ChREBP) reported to coordinate lipid synthesis pathways. ChREBP (α) mRNA upregulation in social isolates was validated with qPCR (Fig. 1A). Interestingly, we also observed significant mRNA upregulation of ChREBP- β , a novel ChREBP isoform recently identified in adipose tissue (7). To further characterize mRNA level changes to glucose metabolism and lipid synthesis genes, we performed qPCR on the glucose transporter gene (*GLUT1*) and several other genes involved in the lipid synthesis pathway (*FASN*, *SCD1*, *ELOVL6*). Of these genes profiled, *GLUT1* and *ELOVL6* were also significantly increased (Fig. 1A). Upregulation of these genes provide further validation for activated glucose metabolism and lipid synthesis pathways in the mammary fat of social isolates (Fig. 1B) and identify ChREBP as an important transcription factor potentially involved in coordinating the metabolic alterations in mammary adipocytes.

Increased polar lipid classes in the mammary fat of socially isolated animals.

The ChREBP gene product has been understudied in adipose tissue; however, recent investigations have suggested that it could play important roles in regulating systemic glucose metabolism through coordinating lipid synthesis in the adipocytes (8). The products of lipid synthesis in adipocytes include neutral lipids that can be stored as triglycerides and polar lipid species that incorporate the products of *de novo* lipid synthesis into their glycerol backbone. Because many of these polar lipids have important signaling roles, some of which are known to influence cancer biology, we performed mass spectrometry-based lipidomics to determine whether increased lipid

synthesis in the mammary adipocytes of socially isolated animals coincides with changes to specific polar lipid classes and/or species.

Lipidomics profiling was performed on polar lipids extracted from the mammary fat pads of TAG mice and identified lipid species from 13 different classes (Fig 2A, B). The distribution of polar lipid classes in grouped and socially isolated mice appeared similar, with the largest percentage of polar lipids being attributed phosphatidylcholine (PC) (Fig. 2A). There was an absolute increase in the amount of polar lipids measured in socially isolated animals; however, this increase was not statistically significant (Fig. 2B). Among the 13 polar lipid classes profiled, we observed significant increases in ether-linked phosphatidylcholine (ePC), ether-linked phosphatidylserine (ePS), and lysophosphatidylcholine (LPC) (Fig.3). Interestingly, when polar lipid species were ranked by significant difference in isolated vs. grouped animals, LPC (16:0) was the highest ranked lipid and three other LPC species (18:0, 18:1 and 16:1) were among the top eleven lipids (Table. 1). These data identify significant changes to specific polar lipid species in socially isolated animals' mammary fat, in particular an increase in LPC lipids.

Increased neutral lipid classes in the mammary fat of socially isolated animals.

The primary roles of the adipocyte involve the storage and mobilization of lipids. Lipids stored within the adipocyte are packaged as neutral triglyceride species, which can be mobilized from the cell as free fatty-acids to provide energy metabolites to other cells and tissues. Additionally, stored neutral lipids can act as precursors to or constituents of other lipid-containing molecules, including polar lipids. To determine whether stored neutral lipids in social isolates mirrored the differences observed in their polar lipid species (i.e. an increase in 16 and 18 carbon, saturated and unsaturated fatty acid chains), lipidomics profiling was performed on neutral lipid extracts from isolated vs. grouped mice.

Of the neutral lipids profiled, we observed significant increases that paralleled the increases in LPC species, with significant increases in 16:0, 16:1, 18:0, and 18:1 containing neutral lipids (Fig. 4, Table 2). We also observed a significant increase in 14:0 containing neutral lipids (Fig. 4, Table 2). Taken together, these data indicate that social isolation is associated with dramatic changes to neutral and polar lipid species in the mammary adipose tissue of socially isolated female mice.

LPC is toxic to TAG breast cancer cells in culture.

Lipidomics analysis identified LPC lipid species as being significantly increased in the mammary adipose tissue of socially isolated TAG mice. Therefore, we sought to determine the effects of exogenous LPC on a TAG cancer cell line derived from an invasive TAG mouse tumor (M6 cells,(9)). In the presence of 2.5% FBS, LPC doses of

100uM and below inhibited proliferation and increased cell death of M6 cells, whereas doses above 100uM resulted in the death of all cells in as little as 8hrs (Fig. 5). Under serum free conditions all LPC concentrations were toxic to the cells (Fig. 5).

LPC in the presence of mammary fat conditioned media promotes TAg breast cancer cell proliferation and protects from serum deprivation-induced cell death.

Typically, LPC is highly unstable in circulation, largely due to its rapid conversion to LPA by the actions of the serum phospholipase-D, autotaxin (10). LPA is an important signaling lipid that has been implicated in cancer cell motility, survival, and proliferation (10). We performed qPCR and Western analyses and did not observe the presence of autotaxin mRNA or protein in the M6 cells (data not shown); thus, we hypothesized that LPC toxicity results from the inability of M6 cells to convert LPC to LPA. Within the tumor microenvironment, mammary adipose tissue could serve as an abundant source of autotoxin (11), therefore we tested the hypothesis that LPC can be converted to LPA to inhibit LPC-mediated toxicity and potentially promote cancer cell proliferation and/or survival.

M6 cells were treated with low doses of LPC (5uM or 10uM) in serum free media or serum free media that had been conditioned with mammary fat from TAg animals. Under serum free conditions, 5uM LPC did not affect the survival or proliferation of the M6 cells; however, 10uM LPC resulted in a marked increase in cell death and decrease in proliferation (Fig. 6A, B). Interestingly, in the presence of mammary fat-conditioned media, LPC inhibited cell death in a dose dependent manner and resulted in slightly increased cell proliferation (Fig. 6C, D). These data suggest that LPC affects cancer cell apoptosis and proliferation after conversion to LPA and that mammary fat conditioned media contains the necessary enzyme for this conversion. To test for the presence of phospholipase-D activity in mammary fat conditioned media, bis-(p-nitrophenyl) phosphate (BNPP; a phospholipase-D substrate that is cleaved to p-nitrophenol) was added to conditioned media or serum free media and the conversion to p-nitrophenol (a yellow product) was determined using spectrophotometry. Following 8 hrs in culture, mammary fat conditioned media showed substantial accumulation of p-nitrophenol when compared to BNPP in serum free media alone (Fig. 7). Taken together, these data suggest that LPC can promote breast cancer cell proliferation and survival following exposure to mammary fat secreted phospholipase-D and conversion to LPA.

LPA promotes survival and proliferation of TAg breast cancer cells under serum free conditions.

The previous experiments revealed that mammary fat secreted enzymes are capable of converting LPC to LPA. Furthermore, LPC transitions from being toxic to TAg cells to being pro-tumorigenic when provided in the presence of mammary fat

conditioned media. These data suggest that LPA derived from LPC is a pro-tumorigenic factor to TAg cells. Therefore, we next determined whether exogenous LPA was sufficient to allow for cancer cell proliferation and/or promote survival under serum free conditions. M6 cells were cultured under serum free conditions +/- increasing doses of LPA. As expected, M6 cells without serum growth factors arrested proliferation and underwent substantial apoptosis (Fig. 8A). Conversely, cells treated with LPA displayed dose dependent increases in proliferation and inhibition of cell death (Fig. 8B). Taken together, these data suggest that LPC can inhibit cancer cell death and promote cancer cell proliferation acting as an intermediate metabolite that is converted to LPA by the actions of mammary fat secreted autotoxin.

Key Research Accomplishments

- We have identified that metabolic gene changes associated with social isolation and increased tumor burden in female mice occur within the mammary adipocytes.
- We have determined that the metabolic gene expression changes in mammary adipocytes and associated with social isolation are not dependent on the background mouse strain.
- We have observed that gene expression changes are specific to the adipocytes of the mammary gland and are not observed in other fat depots.
- The gene expression changes observed in mammary adipocytes of socially isolated animals correlate with functional metabolic changes including increased glucose consumption and increased lipid synthesis.
- In addition to metabolic changes in mammary adipocytes of socially isolated animals, we have observed elevated levels and secretion of Leptin protein in isolated vs. grouped animals' mammary adipose tissue.
- Mammary fat conditioned media from social isolates potentiates the proliferation of cancer cells compared to media made using grouped animal mammary fat, suggesting adipocyte secreted proteins/metabolites are linked to the increased tumor burden observed *in vivo*.
- The findings detailed above have been compiled into a manuscript that has recently been accepted for publication in *Cancer Prevention Research*.
- We have further characterized the metabolic gene expression changes within the mammary adipose tissue of social isolates and have identified upregulation of CHREBP α/β as potentially important transcription factors coordinating increased lipid synthesis within the mammary fat of social isolates.
- We have performed lipidomics profiling of lipids extracted from the mammary adipose tissue of isolated vs. grouped mice and have identified dramatic changes in several polar and neutral lipid species within social isolates.
- Lysophosphatidylcholine (LPC), the major precursor to the important signaling lipid, lysophosphatidic-acid (LPA), is significantly increased in social isolates' mammary fat pads.

- LPC inhibits proliferation of TAg cancer cells at low doses and is toxic to cancer cells at high doses. However, when TAg cancer cells are treated with LPC in the presence of mammary fat conditioned media, LPC is antiapoptotic and proliferative.
- Mammary fat conditioned media has phospholipase-D activity, thus can convert LPC to LPA.
- LPA protects SV40-TAg breast cancer cells from serum starvation-induced apoptosis and allows for proliferation in the absence of serum factors.

Reportable Outcomes

Presentations:

Chronic social stress in a mouse model of triple-negative breast cancer is associated with oncogenic changes in mammary fat. Volden PA, Wonder E, Skor M, Patel F, Conzen SD, Brady MJ. AACR 2013, April 6-10 in Washington, DC.

Mammary adipocyte-specific alterations are associated with paracrine effects on mammary tumorigenesis. Volden PA, Wonder E, Brady MJ, McClintock MK, Conzen SD. AACR 2012, April 2-6 in Chicago, IL

Determining Fatty Acid Aliphatic Chain length by Isotropic Mixing Sachleben JR, Yi R, Volden PA, Conzen SD. Experimental NMR, April 2012 in Miami, FL

Environmental stress and breast cancer biology: What is the link? Conzen SD, Volden PA, Brady MJ, McClintock MK. AACR 2011: 102nd Annual Meeting, April 2-6 in Orlando, FL

Mammary adipocyte-specific metabolic alterations are associated with paracrine effects on mammary tumorigenesis. Paul A. Volden, Erin L. Wonder, Maxwell N. Skor, Christopher M. Carmean, Honggang Ye, Masha Kocherginsky, Eleanor Smith, Steven Kregel, Martha K. McClintock, Matthew J. Brady, and Suzanne D. Conzen. Keystone: Cancer metabolism, 2010, Banff, British Columbia

Environmental stress and breast cancer biology: What is the link? Conzen SD, Volden PA, Brady MJ, McClintock MK. AACR 2011: 102nd Annual Meeting, April 2-6 in Orlando, FL

Publications:

Chronic social isolation is associated with metabolic gene expression changes specific to mammary adipose tissue. Volden PA, Wonder EL, Skor MN, Carmean CM, Ye H, Kocherginsky M, Kregel S, McClintock MK, Brady MJ, Conzen SD. Cancer Prevention Research. In press

The Influence of Glucocorticoid Signaling on Tumor Progression. Volden PA, Conzen SD. Invited mini-review. Brain, Behavior, and Immunity. Brain Behav. Immun. 2013;30 Suppl:S26-31.

Conclusions:

Our previous work has identified an association between chronic stress, metabolic alterations within mammary adipocytes, and increased mammary tumor burden in female TAg mice (6). Because mammary fat conditioned media from socially-isolated vs. group-housed mice potentiates *in vivo* proliferation of TAg breast cancer cells, we hypothesized that mammary adipocyte-specific metabolic alterations in social isolates occur with concomitant pro-tumorigenic changes to proteins and/or metabolites secreted from the mammary adipocytes. Indeed, mammary adipose tissue from social isolates had elevated leptin protein levels and secreted more leptin when cultured *ex vivo* (appendix1, section 1, Fig. 4A, B, C). Interestingly, TAg cancer cells did not express the leptin receptor and, therefore, did not respond to exogenous leptin treatment. This result suggests that other factors secreted from the mammary fat may be involved in the increased tumor burden observed in social isolates.

Our recent investigations focusing on the metabolic alterations in mammary fat have identified LPC as a potential metabolite involved in the larger tumor formation within social isolates. Lipidomics profiling identified significant increases in the LPC lipid class within the mammary adipose tissue of social isolates, the most significant changes occurring in saturated and mono-unsaturated 18- and 16-carbon LPCs. In parallel, we observed increased neutral lipids containing saturated and mono-unsaturated 18- and 16-carbon acyl chains. Additionally, although LPC was toxic to TAg cancer cells in standard culture, we observed enhanced TAg cell proliferation and dose-dependent protection from apoptosis when TAg cells were treated with LPC in the presence of mammary fat conditioned media. An assay of phospholipase-D activity revealed that mammary fat conditioned media contains enzymatic activity predicted to convert LPC to the important signaling lipid, LPA. Similar to what was observed with LPC plus conditioned media, LPA treatment alone dose-dependently protected TAg cells from serum starvation-induced cell death and allowed for proliferation under serum free conditions. Taken together, these data identify LPC and LPA as potentially important lipids involved in social isolation-associated increased mammary tumor burden in TAg female mice.

Ongoing experiments have been designed to elucidate the roles of LPC and LPA within isolated vs. grouped animals, *in vivo*. Gene expression analyses suggest that TAg tumors express LPA receptors; however, we have not observed differences in receptor expression between grouped and isolated animals (data not shown). We will perform lipidomic analysis on serum from grouped and isolated animals to determine whether LPC or LPA differences can be detected in circulation. Furthermore, we will measure LPA and LPC levels in mammary fat-conditioned media from to determine whether LPCs or LPAs are differentially secreted from isolated vs. grouped animals. Further indication that LPA signaling contributes to the increased tumor burden in social

isolates will provide the rationale necessary for *in vivo* experiments aimed at antagonizing the LPC-autotaxin-LPA signaling axis. Indeed, small molecule inhibitors of autotaxin and LPA receptors are currently available and antagonizing autotaxin and/or LPA signaling has shown promise in pre-clinical cancer studies (10, 12).

References

1. Berkman LF, Syme SL. SOCIAL NETWORKS, HOST-RESISTANCE, AND MORTALITY - 9-YEAR FOLLOW-UP-STUDY OF ALAMEDA COUNTY RESIDENTS. *American Journal of Epidemiology*. 1979;109:186-204.
2. Duijts SFA, Zeegers MPA, Van der Borne B. The association between stressful life events and breast cancer risk: A meta-analysis. *International Journal of Cancer*. 2003;107:1023-9.
3. Song M, Lee K-M, Kang D. Breast Cancer Prevention Based on Gene-Environment Interaction. *Molecular Carcinogenesis*. 2011;50:280-90.
4. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98:10869-74.
5. Hermes GL, Delgado B, Tretiakova M, Cavigelli SA, Krausz T, Conzen SD, et al. Social isolation dysregulates endocrine and behavioral stress while increasing malignant burden of spontaneous mammary tumors. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106:22393-8.
6. Williams JB, Pang D, Delgado B, Kocherginsky M, Tretiakova M, Krausz T, et al. A Model of Gene-Environment Interaction Reveals Altered Mammary Gland Gene Expression and Increased Tumor Growth following Social Isolation. *Cancer Prevention Research*. 2009;2:850-61.
7. Herman MA, Peroni OD, Villoria J, Schoen MR, Abumrad NA, Blueher M, et al. A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. *Nature*. 2012;484:333-U66.
8. Shankar K, Harrell A, Liu X, Ronis MJJ, Badger TM. Increased adipocyte de novo lipogenesis underlies high carbohydrate driven obesity: Role for ChREBP. *Faseb Journal*. 2007;21:A693-A.
9. Holzer RG, MacDougall C, Cortright G, Atwood K, Green JE, Jorczyk CL. Development and characterization of a progressive series of mammary adenocarcinoma cell lines derived from the C3(1)/SV40 Large T-antigen transgenic mouse model. *Breast Cancer Research and Treatment*. 2003;77:65-76.
10. Houben AJS, Moolenaar WH. Autotaxin and LPA receptor signaling in cancer. *Cancer and Metastasis Reviews*. 2011;30:557-65.
11. Ferry G, Tellier E, Try A, Gres S, Naime I, Simon MF, et al. Autotaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation - Up-regulated expression with adipocyte differentiation and obesity. *Journal of Biological Chemistry*. 2003;278:18162-9.
12. Prestwich GD, Gajewiak J, Zhang H, Xu X, Yang G, Serban M. Phosphatase-resistant analogues of lysophosphatidic acid: Agonists promote healing, antagonists and autotaxin inhibitors treat cancer. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*. 2008;1781:588-94.

Appendices, section 1:

1 **Chronic social isolation is associated with metabolic gene expression**
2 **changes specific to mammary adipose tissue**

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18
19 **Running title:** Social isolation, adipocytes and mammary cancer

20
21 **Keywords:** adipocyte, chronic stress, breast cancer, microenvironment, neuroendocrine,
22 cortisol

23 **Funding:** This work was supported by NIH R01-CA148814 to SDC and MKM, Idea Award BC
24 061754 U.S. Army W81XWH-07-1-0296 to MKM, and NIH 1 T32 DK087703 and a DOD pre-
25 doctoral fellowship W81XWH-11-1-014901 to Paul Volden. The Diabetes Research and
26 Training Center at the University of Chicago is supported by NIH P60-DK020595. The University
27 of Chicago Comprehensive Cancer Center core facilities are supported by NIH P30-CA014599.

28 **Disclosures:** none

29 **Acknowledgments:** We thank Drs. Wen Zhang and Kathleen Markan for assistance with
30 metabolic assays, Dr. Brian Neel for providing CD1 mice, Joscelyn Hoffman, Hannah You,
31 Bradley Williams and Diana Pang for sharing their expertise with this model, and Graham Bell
32 and the University of Chicago Diabetes Research Training Center for the use of the metabolic
33 caging system.

34 Abstract word count: 248

35 Word count exclusive of abstract: 4,998

36 Figures: 5

37 Tables: 1

1 Abstract

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

24

1 **Introduction**

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

12 Recent models of breast cancer examining the effects of imposed social
13 isolation, a well-defined chronic stressor for female rodents, have found an association
14 with increased mammary tumor growth. For example, in SV40 T-antigen FVB/N (TAg)
15 mice (5) and Sprague-Dawley rats (6), social isolation was associated with larger
16 mammary gland tumor burden and increased tumor invasiveness independently of
17 changes in circulating estrogen and progesterone levels. Furthermore, our laboratories
18 discovered that genes encoding key enzymes regulating lipid metabolism were
19 differentially upregulated in the mammary glands of socially isolated versus group-
20 housed mice, even prior to differences in tumor development (5). These results
21 suggested that changes in lipid metabolism (in the pre-malignant epithelial cells and/or
22 the adjacent adipocytes and stromal cells) could be driving the relatively aggressive
23 mammary tumor growth of the social isolates.

24 In mouse models, social stressors have been linked to obesity (7), disruption of
25 metabolism (8, 9), and diabetes (10), supporting an association between exposure to
26 social stressors, the physiological stress response, and metabolic disorders. However,
27 the mechanisms through which disrupted metabolism promote mammary tumorigenesis
28 are still unclear. A link between metabolic syndrome/obesity and breast cancer has

1 been suggested (11). Obesity is associated with increased local production of estrogen
2 in mammary gland fat likely contributing to estrogen receptor positive (ER+) breast
3 cancer progression (12). However, emerging data also link metabolic diseases to ER
4 negative (ER-) breast cancer, suggesting that factors other than estrogen are involved
5 (13). Indeed, there is increasing evidence that in addition to estrogenic factors,
6 mammary gland tumorigenesis can be influenced by both local and systemic metabolic
7 signaling molecules, including insulin and leptin (14-16). Adding complexity, the stromal
8 compartment and its reciprocal communication with the mammary epithelium is likely an
9 important factor influencing breast cancer (17). Many cell types, including fibroblasts,
10 adipocytes, and immune and endothelial cells compose the mammary stroma.
11 Mammary gland adipocytes are arguably the least well-understood component. The
12 relative lack of studies on mammary adipocytes in breast cancer biology is surprising
13 considering the abundance of mammary gland adipose tissue and the well-established
14 role of fat as an endocrine/paracrine tissue (18).

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

28 Using a global gene expression approach, we previously identified key metabolic
29 genes including acetyl-CoA carboxylase alpha (*Acaca*), hexokinase 2 (*Hk2*), and ATP

1 citrate lyase (*Acly*) as significantly overexpressed in the mammary glands of isolated
2 versus group-housed TAg mice. Interestingly, increased expression of these gene
3 products is associated with the hallmark metabolic changes observed in cancer cells
4 (23). However, in our previous experiments RNA was obtained from whole mammary
5 gland tissue so we could not determine the specific cell type(s) that were contributing to
6 increased metabolic gene expression. Because it has become increasingly clear that
7 mammary epithelial cell proliferation is influenced by adjacent non-epithelial stromal
8 cells (17), we sought to establish the specific cell types contributing to mammary gland
9 metabolic gene expression changes. Our new findings reveal an association between
10 social isolation, the ensuing stress response, and increased mammary gland adipose
11 tissue lipid metabolism, without a measurable concomitant effect on systemic
12 metabolism. While previous studies have implicated increased mammary fat estrogen
13 production in ER+ breast cancers, our results implicate mammary adipocyte function
14 and its secretome as an important modulator in a model of ER-negative breast cancer
15 growth.

16 **Materials and Methods**

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

18 FVB/N mice homozygous for the SV40 TAg transgene (originally provided as
19 hemizygous TAg mice by Jeff Green of the National Cancer Institute's Mouse Models of
20 Cancer Consortium), non-transgenic FVB/N mice (Charles River) and Swiss CD1 mice
21 (Charles River) were weaned at 3 weeks of age and transferred to differential housing
22 as described in the Supplementary Methods section. TAg homozygous animals were
23 maintained and bred to generate TAg homozygous study populations. Female TAg mice
24 were no longer bred following birth of a litter. To minimize confounding influences from
25 estrous cycle hormones on experimental results, all study animals were sacrificed in
26 estrus phase, as determined by vaginal cytology (24). National Institutes of Health and
27 University of Chicago Animal Care Guidelines were followed for all studies. A detailed

1 outline of experiments and setup prior to animal sacrifice are described in supplemental
2 methods.

3 **Measurement of circulating factors and food/caloric consumption**

4 Blood glucose was measured via tail bleed using a Bayer contour glucose meter.
5 Tail blood was collected for plasma isolation using heparinized capillary microvettes
6 (Andwin Scientific) and was diluted for corticosterone measurements 1:50 in buffer
7 provided with a corticosterone ELISA kit (Enzo Life Sciences). Immediately at sacrifice,
8 cardiac puncture was performed to collect blood, and serum was isolated and stored at
9 -80° C. Serum insulin and leptin were measured by ELISA (ALPCO Diagnostics, Crystal
10 Chem.; respectively). Serum free-fatty acids were measured by enzymatic assay (Wako
11 Diagnostics). Food consumed was calculated weekly as initial food mass minus the final
12 mass at week's end. Calories consumed were calculated by multiplying the consumed
13 food mass by the diet's caloric density (Teklad #8904, 3.0kcal/g). Statistical analyses
14 are provided in supplemental methods.

15 **Adipose tissue harvest, collagenase digestion, and centrifugal separation of** 16 **mammary adipocytes**

17 Mice were sacrificed at 15 weeks of age and mammary fat pads with palpable
18 tumors were excluded from experiments. Gonadal fat pads were excised from their
19 adjacent fallopian tubes and snap-frozen. Excised mammary fat pads were immediately
20 placed in 2 mL microcentrifuge tubes containing 700 μ L DMEM with 10% FBS. Details
21 of adipose tissue harvest and adipocyte isolation are provided in supplementary
22 methods and are a modification of the procedure reported previously (25).

23 **Quantitative RT-PCR**

24 mRNA (1ug) was reverse-transcribed using the qScript cDNA synthesis kit
25 (Quanta Biosciences). Q-RT-PCR was performed with PerfeCTa SYBR Green FastMix
26 (Quanta Biosciences). All reactions were performed in a Biorad iCycler iQ real-time

1 PCR system. Details of the statistical analysis are provided in supplemental methods
2 section.

3 **Adipocyte glucose consumption and lipogenesis**

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

11 To compare adipocyte glucose consumption, adipocytes (10 µL) from individual
12 animals (group-housed n=5; socially isolated n=3) were placed into 96 well plates
13 containing 90 µL of DMEM (1 g/L glucose) and 1% BSA. Cells were stimulated with 50
14 nM insulin or vehicle control, incubated for 4 h, and medium was then collected and
15 glucose consumption measured as loss of glucose from the media between 0 and 4 h
16 (26). Statistical analyses are provided in supplemental methods.

17 To assess lipogenesis, 10 µL of adipocytes from individual animals (n=3, each
18 condition) were placed into scintillation vials containing 400 µL of DMEM (1 g/L glucose)
19 and 1% BSA. Cells were treated with vehicle or 10 nM insulin and 100 µL of ¹⁴C-labeled
20 glucose (0.001uCi/mL, 1uCi /Rxn) was immediately added to each vial. Cells were
21 incubated at 37°C for 60 min and 4 mL of Betafluor was added, followed by vortexing.
22 The following day, 3.2 mL of the upper (lipid-containing) fraction was transferred to a
23 new scintillation vial and radioactivity measured using a scintillation counter. Statistical
24 analyses are provided in supplemental methods.

25 **Leptin measurements**

26 Mammary adipocytes were sonicated in 1X PBS containing protease inhibitors,
27 and spun at 4°C and 13,400 X g for 15 min. The infranatant between the top lipid layer

1 and pelleted nuclei that contained soluble proteins was transferred to a new tube. For
2 Western blot analysis, 20 μ L of mammary adipocyte protein lysate from individual
3 animals (n= 5 per housing condition) was boiled with 4X Laemmli buffer for 10 min.
4 Samples were resolved on a 15% SDS gel, transferred to PVDF (Millipore), and
5 immunoblotted with anti-leptin antibody (A-20, Santa Cruz Biotechnology) while anti-
6 cyclophilin-B antibody was used as a loading control (PAI-027, Affinity Bio-Reagents).
7 Leptin ELISA (Crystal Chem Inc.) assays were performed on protein lysates (group-
8 housed, n=7; socially isolated, n=5), following BCA-based protein normalization
9 (Thermo Scientific). Leptin receptor immunoblotting was performed with Abcam 5593
10 (Supp. Fig. 3B). Statistical analyses methods are provided in supplemental methods.

11 For secreted leptin measurements, fat pads from 4 grouped or 4 socially isolated
12 animals were pooled, minced and spun at 100 X g for 1 min. The floating tissue was
13 weighed and 1 g incubated with 10 mL of media (SH30240.01, Hyclone), containing 1%
14 BSA and 1% penicillin/streptomycin (P/S), at 37°C for 24 h. Medium was harvested and
15 sterile-filtered through a 0.22 μ m syringe filter, aliquoted, and stored at -80°C. Media
16 (40ul) was used for ELISA leptin measurements (Crystal Chem Inc.). Statistical
17 analyses are provided in supplemental methods.

18 **Pre-malignant SV40-TAg-mammary epithelial cell proliferation**

19 The SV40-Tag M27H4 mammary cell line (a kind gift of Dr. Cheryl Jurcyk, Boise State
20 University) was originally isolated from a hyperplastic, non-invasive lesion in a 2 month
21 old C3(1)/Tag mouse (27). The cells were authenticated by microscopic morphology
22 and were routinely tested via growth curve comparison to the original series of cell lines
23 (27). Cells (5000/well, ~50% confluence after 16hrs) were seeded in 96-well plates
24 containing DMEM, 10% FBS, and 1% P/S. After 16 hrs, media was removed and cells
25 were washed with PBS and then cultured with either 100 μ L of mammary adipose tissue
26 culture media from grouped or isolated animals (described above), or in control media
27 (serum free, 1% BSA, 1% P/S). Replicate wells from each condition were washed with
28 PBS and fixed with 10% TCA at 24 hour intervals. On the final day, total protein from

1 each well, representing relative cell number, was measured via the SRB method (28).
2 The SRB assay was also used to measure proliferation of M27H4 cells in response to
3 recombinant leptin (Life Technologies). Statistical analyses are provided in
4 supplemental methods.

5 **Results**

6 **Social isolation versus group housing is associated with increased vigilance** 7 **followed by accelerated mammary tumor growth**

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

15 In agreement with our previous work (5), among the mice that had palpable
16 tumors by 18 weeks of age (Supp. Fig 1A), isolated mice developed a significantly
17 larger average tumor burden than group-housed mice (* $p=0.013$, Supp. Fig.1B). Also,
18 we again observed that socially isolated mice had become more vigilant by 16 weeks of
19 age, as demonstrated by a longer time to leave their home base and explore a novel
20 environment ($p<0.0001$; log-rank test, Supp. Fig.1C). These results confirm that
21 exposure to social isolation is associated with increased vigilance and increased
22 mammary tumor growth (5).

23 **Social isolation is associated with metabolic gene expression changes in** 24 **mammary adipocytes**

25 Next, we sought to determine whether the increased tumor burden of socially
26 isolated mice was associated with local and/or systemic changes in lipid metabolism.

1 Our previous studies in the TAg model found that social isolation results in increased
2 mRNA steady-state levels of key genes encoding glucose metabolism and lipid
3 synthesis enzymes (*Hk2*, *Acaca*, and *Acly*) in whole mammary gland extracts from 15
4 week old mice, before differences in tumor formation (5). Increased glucose metabolism
5 and *de novo* lipid synthesis are associated with the hallmark metabolic changes
6 observed in cancer cells. However, *de novo* synthesis of lipids from glucose is also a
7 primary function of adipocytes, and therefore one of or both of these cell populations
8 could account for the observed overall change in mammary gland metabolic gene
9 expression. To separate the cell types, we used a collagenase digestion and
10 centrifugation protocol (25) and isolated the floating adipocytes from other cells in
11 minced mammary glands of 15-week old mice. Representative images of fractionated
12 cells are shown in Figure 1A. mRNA was isolated from each fraction and gene
13 expression was analyzed by Q-RT-PCR.

14 A comparison of the relative gene expression in the adipocyte versus
15 stromal/epithelial fraction (regardless of housing condition) revealed 20-60 fold higher
16 overall metabolic gene expression in the adipocytes (Fig. 1B). Adipocytes from socially
17 isolated TAg mouse mammary glands expressed significantly higher steady-state *Hk2*
18 ($2^{-\Delta\Delta Ct}=1.84$, * $p=0.012$), *Acly* ($2^{-\Delta\Delta Ct}=2.93$, *** $p=0.0001$) and *Acaca* ($2^{-\Delta\Delta Ct}=2.94$,
19 *** $p=0.0001$) mRNA levels compared to mRNA from group-housed mammary gland
20 adipocytes (Fig. 1C). In cells of the non-adipocyte fraction, expression of these genes
21 was not statistically different between isolated and group-housed animals ($p>0.12$ for all
22 three genes, Fig 1D, Supp. Table 1). These results indicate that these metabolic gene
23 expression changes in the mammary gland following social isolation occur primarily in
24 the adipocyte tissue fraction.

25 To determine whether the elevated metabolic gene expression was specific to
26 the mammary gland fat depot or similarly found in other adipose tissue depots, we also
27 harvested gonadal fat attached to the uterus and fallopian tubes. Unlike mammary
28 adipose tissue, there were no significant differences in the gonadal fat metabolic gene
29 expression from the isolated versus grouped mice (Fig. 1E, Supp. Table 1, $p>0.43$ for all

1 three genes), suggesting that the mammary adipocytes were more sensitive to stress-
2 induced upregulation of metabolic gene expression.

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

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

15 To test this possibility, parallel cohorts of chronically isolated and group-housed
16 female TAg mice were placed in individual metabolic cages. This was also a test of the
17 enduring effects of living in groups, as all mice had to be isolated during the metabolic
18 cage studies because grouped metabolic cages are not available. When animals were
19 placed in the individual metabolic cages, we did not detect any systemic metabolic
20 differences between the previously grouped and isolated cohorts (Supp. Fig.2 and
21 Supp. Table 2). In addition, the previously observed differences in food consumption
22 (Table 1) were no longer evident ($p=0.80$ active, $p=0.27$ inactive period, Supp. Table 2),
23 suggesting that the superimposed stress of social isolation in the metabolic cages
24 affected the eating behavior of the grouped mice.

25 In addition to food consumption, animal weight, and metabolic cage analyses, we
26 measured several circulating markers of systemic metabolism while the animals were in
27 their assigned social conditions. As shown in Table 1, at 15 wks of age, we did not
28 observe significant differences in systemic circulating blood glucose, serum insulin,

1 serum free-fatty acids, or serum leptin, even though profound changes were seen in
2 mammary adipose gene expression at this age. Thus, circulating metabolic factor levels
3 did not suggest a significant effect of social isolation on systemic metabolism.

4 **Upregulation of metabolic genes in the mammary gland occurs independently of** 5 **the SV40 TAg transgene and is not limited to the FVB/N mouse strain**

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

14 We measured whole mammary gland gene expression from chronically isolated
15 and group-housed 15-wk-old female WT and CD1 mice. The results recapitulated the
16 upregulation of *Hk2* (WT, * $p=0.02$; CD1, $p=0.09$), *Acly* (WT, *** $p<0.001$; CD1,
17 ** $p=0.007$), and *Acaca* (WT, * $p=0.03$; CD1, * $p=0.01$) steady-state mRNA that we
18 observed in mammary glands from isolated 15-wk old TAg mice (Fig. 2A, B; Supp.
19 Table 3). Moreover, as we had observed in the TAg mice, gene expression in gonadal
20 fat was not significantly different between isolated versus group-housed female CD1
21 mice (Fig. 2C, $p>0.59$ for all genes), and only one of the three metabolic genes was
22 significantly upregulated in the WT visceral fat (*Acaca* * $p=0.04$, *Hk2* $p=0.47$, *Acly*
23 $p=0.10$; Fig. 2D).

24 As was observed in TAg mice, CD1 mice metabolic cage measures (Supp. Fig.2;
25 Supp. Table 2) and other markers of systemic metabolism (Supp. Table 4) were not
26 significantly different between grouped and isolated CD1 mice. Together, these results
27 support the hypothesis that depot-specific upregulation of metabolic gene expression in

1 mammary fat is a broader characteristic of mammary fat from chronically isolated mice,
2 rather than dependent on mammary tumor formation.

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

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

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

20 In a parallel experiment, we assessed the relative amount of radio-labeled
21 glucose incorporated into lipid (a measurement of lipogenesis) in mammary adipocytes
22 from the same set of animals. Under basal conditions, lipogenesis was nearly twice as
23 high in mammary adipocytes from socially isolated mice (Fig. 3C; *p=0.05). Following
24 insulin stimulation, the level of *de novo* lipid synthesis in isolated animals' mammary
25 adipocytes further increased to roughly 3-fold higher than group-housed animals'
26 adipocytes (Fig. 3C; *p=0.05, Wilcoxon rank sum test). Taken together, these results
27 demonstrate that increased *Hk2*, *Acly*, and *Acaca* mRNA expression in mammary
28 adipocytes of socially isolated animals is indeed associated with a concomitant relative

1 increase in both glucose consumption and glucose incorporation into newly synthesized
2 lipids.

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

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

14 Using both Western blot and ELISA analyses, we observed approximately 60-
15 70% more leptin in the mammary adipocytes of social isolates (Fig. 4A, B; ELISA,
16 * $p=0.01$; Western blot, ** $p=0.009$). To determine whether the elevation in intracellular
17 leptin correlated with increased leptin secretion, we cultured mammary adipose tissue
18 from isolated and group-housed animals for 24 hours under serum-free conditions. The
19 media was then harvested and its leptin content was assessed. As observed in Fig. 4C,
20 leptin levels were elevated in the mammary adipose tissue culture media from the
21 socially isolated animals relative to media from group-housed mouse mammary adipose
22 tissue (* $p=0.02$). Thus, social isolation and the ensuing stress response appear to result
23 in increased mammary adipocyte leptin protein expression and secretion. In contrast,
24 systemic levels were not significantly affected (Table 1), again indicating an effect of
25 social isolation and associated neuroendocrine responses to stressors on gene
26 expression specifically in the mammary adipose tissue microenvironment, as opposed
27 to a generalized effect on all fat depots.

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

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

10 Conditioned media was applied to an SV40 TAg transgenic mammary epithelial
11 cell line (M27H4) derived from a *in situ* hyperplastic lesion (27). Media derived from
12 culturing the mammary adipose tissue, regardless of animal housing, was sufficient to
13 drive cancer cell growth without serum (Fig. 4D), indicating that secreted factors and/or
14 metabolites from mammary adipose tissue can support cancer cell proliferation.
15 Interestingly, media derived from culturing the mammary adipose tissue from socially
16 isolated animals resulted in significantly more M27H4 cell proliferation than media from
17 group-housed mouse mammary adipose tissue (**p<0.0001). However, addition of
18 exogenous recombinant purified leptin (final concentration 0.1ng/mL-10ug/mL) did not
19 affect cell proliferation (Supp. Fig. 3A), suggesting that factor(s) other than leptin drive
20 proliferation of the epithelial cells in culture and may also contribute to the increased
21 tumor burden observed in socially isolated animals. Thus, isolated animals' mammary
22 adipose tissue appears to be enriched in the production of proliferative factors.
23 Furthermore, differences in the secretome components (including leptin) likely
24 contribute to the larger tumor growth seen in socially isolated versus group-housed
25 mice.

26 **Discussion**

27 Although association studies examining social stressors and human cancer risk
28 have shown mixed results, several rodent models suggest that the social stress

1 response can contribute to cancer progression (5, 6, 38-40). Because the biology of the
2 stress response involves complex changes in physiology, identifying the precise
3 aspects of the stress response that influence cancer biology is challenging. Here, we
4 identify a relationship between exposure to a chronic social stressor, altered mammary
5 adipose tissue metabolism, and breast cancer progression. Our results revealing
6 metabolic alterations within the adipocytes of the mammary gland microenvironment
7 expand the importance of maintaining metabolic homeostasis in cancer prevention.

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

24 Adipose tissue secretes numerous hormones, growth factors and adipokines,
25 some of which have been linked to both inflammation (43, 44) and cancer progression
26 (45-48). Of note, we observed elevated leptin secretion from the mammary fat of
27 socially isolated animals. Despite this observation, the mouse mammary epithelial
28 cancer cell line used in this study did not proliferate more when exposed to recombinant
29 mouse leptin (Supp. Fig. 3A). This is likely because the mouse cell line does not

1 express detectable levels of the leptin receptor long isoform (Supp. Fig. 3B) required for
2 full activity (49). However, expression of the leptin receptor long isoform was observed
3 in tumor samples from TAg mice (Supp. Fig.3B). Thus, we cannot rule out an increase
4 in the local mammary leptin concentration playing an important role in the increased
5 tumor burden observed *in vivo* in socially isolated animals. Indeed, several studies
6 support a role for increased leptin in breast cancer risk (14, 36).

7 The M27H4 cell line we used in this study was chosen because of its derivation
8 from a TAg mouse mammary *carcinoma in situ*. However, mouse immortalized cells in
9 culture inevitably undergo genotypic and phenotypic changes over time. We are
10 currently attempting to identify the additional factor(s) in the conditioned media from
11 adipose tissue that contribute to the differential effects on epithelial cell proliferation,
12 and in the future will test the requirement for these factors on a variety of mammary
13 epithelial cell lines and for their presence *in vivo*.

14 The finding that changes in metabolic gene expression are more prominent in
15 mammary versus visceral fat depots was completely unanticipated. However, given the
16 critical role of lactation in reproductive fitness, heightened transcriptional
17 responsiveness of the mammary fat to stress hormones may allow preservation of milk
18 production during times of chronic environmental stress exposure (50). In fact, the
19 depot-specificity of adipose tissue adipokine secretion and hormone responsiveness is
20 increasingly appreciated (51). Our observations that mammary adipose tissue displays
21 a unique physiological association with chronic environmental stress exposure is in line
22 with the current appreciation for 'depot-specificity' in adipose tissue biology (51).
23 Furthermore, interactions between adipocytes and cancer epithelium have been
24 increasingly reported in the literature (52); however, mammary fat as an independent
25 depot and its specific influences on breast cancer biology remain largely
26 uncharacterized. Our data raise important questions about local crosstalk between
27 mammary adipocytes and epithelium in the *in situ* stages of breast cancer, when
28 preventive intervention may be most relevant. For example, do metabolic changes in
29 mammary adipocytes, independent of obesity, contribute to tumor incidence and

1 progression? Can mammary adipocyte biology be altered through a dietary intervention
2 (e.g. increased omega-3 fatty acids) or with small molecules (e.g. metformin) to reduce
3 secretion of pro-tumorigenic factors and cancer progression? Answers to these and
4 other questions will require in-depth studies of mammary fat biology and biochemistry.

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

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

References

- 1
2
3 1. Berkman LF, Syme SL. Social networks, host-resistance, and mortality - 9-year
4 follow-up-study of alameda county residents. *American Journal of Epidemiology*.
5 1979;109:186-204.
- 6 2. Duijts SFA, Zeegers MPA, Van der Borne B. The association between stressful
7 life events and breast cancer risk: a meta-analysis. *International Journal of Cancer*.
8 2003;107:1023-9.
- 9 3. Song M, Lee K-M, Kang D. Breast cancer prevention based on gene-
10 environment interaction. *Molecular Carcinogenesis*. 2011;50:280-90.
- 11 4. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene
12 expression patterns of breast carcinomas distinguish tumor subclasses with clinical
13 implications. *Proceedings of the National Academy of Sciences of the United States of*
14 *America*. 2001;98:10869-74.
- 15 5. Williams JB, Pang D, Delgado B, Kocherginsky M, Tretiakova M, Krausz T, et al.
16 A Model of Gene-environment interaction reveals altered mammary gland gene
17 expression and increased tumor growth following social isolation. *Cancer Prevention*
18 *Research*. 2009;2:850-61.
- 19 6. Hermes GL, Delgado B, Tretiakova M, Cavigelli SA, Krausz T, Conzen SD, et al.
20 Social isolation dysregulates endocrine and behavioral stress while increasing
21 malignant burden of spontaneous mammary tumors. *Proceedings of the National*
22 *Academy of Sciences of the United States of America*. 2009;106:22393-8.
- 23 7. Bartolomucci A, Cabassi A, Govoni P, Ceresini G, Cero C, Berra D, et al.
24 Metabolic consequences and vulnerability to diet-induced obesity in male mice under
25 chronic social stress. *Plos One*. 2009;4.
- 26 8. Chuang JC, Cui HX, Mason BL, Mahgoub M, Bookout AL, Yu HG, et al. Chronic
27 social defeat stress disrupts regulation of lipid synthesis. *Journal of Lipid Research*.
28 2010;51:1344-53.
- 29 9. Rodriguez-Sureda V, Lopez-Tejero MD, Llobera M, Peinado-Onsurbe J. Social
30 stress profoundly affects lipid metabolism: Over-expression of SR-BI in liver and
31 changes in lipids and lipases in plasma and tissues of stressed mice. *Atherosclerosis*.
32 2007;195:57-65.
- 33 10. Nonogaki K, Nozue K, Oka Y. Social isolation affects the development of obesity
34 and type 2 diabetes in mice. *Endocrinology*. 2007;148:4658-66.

- 1 11. Lorincz AM, Sukumar S. Molecular links between obesity and breast cancer.
2 Endocrine-Related Cancer. 2006;13:279-92.
- 3 12. Subbaramaiah K, Howe LR, Bhardwaj P, Du B, Gravaghi C, Yantiss RK, et al.
4 Obesity is associated with inflammation and elevated aromatase expression in the
5 mouse mammary gland. Cancer Prevention Research. 2011;4:329-46.
- 6 13. Davis AA, Kaklamani VG. Metabolic syndrome and triple-negative breast cancer:
7 a new paradigm. International journal of breast cancer. 2012;2012:809291.
- 8 14. Park J, Kusminski CM, Chua SC, Scherer PE. Leptin receptor signaling supports
9 cancer cell metabolism through suppression of mitochondrial respiration in vivo.
10 American Journal of Pathology. 2010;177:3133-44.
- 11 15. Vona-Davis L, Rose DP. Adipokines as endocrine, paracrine, and autocrine
12 factors in breast cancer risk and progression. Endocrine-Related Cancer. 2007;14:189-
13 206.
- 14 16. Daling JR, Malone KE, Doody DR, Johnson LG, Gralow JR, Porter PL. Relation
15 of body mass index to tumor markers and survival among young women with invasive
16 ductal breast carcinoma. Cancer. 2001;92:720-9.
- 17 17. Kim JB, Stein R, O'Hare MJ. Tumour-stromal interactions in breast cancer: The
18 role of stroma in tumourigenesis. Tumor Biology. 2005;26:173-85.
- 19 18. Scherer PE. Adipose tissue - From lipid storage compartment to endocrine
20 organ. Diabetes. 2006;55:1537-45.
- 21 19. Park J, Euhus DM, Scherer PE. Paracrine and endocrine effects of adipose
22 tissue on cancer development and progression. Endocr Rev. 2011;32:550-70.
- 23 20. Zhang YY, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional
24 cloning of the mouse obese gene and its human homolog. Nature. 1994;372:425-32.
- 25 21. Cirillo D, Rachiglio AM, Ia Montagna R, Giordano A, Normanno N. Leptin
26 signaling in breast cancer: an overview. Journal of Cellular Biochemistry. 2008;105:956-
27 64.
- 28 22. Wajchenberg BL. Subcutaneous and visceral adipose tissue: Their relation to the
29 metabolic syndrome. Endocrine Reviews. 2000;21:697-738.
- 30 23. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. Cell.
31 2008;134:703-707.

- 1 24. Caligioni CS. Assessing reproductive status/stages in mice. *Current protocols in*
2 *neuroscience / editorial board, Jacqueline N Crawley [et al]. 2009;Appendix 4:41-*
3 *Appendix I.*
- 4 25. Jurczak MJ, Danos AM, Rehrmann VR, Allison MB, Greenberg CC, Brady MJ.
5 *Transgenic overexpression of protein targeting to glycogen markedly increases*
6 *adipocytic glycogen storage in mice. American Journal of Physiology-Endocrinology and*
7 *Metabolism. 2007;292:E952-E63.*
- 8 26. Yuen VG, McNeill JH. Comparison of the glucose oxidase method for glucose
9 *determination by manual assay and automated analyzer. Journal of Pharmacological*
10 *and Toxicological Methods. 2000;44:543-6.*
- 11 27. Holzer RG, MacDougall C, Cortright G, Atwood K, Green JE, Jorcyk CL.
12 *Development and characterization of a progressive series of mammary*
13 *adenocarcinoma cell lines derived from the C3(1)/SV40 Large T-antigen transgenic*
14 *mouse model. Breast Cancer Research and Treatment. 2003;77:65-76.*
- 15 28. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity
16 *screening. Nature Protocols. 2006;1:1112-6.*
- 17 29. Green JE, Shibata MA, Yoshidome K, Liu ML, Jorcyk C, Anver MR, et al. The
18 *C3(1)/SV40 T-antigen transgenic mouse model of mammary cancer: ductal epithelial*
19 *cell targeting with multistage progression to carcinoma. Oncogene. 2000;19:1020-7.*
- 20 30. Leong H, Mathur PS, Greene GL. Inhibition of mammary tumorigenesis in the
21 *C3(1)/SV40 mouse model by green tea. Breast Cancer Research and Treatment.*
22 *2008;107:359-69.*
- 23 31. La Merrill M, Baston DS, Denison MS, Birnbaum LS, Pomp D, Threadgill DW.
24 *Mouse breast cancer model-dependent changes in metabolic syndrome-associated*
25 *phenotypes caused by maternal dioxin exposure and dietary fat. American Journal of*
26 *Physiology-Endocrinology and Metabolism. 2009;296:E203-E10.*
- 27 32. Walker CG, Bryson JM, Hancock DP, Caterson ID. Leptin secretion is related to
28 *glucose-derived lipogenesis in isolated adipocytes. International Journal of Obesity.*
29 *2007;31:723-9.*
- 30 33. Mueller WM, Gregoire FM, Stanhope KL, Mobbs CV, Mizuno TM, Warden CH, et
31 *al. Evidence that glucose metabolism regulates leptin secretion from cultured rat*
32 *adipocytes. Endocrinology. 1998;139:551-8.*
- 33 34. Gonzalez-Perez RR, Xu YB, Guo SC, Watters A, Zhou WQ, Leibovich SJ. Leptin
34 *upregulates VEGF in breast cancer via canonic and non-canonical signalling pathways*
35 *and NF kappa B/HIF-1 alpha activation. Cellular Signalling. 2010;22:1350-62.*

- 1 35. Gonzalez RR, Watters A, Xu YB, Singh UP, Mann DR, Rueda BR, et al. Leptin-
2 signaling inhibition results in efficient anti-tumor activity in estrogen receptor positive or
3 negative breast cancer. *Breast Cancer Research*. 2009;11:R36.
- 4 36. Ray A, Nkhata KJ, Cleary MP. Effects of leptin on human breast cancer cell lines
5 in relationship to estrogen receptor and HER2 status. *International Journal of Oncology*.
6 2007;30:1499-509.
- 7 37. Hu X, Juneja SC, Maihle NJ, Cleary MGP. Leptin - A growth factor in normal and
8 malignant breast cells and for normal mammary gland development. *Journal of the*
9 *National Cancer Institute*. 2002;94:1704-11.
- 10 38. Costanzo ES, Sood AK, Lutgendorf SK. Biobehavioral Influences on Cancer
11 Progression. *Immunology and Allergy Clinics of North America*. 2011;31:109-32.
- 12 39. Boyd AL, Salleh A, Humber B, Yee J, Tomes L, Kerr LR. Neonatal experiences
13 differentially influence mammary gland morphology, estrogen receptor alpha protein
14 levels, and carcinogenesis in balb/c mice. *Cancer Prevention Research*. 2010;3:1398-
15 408.
- 16 40. Hasen NS, O'Leary KA, Auger AP, Schuler LA. Social isolation reduces
17 mammary development, tumor incidence, and expression of epigenetic regulators in
18 wild-type and p53-heterozygotic mice. *Cancer Prevention Research*. 2010;3:620-9.
- 19 41. Brunner EJ, Hemingway H, Walker BR, Page M, Clarke P, Juneja M, et al.
20 Adrenocortical, autonomic, and inflammatory causes of the metabolic syndrome -
21 Nested case-control study. *Circulation*. 2002;106:2659-65.
- 22 42. Backe E-M, Seidler A, Latza U, Rossnagel K, Schumann B. The role of
23 psychosocial stress at work for the development of cardiovascular diseases: a
24 systematic review. *International Archives of Occupational and Environmental Health*.
25 2012;85:67-79.
- 26 43. Faggioni R, Feingold KR, Grunfeld C. Leptin regulation of the immune response
27 and the immunodeficiency of malnutrition. *Faseb Journal*. 2001;15:2565-71.
- 28 44. Otero M, Lago R, Gomez R, Lago F, Dieguez C, Gomez-Reino JJ, et al.
29 Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and
30 visfatin in patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases*.
31 2006;65:1198-201.
- 32 45. Byeon JS, Jeong JY, Kim MJ, Lee SM, Nam WH, Myung SJ, et al. Adiponectin
33 and adiponectin receptor in relation to colorectal cancer progression. *International*
34 *Journal of Cancer*. 2010;127:2758-67.

- 1 46. Riolfi M, Ferla R, Del Valle L, Pina-Oviedo S, Scolaro L, Micciolo R, et al. Leptin
2 and its receptor are overexpressed in brain tumors and correlate with the degree of
3 malignancy. *Brain Pathology*. 2010;20:481-9.
- 4 47. Jarde T, Caldefie-Chezet F, Damez M, Mishellany F, Penault-Llorca F, Guillot J,
5 et al. Leptin and leptin receptor involvement in cancer development: A study on human
6 primary breast carcinoma. *Oncology Reports*. 2008;19:905-11.
- 7 48. Iyengar P, Combs TP, Shah SJ, Gouon-Evans V, Pollard JW, Albanese C, et al.
8 Adipocyte-secreted factors synergistically promote mammary tumorigenesis through
9 induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization.
10 *Oncogene*. 2003;22:6408-23.
- 11 49. Gorska E, Popko K, Stelmaszczyk-Emmel A, Ciepiela O, Kucharska A, Wasik M.
12 Leptin receptors. *European Journal of Medical Research*. 2010;15: 50-54.
- 13 50. Hovey RC, Aimo L. Diverse and active roles for adipocytes during mammary
14 gland growth and function. *Journal of Mammary Gland Biology and Neoplasia*.
15 2010;15:279-90.
- 16 51. Wronska A, Kmiec Z. Structural and biochemical characteristics of various white
17 adipose tissue depots. *Acta Physiologica*. 2012;205:194-208.
- 18 52. Nieman KM, Romero IL, Van Houten B, Lengyel E. Adipose tissue and
19 adipocytes support tumorigenesis and metastasis. *Biochimica et Biophysica Acta (BBA)*
20 - Molecular and Cell Biology of Lipids. 2013; in press.
- 21 53. Macfarlane DP, Forbes S, Walker BR. Glucocorticoids and fatty acid metabolism
22 in humans: fuelling fat redistribution in the metabolic syndrome. *Journal of*
23 *Endocrinology*. 2008;197:189-204.
- 24 54. Wu W, Chaudhuri S, Brickley DR, Pang D, Karrison T, Conzen SD. Microarray
25 analysis reveals glucocorticoid-regulated survival genes that are associated with
26 inhibition of apoptosis in breast epithelial cells. *Cancer Research*. 2004;64:1757-64.

27

1 **Figure Legends**

2 **Fig. 1.** Mammary gland cell fractionation followed by Q-RT-PCR measurement of gene
 3 expression from isolated and group-housed mice. Collagenase treatment followed by
 4 centrifugation was used to separate SV40 TAg mammary gland adipocytes (left panel)
 5 from the non-adipocytes (epithelial/stromal cells, right panel) (A). Relative *Hk2*, *Acly*,
 6 and *Acaca* gene expression in the adipocytes (white bars) vs. non-adipocyte (black)
 7 cells (B). Expression of *Hk2*, *Acly*, and *Acaca* normalized to beta-actin (*Actb*) mRNA
 8 expression in mammary adipocytes (C), non-adipocytes (epithelial/stromal cells) (D),
 9 and in visceral fat (E) in isolated (white bars) vs. grouped mice (black). Error bars
 10 indicate standard deviation. In panels C-E; * $p < 0.05$, *** $p < 0.001$. N = 4 and 7 for
 11 grouped and isolated animals, respectively.

12 **Fig. 2.** Relative mammary gland and visceral fat depot gene expression in differentially
 13 housed WT and CD1 mice. *Hk2*, *Acly*, and *Acaca* gene expression from 15 week old
 14 CD1 mammary glands (A; n=4 grouped, 5 isolated) or WT mammary glands (B; n=11
 15 grouped, 9 isolated). *Hk2*, *Acly*, and *Acaca* gene expression at 15 weeks in CD1 mice
 16 gonadal adipose tissue (C; n=3 grouped, 4 isolated) or in WT mice gonadal adipose
 17 tissue (D; n=12 grouped, 9 isolated). Socially Isolated mRNA expression (white bars)
 18 relative to group-housed mRNA expression (black). Error bars indicate standard
 19 deviation. In all panels; * $p < 0.05$, *** $p < 0.001$, NS=not significant.

20 **Fig. 3.** Glucose consumption and lipogenesis in isolated mammary adipocytes from
 21 differentially housed TAg mice. A, *Hk2*, *Acly*, and *Acaca* gene products involved in
 22 glucose and lipid metabolism. B, mammary adipocytes from group housed (n=5) and
 23 socially isolated (n=3) animals were cultured in low glucose media, +/- insulin, for 4
 24 hours. Media was then harvested and glucose in the media was measured via the
 25 glucose oxidase method and consumption assessed by a comparison to glucose in
 26 fresh (not used for culture) media. C, mammary adipocytes were purified from group
 27 housed and socially isolated animals (n=3 per group) and immediately cultured with ^{14}C
 28 glucose, +/- insulin, for 1hr. The lipid fraction was extracted from the adipocytes and

1 ^{14}C , representing the incorporation of labeled glucose into lipid species, was counted
2 using a scintillation counter. Average basal lipogenesis and glucose consumption
3 values for grouped animals were normalized to 1. All other measurements are reported
4 as relative to this value. $p \leq 0.05$, Wilcoxon rank sum test.

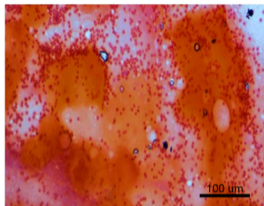
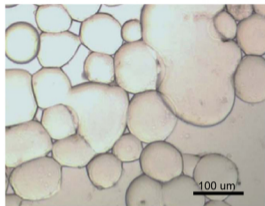
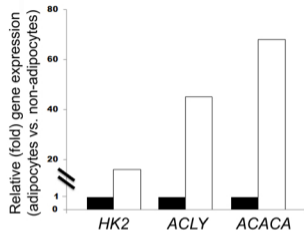
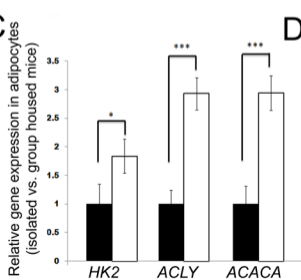
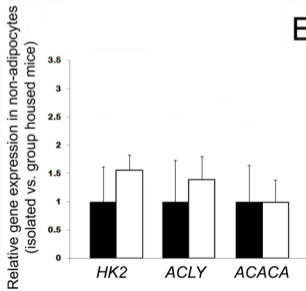
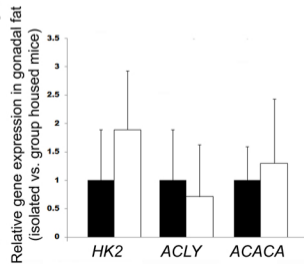
5 **Fig. 4.** Analysis of mammary adipose tissue lysates and secreted factors from
6 differentially housed TAg mice. A, Representative Western blot showing grouped and
7 socially isolated mouse mammary adipocyte lysates probed with antibodies targeting
8 leptin, adiponectin, and actin proteins on the same blot. B, Relative leptin expression in
9 mammary adipocyte lysates as measured by Western blot densitometry ($n=5$ per group)
10 and ELISA ($n=7$ grouped, 5 isolated). C, Mammary adipocyte-secreted leptin from a
11 24hr adipocyte culture media, measured by ELISA ($n=4$ per group). Error bars indicate
12 standard deviation; $*p < 0.05$, $**p < 0.01$, Wilcoxon rank sum test. D, Serum-free media
13 incubated with isolated and group-housed mammary adipose tissue applied to the
14 M27H4 SV40-TAg mammary epithelial cell line followed by measurement of cellular
15 protein over time (SRB assay) reflecting total cell number. Serum-free DMEM with 1%
16 BSA was used as a control. $***p < 0.0001$ isolated vs. grouped, error bars indicate
17 standard deviation.

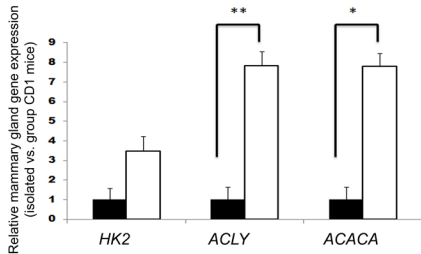
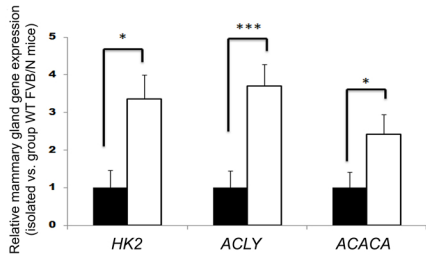
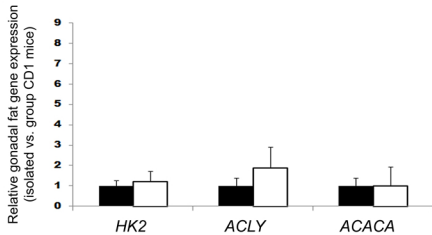
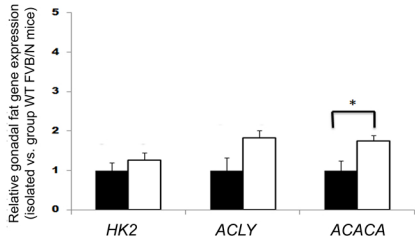
18 **Fig. 5.** Working model showing interactions between the social environment (exposure
19 to a chronic stressor), the individual's ensuing stress response, and the neuroendocrine
20 axis with mammary tumorigenesis. The HPA axis and sympathetic nervous system
21 mediate the physiological response to chronic stressors such as social isolation.
22 Altered neuroendocrine signaling in the mammary microenvironment results in
23 metabolic changes within mammary adipocytes. These include changes in the
24 mammary adipocyte secretome (e.g. increased leptin production) which can promote
25 cancer cell proliferation. Neuroendocrine signaling (e.g. via glucocorticoids) could also
26 act directly on the mammary epithelium and initiate oncogenic pathways.

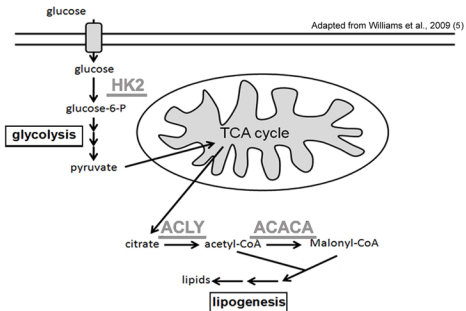
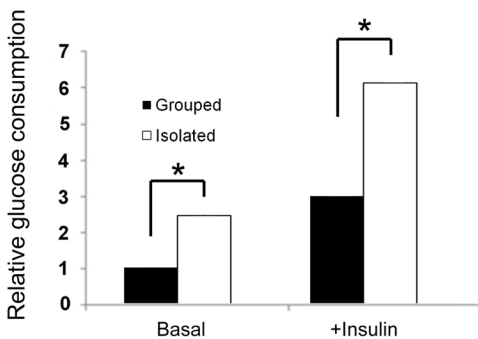
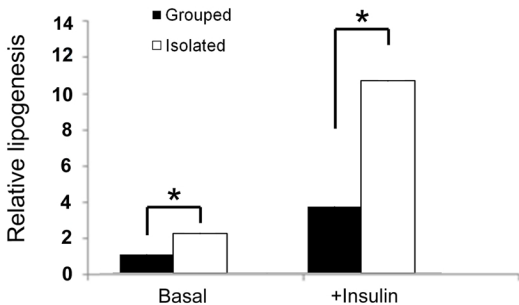
27 **Table 1.** Measurements of circulating metabolic parameters, food consumption, and
28 weights in grouped vs. isolated TAg female mice. Circulating markers were obtained

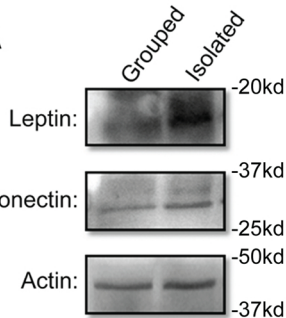
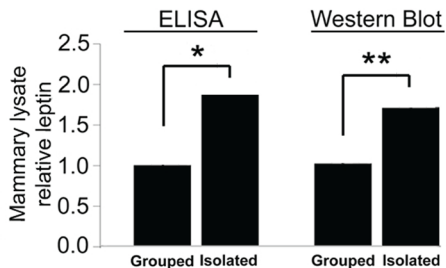
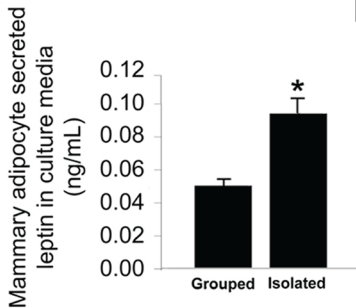
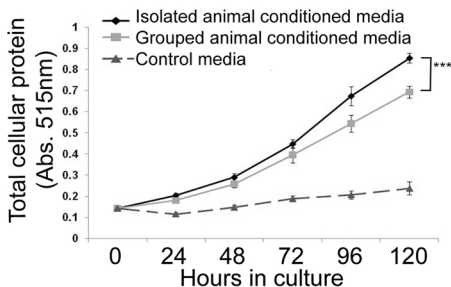
1 from 15wk old animals (9 group-housed, 8 isolated). Food consumption and weights (16
2 group-housed, 15 isolated) are divided into pre- and post-tumor time periods to
3 investigate the effects of tumor burden on metabolism. Data indicate means +/-
4 standards deviation, with p-value obtained using a student's T-test.

5

A**B****C****D****E**

A**B****C****D**

A**B****C**

A**B****C****D**

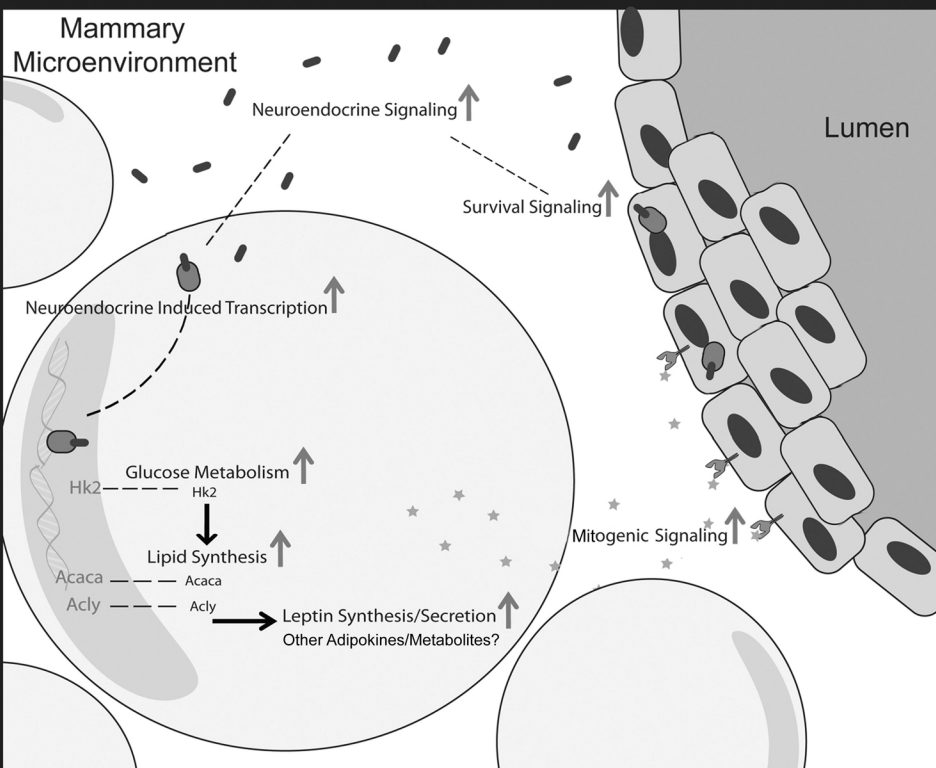
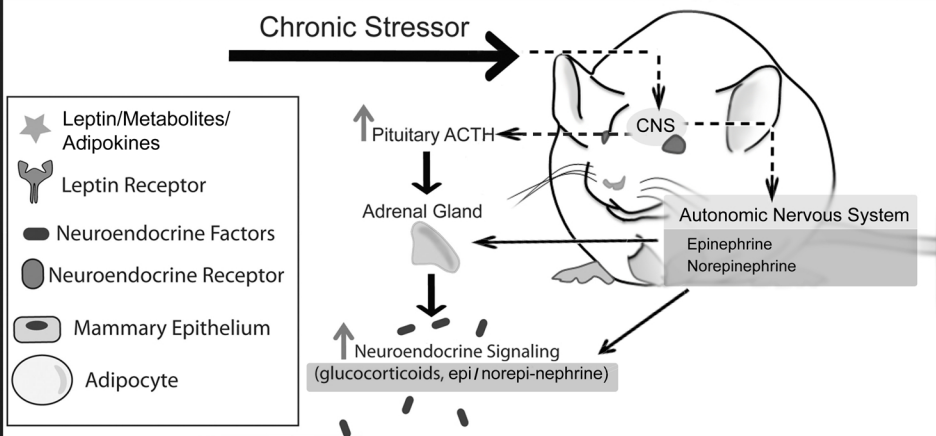


Table 1: Measurements of circulating metabolic parameters, food consumption, and weights in grouped vs. isolated TAg female mice.

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

Appendices, section 2:

Figure Legends:

Fig. 1: Activation of glycolysis and lipid synthesis pathways in socially isolated animals' mammary adipose tissue. A, relative expression of metabolic genes in socially isolated animals' mammary fat (black bars) compared to group-housed animals' mammary fat (grey). B, Pathway map of metabolic genes profiled in mammary fat from grouped and socially isolated animals. Green dashed lines indicate putative ChREBP- α direct target genes based on ChIP analysis. Red asterisks indicate genes identified as being significantly increased in socially isolated animals. Error bars indicate standard deviation; * $p \leq 0.05$, ** $p \leq 0.01$.

Fig. 2: Lipidomics profiling of polar lipids. A, Distribution of polar lipid classes identified in mammary fat of grouped vs. socially isolated TAG mice. B, Absolute quantification of total polar lipids in mammary fat of grouped vs. isolated mice.

Fig. 3: Specific polar lipid classes are increased in the mammary fat of socially isolated animals. Relative polar lipid concentrations for lipid classes profiled. Error bars indicate standard deviation; * $p \leq 0.05$ with Bonferroni correction.

Fig. 4: Specific neutral lipids are increased within the mammary fat of socially isolated animals. Relative neutral lipid concentrations in grouped vs. socially isolated TAG mice. * $p \leq 0.05$, ** $p \leq 0.01$.

Fig. 5: LPC is toxic to M6 breast cancer cells. M6 cells were incubated with the indicated doses of LPC 16:0 in the presence or absence of 2.5% FBS and percentage of dead cells was measured after 8hrs using YOYO-1 iodide staining.

Fig. 6: LPC in the presence of mammary fat conditioned media promotes M6 cell survival and proliferation. M6 cells were incubated with the indicated doses of LPC in the presence or absence of mammary fat conditioned media. Proliferation was measured as change in confluence with respect to time using the IncucyteTM live cell imaging system. Dead cells (YOYO-1 iodide positive) were counted using the IncucyteTM live cell imaging software and normalized to confluence. A, M6 cells' change in confluence with LPC doses and serum free conditions. B, dead cell count normalized to confluence with LPC doses and serum free conditions. C, M6 cells' change in confluence with LPC doses and mammary fat conditioned media. B, dead cell count normalized to confluence with LPC doses and mammary fat conditioned media.

Fig. 7: Mammary fat conditioned media contains phospholipase-D activity. BNPP was added to serum free media and mammary fat conditioned media. Conversion of BNPP

to PNP, an indication of phospholipase-D mediated BNPP cleavage, was determined by yellow product formation and 410nm absorbance.

Fig. 8. LPA promotes survival and proliferation of TAg breast cancer cells under serum free conditions. A, proliferation of M6 breast cancer cells in serum free media with increasing concentrations of LPA 18:1. B, Count of dead cells (YOYO-1 iodide positive) cultured under serum-free conditions with increasing concentrations LPA 18:1

Table 1: Top 20 significantly changed polar lipids ranked according to P-value.

Table 2: Concentrations of neutral lipid classes in grouped vs. socially isolated TAg mice.

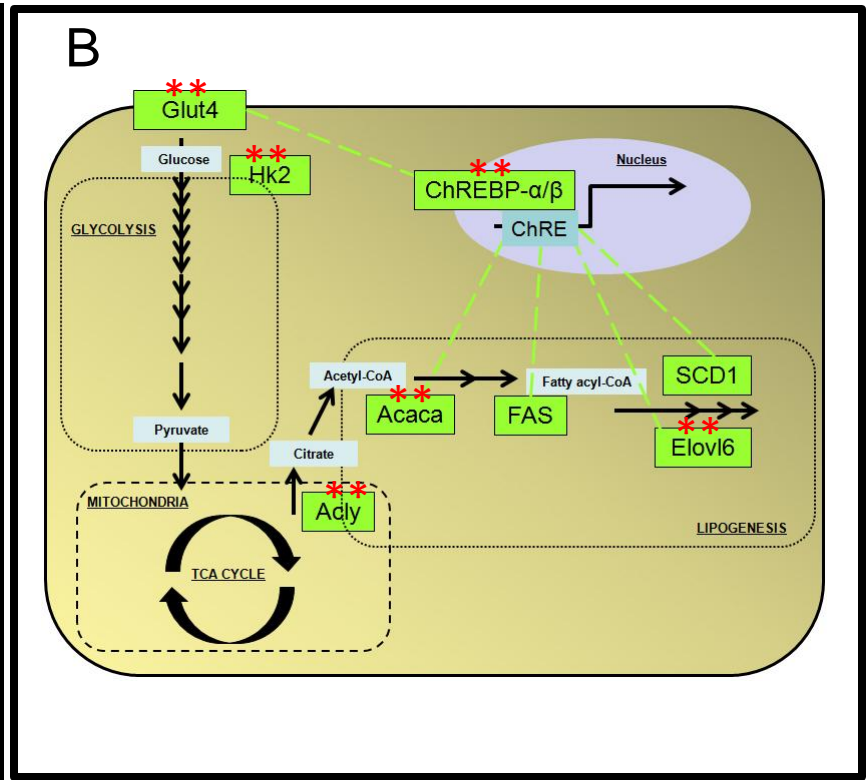
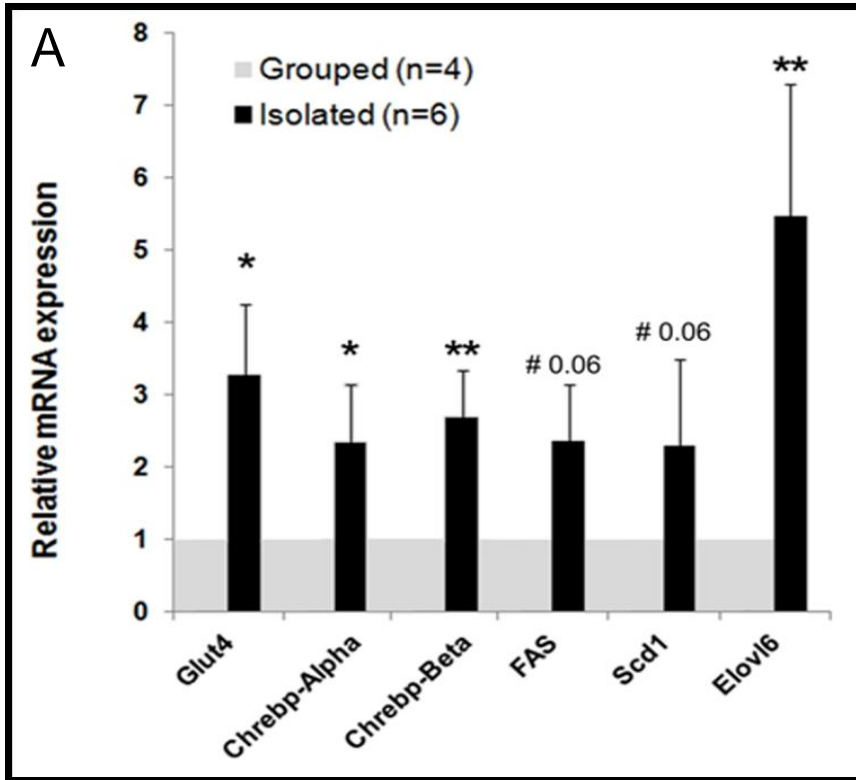


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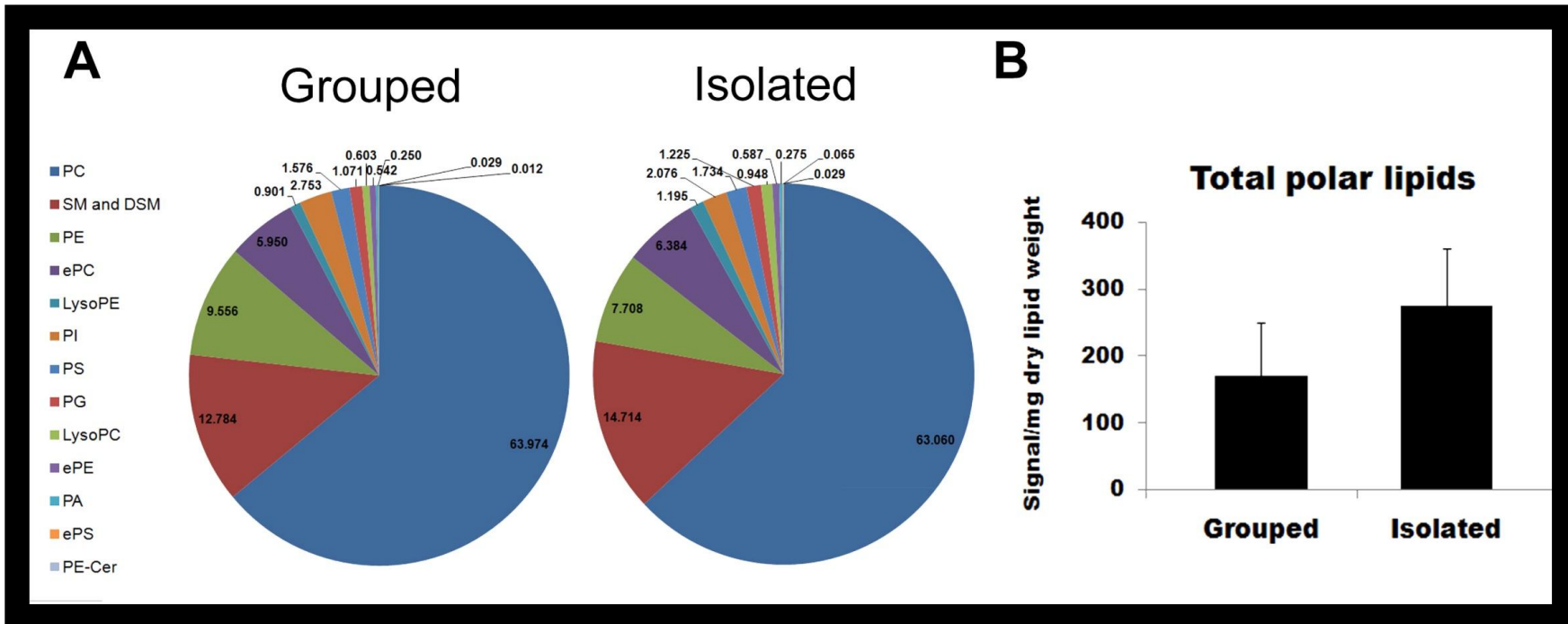


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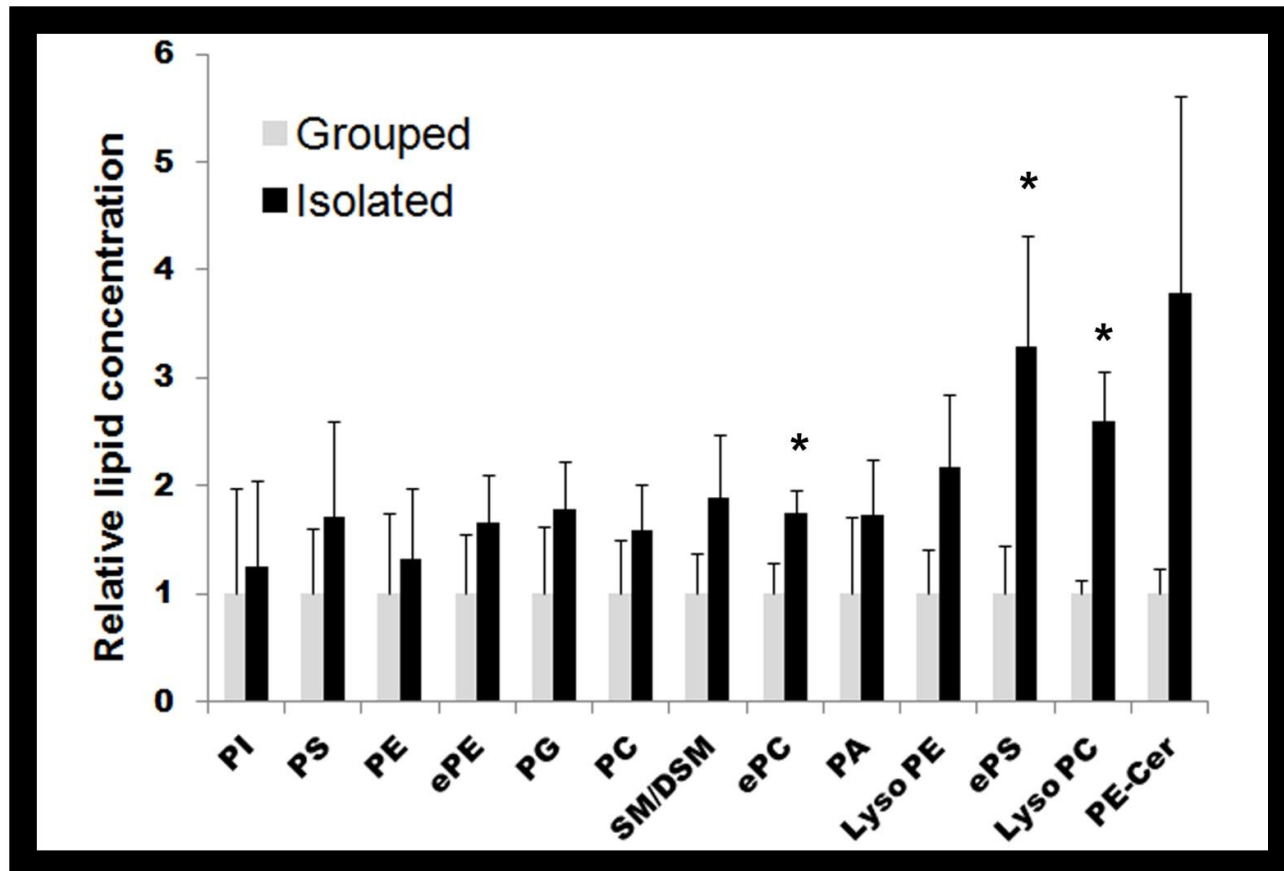


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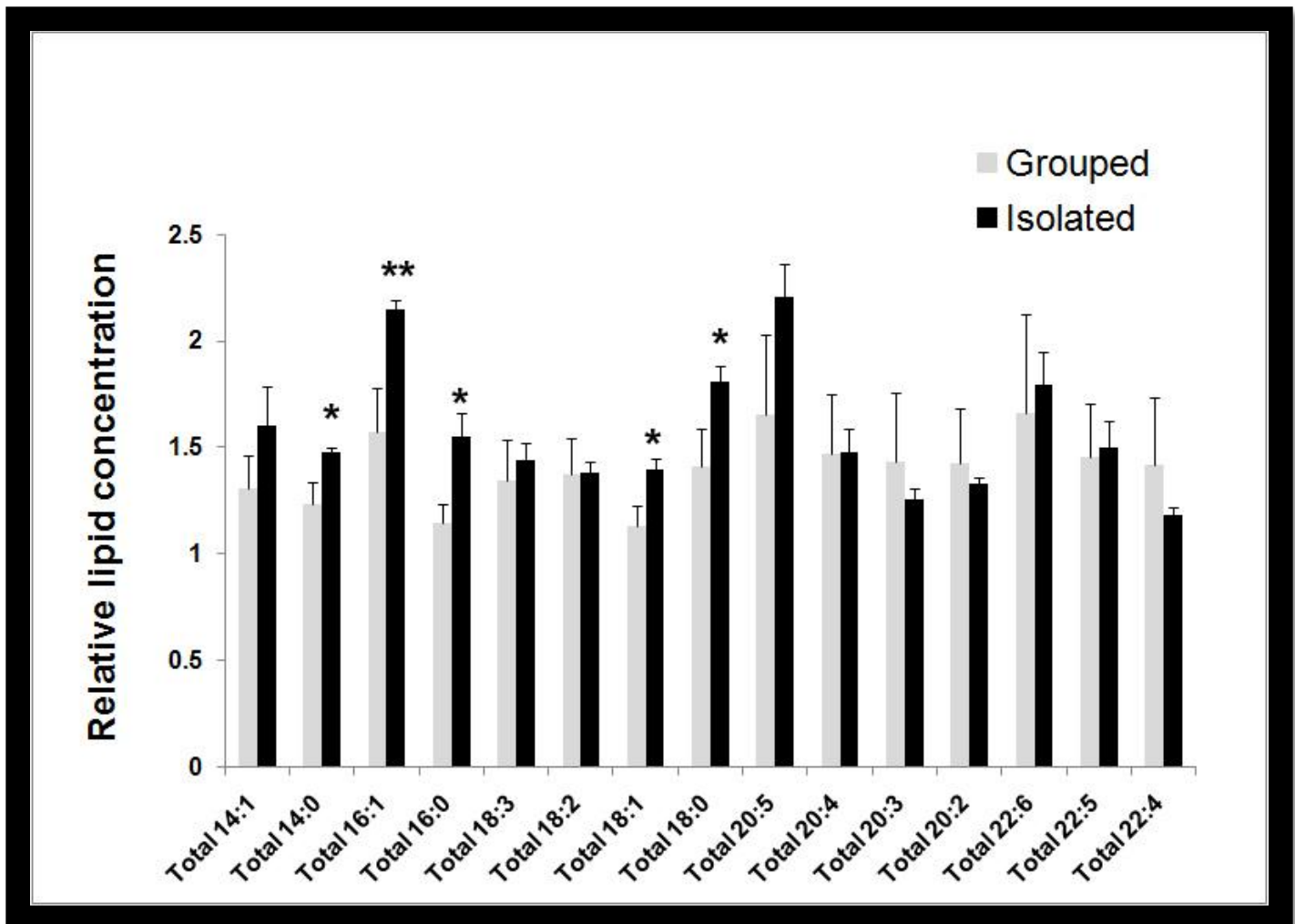


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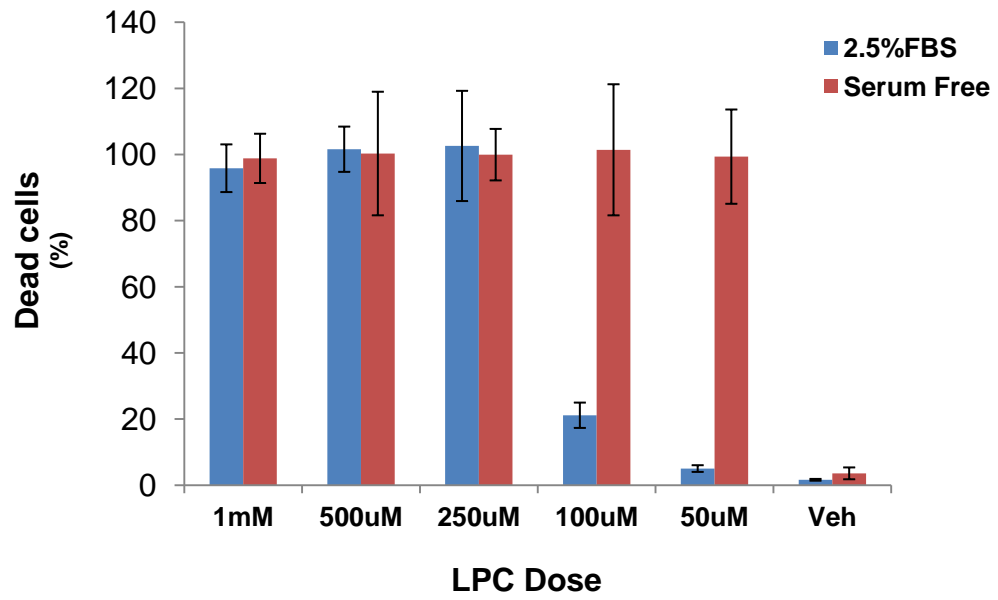


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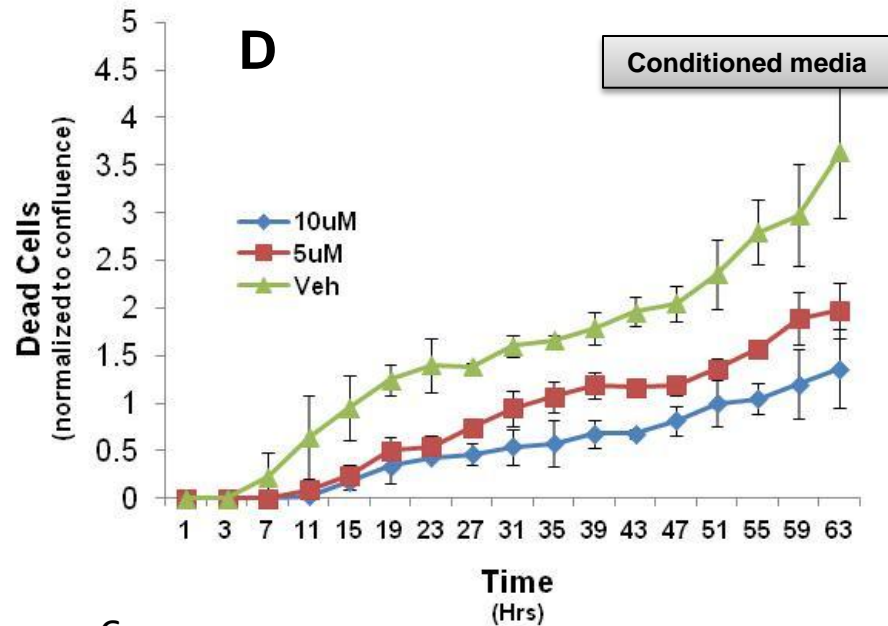
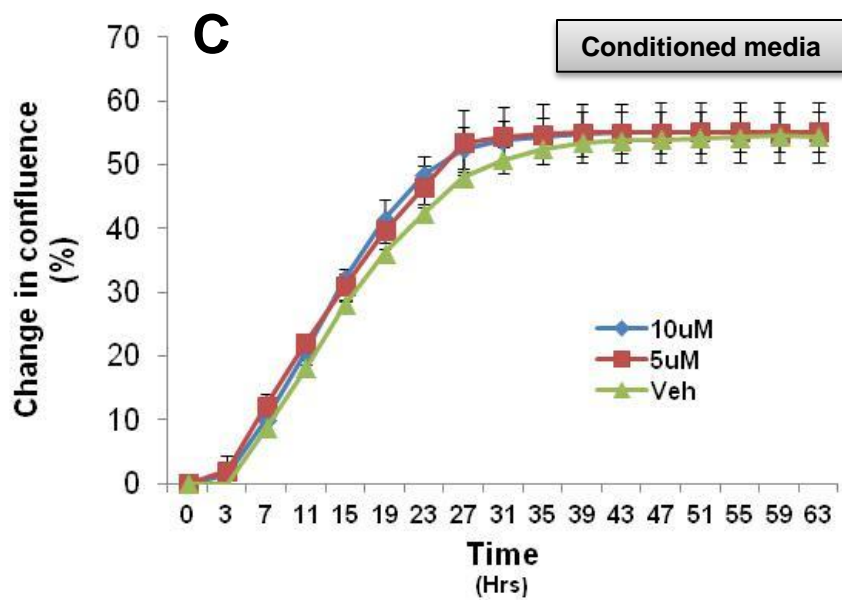
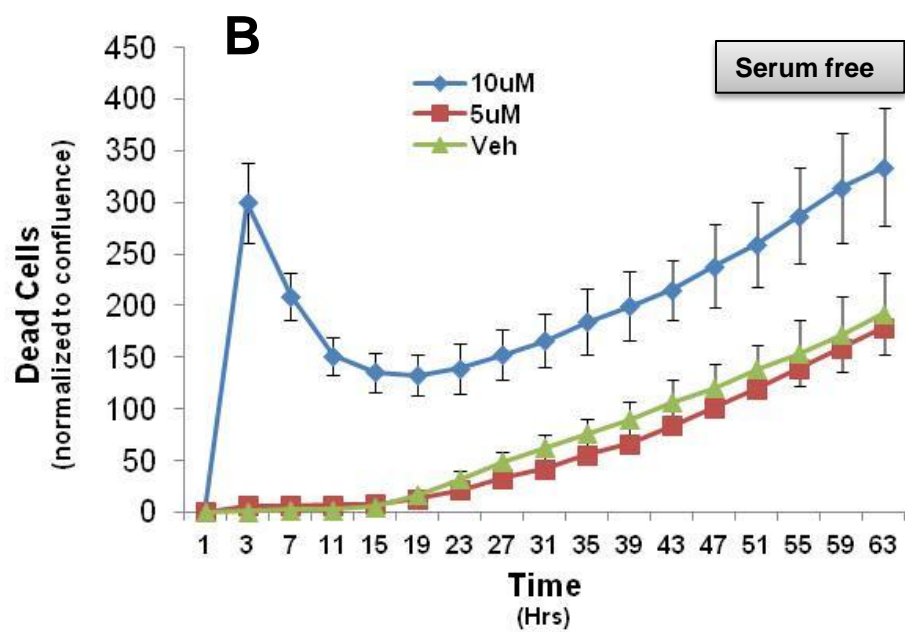
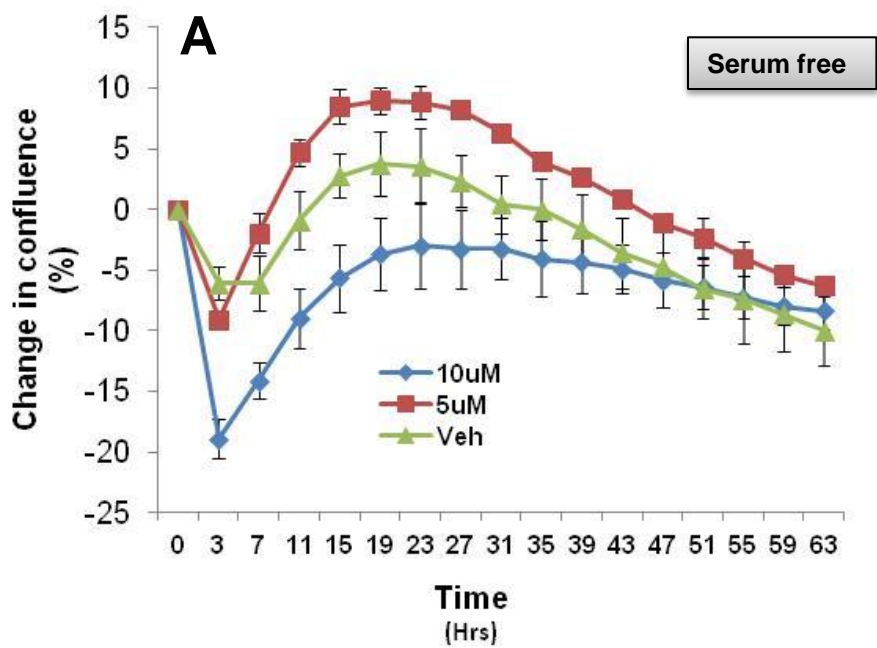


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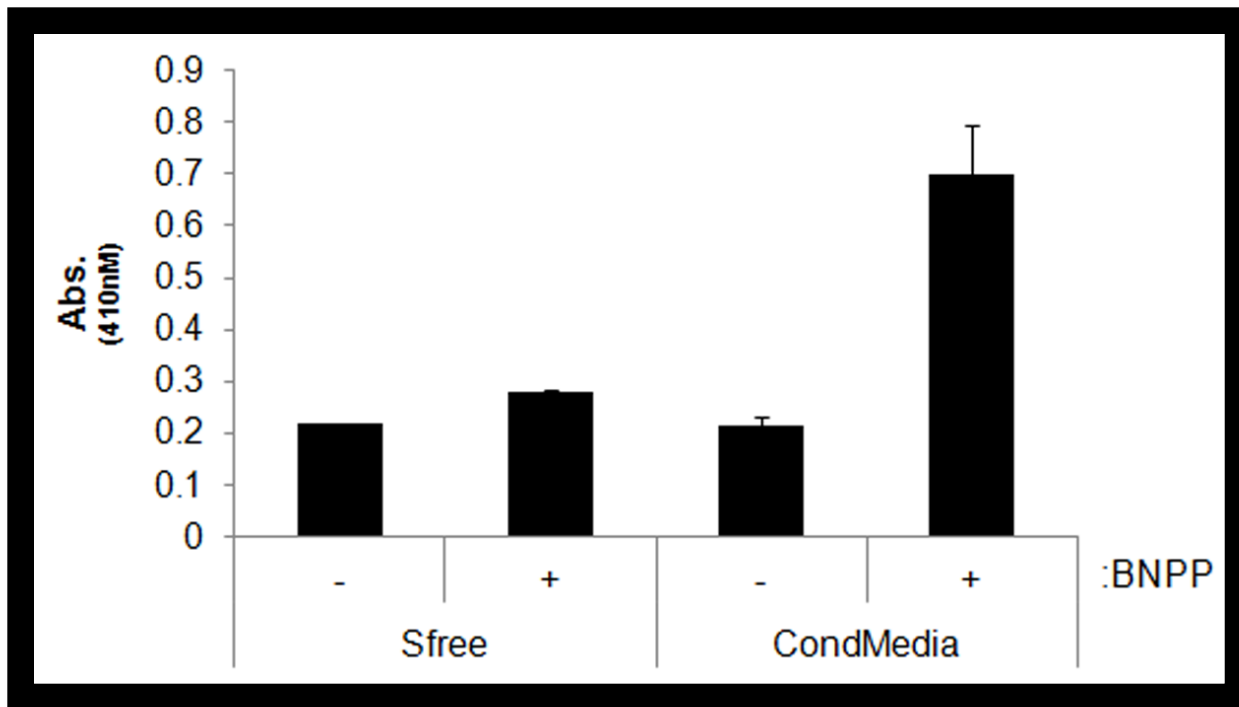


Figure 7.

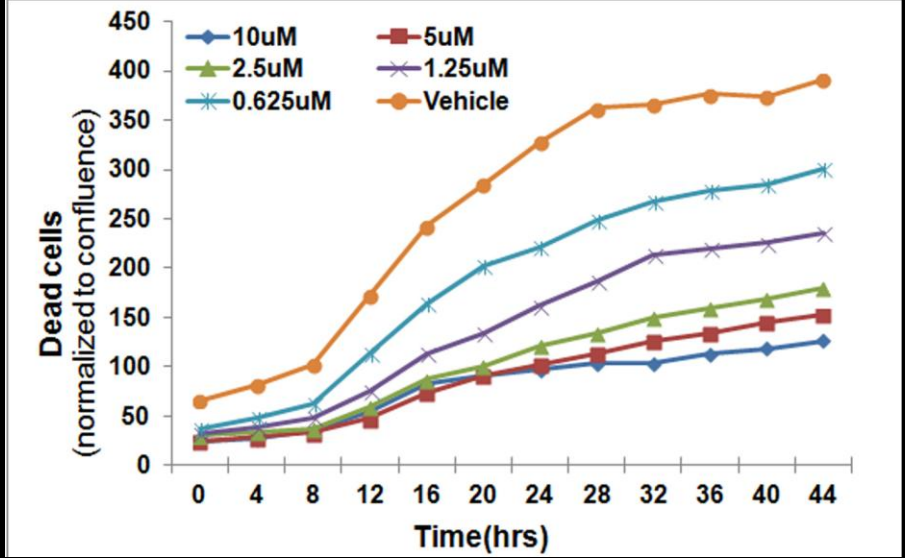
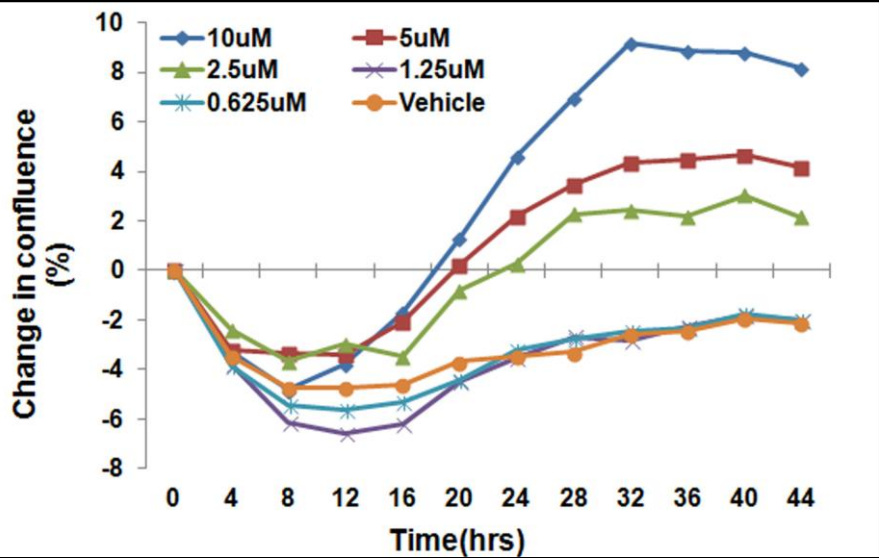


Figure 8.

<u>Rank</u>	<u>Mass</u>	<u>Compound Formula</u>	<u>Compound Name</u>	<u>p-val</u>
1	496.3	C24H50O7PN	LPC(16:0)	0.0001490
2	870.7	C50H96O8PN	PC(42:2)	0.0001551
3	734.5	C38H72O10PN	PS(32:1)	0.0001824
4	522.3	C26H52O7PN	LPC(18:1)	0.0002745
5	480.3	C23H46O7PN	LPE(18:1)	0.0003017
6	494.3	C24H48O7PN	LPC(16:1)	0.0003116
7	840.6	C48H90O8PN	PC(40:3)	0.0005223
8	818.6	C44H84O10PN	PS(38:1)	0.0005362
9	828.6	C47H90O8PN	PE(42:2)	0.0006506
10	798.6	C45H84O8PN	PE(40:3)	0.0006913
11	524.4	C26H54O7PN	LPC(18:0)	0.0007365
12	814.5	C47H76O8PN	PE(42:9)	0.0009465
13	664.5	C35H70O8PN	PE(30:0)	0.0010830
14	802.6	C44H84O9PN	ePS(38:2)	0.0013139
15	712.5	C39H70O8PN	PE(34:4)	0.0013176
16	788.6	C44H86O8PN	PC(36:1)	0.0013196
17	862.6	C48H80O10PN	PS(42:7)	0.0014234
18	948.5	C51H79O13P	PI(42:10)	0.0016986
19	822.6	C48H88O7PN	ePC(40:5)	0.0017553
20	850.6	C49H88O8PN	PE(44:5)	0.0017829

Table 1.

	Grouped	Isolated	Pvalue
Total 14:1	1.359026	1.661565	0.219989
Total 14:0	28.37567	33.98356	0.022288
Total 16:1	69.50874	94.73596	0.007754
Total 16:0	358.9951	486.9224	0.012314
Total 18:3	20.59789	22.04222	0.587854
Total 18:2	287.045	288.5631	0.960902
Total 18:1	403.9124	499.0571	0.020276
Total 18:0	110.649	142.2067	0.035092
Total 20:5	1.294511	1.724969	0.133205
Total 20:4	3.041031	3.052704	0.982378
Total 20:3	1.667471	1.457382	0.49942
Total 20:2	2.269884	2.123427	0.66278
Total 22:6	2.229097	2.415044	0.73185
Total 22:5	1.032384	1.066587	0.840775
Total 22:4	0.123311	0.10279	0.369624

Table 2.