IGF1 dependence of dietary energy balance effects on murine Met1 mammary tumor progression, epithelial-to-mesenchymal transition, and chemokine expression

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Abstract

Luminal breast tumors with little or no estrogen receptor α expression confer poor prognosis. Using the Met1 murine model of luminal breast cancer, we characterized the IGF1-dependency of diet-induced obesity (DIO) and calorie restriction (CR) effects on tumor growth, growth factor signaling, epithelial-to-mesenchymal transition (EMT), and chemokine expression. Liver-specific IGF1-deficient (LID) and littermate control (LC) mice were administered control, DIO, or 30% CR diets for 3 months before orthotopic injection of Met1 cells. Tumors grew for 1 month and then were assessed for Akt pathway activation and mRNA expression of chemokine and EMT constituents. LID mice, regardless of diet, displayed reduced Met1 tumor growth and downregulated Akt, EMT, and chemokine pathways. CR, relative to control, reduced serum IGF1 and Met1 tumor growth in LC (but not LID) mice. DIO, relative to control, increased Met1 tumor growth and chemokine expression in LID mice, and had no effect on serum IGF1 or pAkt or cyclin D1 expression in either genotype. Thus, circulating IGF1 (in association with Akt, EMT, and chemokines) regulated Met1 tumor growth. While the anticancer effects of CR were largely IGF1-dependent, the procancer effects of DIO manifested only when circulating IGF1 levels were low. Thus, in a murine model of luminal breast cancer, IGF1 and its downstream signaling pathway, EMT, and chemokines present possible mechanistic regulatory targets. Transplanted MMTV1 Wnt1 mammary tumor growth was also reduced in LID mice, relative to LC mice, suggesting that the IGF1 effects on mammary tumor growth are not limited to Met1 tumors.

Key Words

- energy balance
- epithelial-to-mesenchymal transition
- IGF1
- chemokine
- calorie restriction

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Introduction

The prevalence of obesity, an established risk and progression factor for estrogen receptor α (ER α)-positive luminal A breast cancer, has dramatically increased in the USA and many other parts of the world over the past 25 years (Bianchini *et al.* 2002, Wu *et al.* 2003). Little is known, however, about the effects of obesity or energy balance modulation on progression of luminal breast tumors with little or no ER α expression (referred to hereafter as ER α^{low}), which relative to luminal A tumors confer a worse prognosis, typically respond poorly to tamoxifen treatment, and display activation of growth factor signaling pathways (Loi *et al.* 2009). The identification of mechanistic targets and intervention strategies for preventing or controlling ER α^{low} luminal breast cancers are urgently needed due to the lack of targeted therapies for these aggressive breast cancers (Uray & Brown 2011).

Calorie restriction (CR) is a low calorie diet regimen that prevents obesity and decreases progression of mammary and many other tumor types in various animal models (Nunez *et al.* 2008, Hursting & Berger 2010, Hursting *et al.* 2010). The effects of dietary energy balance alterations, such as CR and diet-induced obesity (DIO), on ER α^{low} luminal breast cancer are poorly characterized, at least in part due to a paucity of relevant animal models of this intrinsic subtype of breast cancer. Comparative oncogenomic studies on human and mouse mammary tumors show that tumors from the polyoma middle-T antigen transgenic mice cluster closely with human luminal B breast tumors and share many of the pathological and molecular hallmarks of human luminal B tumors (Herschkowitz *et al.* 2007, Fluck & Schaffhausen 2009, Zhu *et al.* 2011). Furthermore, the Met1 cell line, derived from a spontaneous tumor from a polyoma middle-T antigen transgenic mouse, progressively loses ER and progesterone receptor positivity with tumor progression, therefore providing a rapid and relevant model for studying ER α^{low} luminal mammary tumor progression when orthotopically transplanted into syngeneic mice (Lin *et al.* 2003, Borowsky *et al.* 2005, Namba *et al.* 2006, Bonuccelli *et al.* 2009).

Findings from models of luminal A, basal-like, and Her2-positive rodent mammary tumor models (Nickerson *et al.* 1999, Wu *et al.* 2003, Dunlap *et al.* 2012), as well as from a broad spectrum of colon, prostate, pancreatic, skin, and other epithelial cancer models (Moore *et al.* 2008b, Olivo-Marston *et al.* 2009, Lashinger *et al.* 2011), indicate that circulating levels of insulin-like growth factor 1 (IGF1) are central to the dietary energy balance–cancer link.

Limited evidence suggests plausible relationships between serum IGF1 levels, tumor Akt signaling, tumor expression of epithelial-to-mesenchymal transition (EMT) and chemokine genes, and mammary tumor progression (Kim *et al.* 2007, Graham *et al.* 2008, Sivakumar *et al.* 2009, Lorenzatti *et al.* 2011, Walsh & Damjanovski 2011). Alterations in systemic IGF1 can modulate cellular signaling through receptor tyrosine kinases and induce changes in downstream proliferation and survival regulators such as Akt and the mammalian target of rapamycin (mTOR; Moore *et al.* 2008a). Akt and mTOR signaling pathway components are frequently altered in human cancers (Fresno Vara *et al.* 2004) and are upregulated by DIO and downregulated by CR in mammary and other tumors (Moore *et al.* 2008a, De Angel *et al.* 2012). Akt activation can induce EMT (Nath *et al.* 2008), while Akt inhibitors can suppress EMT (Hong *et al.* 2009). Characteristics of EMT, an orchestrated program in which cell–cell and cell–extracellular matrix interactions are altered to enhance tumor progression and initiate local invasion and metastasis (Thiery 2003), include the progressive loss of epithelial markers, such as E-cadherin, and gain of mesenchymal markers, such as snail, slug, vimentin, N-cadherin, twist, transforming growth factor B1 (Tgfb1), and matrix metalloproteinases (Mmps) (Rucklidge *et al.* 1994). Increased body adiposity (Calabro & Yeh 2007, Subbaramaiah *et al.* 2011) and tumoral Akt activation (Balkwill 1998, 2004, Roca *et al.* 2008) are also associated with increased tumoral chemokine expression. Chemokines, which are a subfamily of secreted cytokines from adipocytes, macrophages, and other cells that stimulate directed chemotaxis in nearby responsive cells, increase inflammation, tumor progression, and invasion in multiple tumor types (Balkwill 2004, Kulbe *et al.* 2004, Szlosarek & Balkwill 2004).

Modulation of dietary fat content has been demonstrated to be effective in regulating ER negative breast cancers (Chlebowski *et al.* 2006), and IGF1 inhibitors are efficacious against triple negative breast cancer cells (Litzenburger *et al.* 2011). Unfortunately, the effects of energy balance and/or IGF1 modulation on ER α^{low} luminal mammary tumor progression are poorly understood. The purpose of this study was to test the hypothesis that DIO enhances, and CR suppresses, ER α^{low} luminal mammary tumor progression, at least in part, through IGF1-dependent pathways. To test this hypothesis, we compared the effects of DIO and CR on progression of orthotopically transplanted Met1 tumors, systemic IGF1

(and other serum hormones), tumoral Akt activation, and EMT and chemokine gene expression in mice without genetic reduction of circulating IGF1.

Materials and methods

Mice

All procedures involving animals were approved and monitored by the National Cancer Institute Animal Care and Use Committee or the University of Texas Institutional Animal Care and Use Committee. All diets were purchased from Research Diets, Inc. (New Brunswick, NJ, USA). Liver-specific IGF1-deficient (loxP+/+ Cre+/-; LID) mice (Yakar *et al.* 1999), backcrossed to FVB/N mice for greater than ten generations, were originally obtained from Dr Derek LeRoith (NIDDK, Bethesda, MD, USA), and maintained in Dr Hursting's breeding colony at the NCI-Frederick National Laboratory (Frederick, MD, USA) or his colony at the University of Texas, Austin (for the Wnt1 study). For these studies, female LID mice and floxed IGF1 littermate control (loxP+/+ Cre-/-; LC) mice were obtained at 3 months of age and were placed on a control diet (modified AIN-76A, #D12450B) for 1 week following receipt. Mice were individually housed with free access to water and exposed to a 12 h light:12 h darkness cycle.

Diet treatments and Met1 tumor transplantation, monitoring, and sample collection

Female LID and LC mice were randomized ($n=10/\text{group}$) to receive: i) control diet fed *ad libitum* (#D12450B), ii) a 30% CR regimen (#D0302702), or iii) a DIO regimen fed *ad libitum* (#D12492). The CR diet was balanced so that all essential nutrients would be consumed at the control level when dietary intake was restricted by 30% relative to controls. Food intake was measured biweekly and body weights were recorded weekly. Mice consumed the experimental diets for 3 months before syngeneic orthotopic transplantation of 1×10^5 Met1 mammary tumor cells into the fourth mammary fat pad. The Met1 cell line (a gift from Dr William Mueller) was originally derived from mammary carcinomas in FVB/N-Tg (MMTV-*PyVmT*) mice (Borowsky *et al.* 2005), and was maintained in complete media (low glucose DMEM, supplemented with 10% fetal bovine serum, penicillin-streptomycin and glutamine) at 37 °C in a humidified 5% CO₂ incubator. In preparation for transplantation, cells were washed with PBS, trypsinized, and viable cells were quantified by trypan blue exclusion using a hemacytometer (Fisher Scientific, Waltham, MA, USA).

Mice continued to consume the experimental diets for another month after transplantation and before euthanasia. Tumors were measured in three dimensions using electronic calipers twice weekly by one technician, and tumor volume ($4/3\pi lwh$) was calculated. At the end of the treatment period, mice were fasted for 12 h and then anesthetized using isoflurane. Blood was collected by cardiac puncture and the mice were then killed by cervical dislocation. Blood was allowed to coagulate at room temperature for 30 min and then centrifuged at 10 000 *g* for 5 min to obtain serum samples that were stored at -80 °C until analyzed for hormones and adipokines. *Ex vivo* caliper measurements of mammary tumors were taken (by the same technician) before tumor tissue was snap frozen in liquid nitrogen and stored at -80 °C until analyzed for protein and/or gene expression.

Serum hormones and adipokines

Serum leptin, insulin, and adiponectin concentrations ($n=10/\text{group}$) from Met1 tumor bearing mice were measured using a Luminex-based LINCoplex bead array assay (Millipore, Billerica, MA, USA) on a Bio-Rad BioPlex multianalyte detection system (Bio-Rad, Inc.). Serum IGF1 ($n=10/\text{group}$) was measured using an ELISA (R&D Systems, Minneapolis, MN, USA) on a Synergy 2 plate reader (BioTek, Winooski, VT, USA).

Phosphorylated protein expression analyses

Met1 mammary tumor tissue was homogenized and lysed in a radioimmunoprecipitation assay buffer (Sigma-Aldrich) with a protease inhibitor cocktail tablet (Roche Applied Sciences) and phosphatase inhibitor cocktails I and II (Sigma-Aldrich). Expression of phosphorylated (p) Akt (Ser473), pErk (Thr185/Tyr187), and pStat3 (Tyr705) proteins was quantified on tumor lysates (20 µg) from a random sample of six mice per diet-genotype group, using Luminex-based Milliplex Map bead array assays (Millipore) on a Bio-Rad BioPlex multianalyte detection system following manufacturer's instructions.

Gene expression analyses

RNA was extracted from Met1 mammary tumor tissue from random samplings of each diet-genotype group ($n=6$ mice/sampling) using an RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. cDNA was synthesized from extracted RNA using the high-capacity cDNA RT Kit (Applied Biosystems) with the addition of an RNase

inhibitor (Ambion) as per manufacturer's directions. Gene expression for cyclin D1, a panel of EMT markers, and a panel of chemokines was measured by real-time quantitative reverse transcriptase PCR (qRT-PCR) using the TaqMan gene expression assay with TaqMan universal PCR mastermix (Applied Biosystems) on an EPgradient S Realplex 4 thermocycler (Eppendorf, Hauppauge, NY, USA). Gene expression is reported for LID mice relative to LC mice. In addition, an inflammatory gene superarray analysis was performed in duplicate for 176 inflammation-related genes on a random subsampling of extracted tumor RNA from three LID DIO mice and three LID control diet-fed mice, using an ABI 7900HT FAST qPCR thermocycler (Applied Biosystems). Gene expression is reported relative to LID control.

MMTV-Wnt1 tumor transplantation, monitoring, and sample collection

Female LID and LC mice (same as described above; $n=10$ /group) were obtained at 3 months of age from Dr Hursting's colony at the University of Texas, Austin. Mice were individually housed with free access to water and exposed to a 12 h light:12 h darkness cycle and allowed to acclimate for 1 week before syngeneic orthotopic transplantation of Wnt1 mammary tumor brei, as previously described (Nunez *et al.* 2008). In brief, a suspension of MMTV-Wnt1 mammary tumor cells was derived (Varticovski *et al.* 2007) from six spontaneously developed mammary carcinomas in MMTV-Wnt1 FVB/NJ (002934; Jackson Laboratory, Bar Harbor, ME, USA, <http://www.jax.org>). MMTV-Wnt1 FVB/NJ mice were euthanized with CO₂, and tumors were collected aseptically using blunt dissection, trimmed of extraneous tissues,

mechanically dissociated by mincing and passage through a 40-micron mesh sterile screen, and suspended in serum-free RPMI 1640 (Quality Biological, Gaithersburg, MD, USA). Cells were further dissociated by serial passage through a syringe with 18-gauge needles. The cell suspension was washed twice and resuspended in serum-free RPMI 1640 medium, and viable cell counts were determined by Hemocytometer counting, following 0.4% trypan blue staining. Cells were resuspended at 2×10^6 cells/ml in 10% DMSO cell-freezing medium and cryopreserved using stepped rate freezing. For orthotopic implantation, 1×10^5 cells were implanted s.c. in 50 μ l serum-free RPMI 1640 medium into the 4th mammary fat pad of recipient LID and LC mice fed control diet (modified AIN-76A, #D12450B). Mice continued to consume the control diet for another month after transplantation and before euthanasia. Tumors were measured in three dimensions using electronic calipers twice weekly by one technician, and tumor volume ($4/3\pi lwh$) was calculated. At the end of the 4-week period after tumor transplantation, mice were fasted for 12 h and then anesthetized using isoflurane. Blood was collected by cardiac puncture and the mice were then killed by cervical dislocation. Blood was allowed to coagulate at room temperature for 30 min and then centrifuged at 10 000 g for 5 min to obtain serum samples that were stored at -80°C until analyzed for hormones and adipokines. *Ex vivo* caliper measurements of mammary tumors were recorded.

Statistical analysis

Summarized data are expressed as means $\pm$ s.d., and analyses were performed using SAS 9.2 (Cary, NC, USA). Molecular analysis could not be performed on all LID

Table 1 Diet and genotype affect body weight and serum hormone levels.

Parameter	Littermate control			LID			P value
	DIO	Control	CR	DIO	Control	CR	Genotype
Final body weight (g)	31.2 $\pm$ 1.98 ^b	24.4 $\pm$ 0.44 ^a	17.6 $\pm$ 0.30 ^c	23.1 $\pm$ 0.87 ^b	20.6 $\pm$ 1.23 ^a	18.3 $\pm$ 0.37 ^c	<0.01
Energy intake (kcal)	90.7 $\pm$ 5.53 ^b	73.6 $\pm$ 2.33 ^a	51.5 $\pm$ 1.63 ^c	85.7 $\pm$ 2.40 ^a	79.2 $\pm$ 4.58 ^a	55.4 $\pm$ 3.20 ^b	0.61
IGF1 (pg/ml)	534 $\pm$ 74.1 ^a	507 $\pm$ 57.5 ^a	332 $\pm$ 22.8 ^b	106.2 $\pm$ 12.8 ^a	110 $\pm$ 42.3 ^a	71.7 $\pm$ 6.66 ^b	<0.01
Insulin (pg/ml)	870 $\pm$ 183 ^b	552 $\pm$ 21.5 ^a	398 $\pm$ 13.6 ^c	1750 $\pm$ 229 ^a	1880 $\pm$ 195 ^a	1610 $\pm$ 318 ^a	<0.01
Leptin (pg/ml)	2900 $\pm$ 961 ^b	846 $\pm$ 175 ^a	358 $\pm$ 89.4 ^c	4450 $\pm$ 1390 ^a	3630 $\pm$ 487 ^a	947 $\pm$ 88.8 ^b	<0.01
Adiponectin (μ g/ml)	57.0 $\pm$ 9.02 ^a	46.7 $\pm$ 5.79 ^a	53.1 $\pm$ 8.05 ^a	78.3 $\pm$ 10.1 ^b	130 $\pm$ 9.12 ^a	98.3 $\pm$ 11.1 ^b	<0.01
Leptin:adiponectin	0.06 $\pm$ 0.03 ^b	0.02 $\pm$ 0.01 ^a	0.01 $\pm$ 0.01 ^c	0.07 $\pm$ 0.05 ^b	0.03 $\pm$ 0.01 ^a	0.01 $\pm$ 0.01 ^c	0.25

Serum was collected at the conclusion of the study from all experimental groups. All serum parameters, excluding IGF1 (R&D Systems, Minneapolis, MN, USA), were analyzed by Millipore multiplex kits ($n=10$, plated in triplicate). Data represent mean $\pm$ s.d. values. ANOVA followed by Tukey's *post hoc* analysis was used to distinguish statistical differences and considered significantly different when $P<0.05$ as indicated by different letters. No significant interactions were detected.

tumors due to the size of the tumor at excision; therefore samples were randomly selected from all other groups for analysis. Met1 tumor growth was analyzed by repeated measures ANOVA. Body weight, feed intake, serum adipokines, tumor volume, protein expression, and mRNA gene expression were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. When significant interactions were detected, analysis of simple effects was completed. Differences in relative gene expression using the super array were considered biologically significant when a minimum of a twofold expression difference occurred, and $P \leq 0.05$ was achieved. *A priori* questions addressing the effects of diet on each genotype independently were analyzed by one-way ANOVA followed by Tukey's *post hoc* analysis. Final MMTV-Wnt1 tumor volume was assessed by Student's *t*-test. Differences were considered statistically significant at $P \leq 0.05$.

Results

Body weight, energy intake, and serum hormone levels from Met1 tumor bearing mice

Final body weights and serum hormone levels after 4 weeks of tumor growth (4 months of diet treatment) and average weekly energy intake throughout the study were analyzed for effects of genotype and/or diet (Table 1). LID mice weighed less than LC mice on DIO or control diet ($P < 0.01$), and LID and LC mice showed the same rank order in weight in response to the three dietary interventions ($\text{CR} < \text{control} < \text{DIO}$; $P < 0.01$ within each genotype). By design, total caloric intake also significantly varied by dietary group ($P < 0.01$) in the same rank order as weight, and genotype had no effect.

Circulating IGF1 levels were lower in LID mice relative to LC mice, regardless of diet ($P < 0.01$). In both LID and LC mice, CR reduced IGF1 levels ($P < 0.01$), but DIO had no significant effect, relative to control. CR and DIO were significantly different from each other in both LID and LC mice ($P < 0.01$ in both).

Serum concentrations of insulin, leptin, and adiponectin were elevated ($P < 0.01$ each) in LID mice as compared with LC mice (Table 1). Relative to control, CR decreased and DIO increased insulin and leptin levels in LC mice; in LID mice, CR decreased ($P < 0.01$), but DIO had no significant effect on leptin, and neither diet affected insulin levels. While no diet effect on adiponectin was observed in LC mice, CR and DIO (relative to control) both decreased adiponectin in LID mice ($P = 0.02$). Lastly, relative to control, CR reduced, and DIO increased, the

ratio of leptin to adiponectin in both genotypes ($P < 0.01$ for both).

Met1 mammary tumor volume

Met1 tumor volume at 3 weeks and study completion was significantly lower in LID mice relative to LC mice,

Figure 5

Effect of a 3 month dietary energy modulation regimen on Met1 murine mammary tumor growth in littermate control (LC) and LID mice. Injected tumor cells were allowed to grow for 4 weeks and tumors were measured biweekly by electronic calipers ($n = 10$). (A) Tumors were palpable and measured at 1 week for all groups except LID control and CR which first appeared at 2.5 weeks. Final *ex vivo* tumor volume was also calculated (B) Data are presented as means $\pm$ s.d. values. Growth was analyzed by repeated measures ANOVA and final tumor measurement was analyzed by two-way ANOVA followed by Tukey's *post hoc* analysis; statistically different when $P < 0.05$. Genotype, $P < 0.01$; diet, $P < 0.01$; interaction of diet and genotype, $P = 0.79$. To address whether diet modulated tumor growth in LID mice (*a priori* question), one-way ANOVA followed by Tukey's *post hoc* test was used; diet, $P < 0.01$. Significant dietary effects relative to the respective genotype control are indicated by an asterisk and the effect of the LID genotype relative to littermate control is indicated by a bar with asterisk.

irrespective of diet ($P < 0.01$; Fig. 1). Regarding diet effects, in LC mice, relative to control, CR significantly reduced ($P = 0.01$), but DIO had no effect, on final mammary tumor volume. In LID mice, DIO increased final tumor volume ($P < 0.01$), but CR had no significant effect, relative to control. After only 4 weeks of tumor growth, macroscopic Met1 metastases were undetectable in lung or liver tissue.

Cell signaling proteins in Met1 tumor tissue

Phosphorylated tumoral levels of Akt (Fig. 2A) and other signaling molecules in response to dietary energy balance interventions were compared between LID and

LC mice. Tumors from LID mice had significantly reduced expression of pAkt relative to tumors from LC mice ($P < 0.05$). No significant diet effect was found for either genotype (LC, $P = 0.68$; LID, $P = 0.14$). Expression of pErk(Thr185/Tyr187) and pSTAT3(Tyr705) in tumor tissue was not modulated by diet or genotype (data not shown).

Cyclin D1, EMT, and cytokine gene expression in Met1 tumor tissue

The mRNA expression of cyclin D1 and several EMT-related genes was significantly altered in LID mice as compared with LC mice. Specifically, relative mRNA expression of cyclin D1 (Fig. 2B), a key downstream target of pAkt, mirrored the relative pAkt protein expression (Fig. 2A) in terms of diet and genotype effects. For EMT genes (Fig. 3), relative to expression in LC tumors, the expression of *Mmp2*, which is responsible for matrix degradation, was downregulated in LID tumors, as were *Stat3*, *Cdh2*, *Fn1*, *Vim*, *Snai1* and *Twist1* (each $P < 0.01$). In contrast, the epithelial marker, E-cadherin was upregulated in LID mouse tumors ($P = 0.01$). There were no differences between LID and LC mice in mRNA expression of the EMT-related genes *Snai2*, *Zeb1*, *Foxc2*, or *Pou5f1* (data not shown).

The dietary regimens significantly impacted the expression of EMT markers in mammary tumor tissue from LC, but not LID mice, with the exception of E-cadherin (Fig. 3). In LC mice (but not LID mice), and relative to control, DIO upregulated the expression of *Mmp9*, *twist*, *Tgfb1*, and vimentin ($P < 0.03$ for each). The expression of the epithelial marker, E-cadherin, was downregulated by DIO, compared with control, in both LC and LID mice (each $P < 0.01$). In LID mice (but not LC mice), and relative to control, CR upregulated E-cadherin expression ($P < 0.01$). CR had no significant effects on other EMT genes in either LC or LID mice. Neither DIO nor CR altered the mRNA expression of the EMT-related genes *Snai1* (Fig. 3), *Cdh2* (Fig. 3), *Snai2*, *Zeb1*, *Foxc2*, or *Pou5f1* (data not shown) in LC or LID mice.

In mammary tissue from LID mice, inflammatory marker mRNA superarray analysis identified 17 significantly upregulated genes in response to DIO relative to control diet (Table 2). All changes in gene expression were confirmed by qRT-PCR. The majority (10/17) of the modulated genes was chemokine ligands ($n = 9$) or receptor ($n = 1$), and the expression of these ten genes was further evaluated in tumors from LC and LID mice in response to the dietary regimens (Fig. 4). The expression of

Figure 2

Genetic reduction in IGF1 (LID) significantly reduced activation of AKT (A) and mRNA expression of downstream target, cyclin D1 (B), relative to littermate control mice. Protein expression was measured by Luminex-based LINCplex bead array assay and mRNA expression ($n = 6$) was measured by qRT-PCR using TaqMan primer/probe system. Differences in sample size were dictated by tissue availability at the time assay was conducted. Bars represent means $\pm$ s.d. values. Phosphorylated AKT was normalized to total AKT expression. Significant differences determined by two-way ANOVA followed by Tukey's *post hoc* analysis are indicated by a bar and asterisk; $P < 0.05$ considered statistically different.

Figure 3

mRNA expression of epithelial-to-mesenchymal transition (EMT) genes. Relative expression was measured by qRT-PCR using the TaqMan primer/probe system. Gene expression is standardized to β -actin and results are normalized to the expression levels of littermate control mice that consumed control diets. Bars represent mean $\pm$ s.d. values ($n=6$ /group).

Data are analyzed by two-way ANOVA followed by Tukey's *post hoc* analysis and considered statistically different when $P < 0.05$. Significant dietary effects relative to the respective genotype control are indicated by an asterisk and the effect of the LID genotype relative to littermate control is indicated by a bar with asterisk.

Ccl1, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl6*, *Ccl17*, *Cxcl2*, *Cxcl9*, and *Ccr5*, but not *Cxcl5*, was significantly reduced in tumors from LID mice relative to LC mice ($P < 0.03$ for each). In LID mice, DIO, compared with control, increased the tumoral

expression of each of these genes, and for several of them, to levels near that in LC mice. Tumoral chemokine expression was not significantly affected by DIO or CR in LC mice, or by CR in LID mice.

Table 2 RNA superarray analysis identified upregulation of 17 genes in response to DIO relative to control fed LID mice ($n=3/\text{group}$).

Gene	Fold change	P value
<i>CCL1</i>	9.9	<0.01
<i>CCL3</i>	2.5	0.04
<i>CCL4</i>	2.4	0.04
<i>CCL5</i>	3.0	<0.01
<i>CCL6</i>	2.4	0.02
<i>CCL17</i>	2.1	0.04
<i>CXCL2</i>	2.8	<0.01
<i>CXCL5</i>	2.0	<0.01
<i>CXCL9</i>	3.7	<0.01
<i>CCR5</i>	2.2	<0.01
<i>Itgb2</i>	2.0	<0.01
<i>Ltb</i>	2.7	0.02
<i>Il2rb</i>	2.3	0.04
<i>Il2rg</i>	2.1	<0.01
<i>Vegfa</i>	2.6	0.02
<i>Cd40lg</i>	4.4	0.04
<i>Bmp7</i>	2.3	0.02

MMTV-Wnt1 mammary tumor

The effects of genetically reduced IGF1 levels were also assessed in a model of basal-like ER α low breast cancer. As observed in the Met1 study, in LID mice relative to LC mice serum IGF1 was 70% lower, while insulin and leptin levels were significantly elevated (data not shown). After 4 weeks of tumor growth, Wnt1 tumors in LID mice on control diet were 57% smaller than tumors in LC mice on control diet ($P<0.01$; Fig. 5). By comparison, Met1 tumors were 95% smaller in LID mice on control diet relative to LC mice on control diet ($P<0.01$; Fig. 1).

Discussion

In this study, we characterized the effects of dietary energy balance interventions (CR and DIO) on Met1 mammary tumor growth, growth factor signaling, and expression of EMT and chemokine genes in LID and LC mice. We found that i) IGF1 is a critical component modulating Met1 tumor growth, Akt activation, EMT, and chemokine gene expression, regardless of diet; ii) in conditions of low circulating IGF1, the effects of CR on tumor growth are repressed; and iii) DIO increases tumor growth only when circulating IGF1 levels are low and potentially by elevating the expression of chemokines. Our results implicate components of the IGF1/Akt, EMT, and chemokine pathways as primary targets for reducing ER α ^{low} luminal mammary tumor growth. In addition, the impact of reduced IGF1 levels on tumor growth was similar in

transplanted MMTV-Wnt1 mammary tumors (a model of ER α low basal-like breast cancer), suggesting these findings may have relevance beyond the Met1 mammary tumor model.

Circulating IGF1 levels affect tumor growth in a variety of animal models. Reduced circulating levels of IGF1 in LID mice, relative to LC mice, suppress development of carcinogen-induced skin and colon tumors (Moore *et al.* 2008b, Olivo-Marston *et al.* 2009) and growth of transplanted colon and pancreatic adenocarcinomas (Wu *et al.* 2002, Lashinger *et al.* 2011). Reduced circulating levels of IGF1 also decrease development of mammary tumors induced either by exposure to the carcinogen 7,12-dimethylbenz (a)anthracene (DMBA) or by crossing LID mice with C3(1)/SV40 large T-antigen transgenic mice, a model of basal-like triple-negative breast cancer (Wu *et al.* 2003, Herschkowitz *et al.* 2007, Zhu *et al.* 2011). Here we further demonstrate that reduced circulating levels of IGF1 also decrease tumor growth in orthotopically transplanted ER α ^{low} Met1 luminal-type mammary tumors and Wnt1 basal-like mammary tumors. The current study is the first, to our knowledge, to show that reduction in circulating IGF1 levels (either genetically or by CR), despite elevated circulating levels of insulin, decreases ER α ^{low} luminal mammary tumor growth. We also demonstrate in the Met1 ER α ^{low} luminal B breast cancer model that, despite enhanced serum insulin levels, reduced circulating levels of IGF1 decrease Akt signaling (pAkt and cyclin D1) and EMT and chemokine gene expression, all of which regulate tumor growth, progression, and/or metastatic potential in other cancer models (Ahmad *et al.* 1999, Mira *et al.* 1999, 2001, Akekawatchai *et al.* 2005, Singh *et al.* 2009, Wu *et al.* 2010, Durfort *et al.* 2012).

Pharmacological inhibitors of the IGF1 pathway effectively diminish breast tumor growth in several preclinical models of breast cancer (Maloney *et al.* 2003, Haluska *et al.* 2006, Gualberto & Pollak 2009, Sabbatini *et al.* 2009, Sun *et al.* 2011). Unfortunately, clinical application has been limited by safety concerns (Gualberto & Pollak 2009) and efficacy due to high phosphorylation of IGF1/insulin receptors in all breast cancer subtypes (Law *et al.* 2008). Consequently, identifying effective targeted or complimentary interventions with fewer side effects could prove valuable. Our findings suggest that CR (in LC mice) partially mimics liver IGF1 deficiency on Met1 tumor growth, systemic IGF1 levels, tumoral Akt pathway signaling, and EMT and chemokine gene expression. In addition, the inhibitory effects of CR on tumor growth are repressed in LID mice, which provide further evidence that the anticancer effects of CR (at least

Figure 4

mRNA expression of chemokine ligands is reduced in Met-1fbv2 tumor tissue of LID mice but expression levels are rescued by the DIO regimen as measured by qRT-PCR using the TaqMan primer/probe system. Bars represent mean $\pm$ s.d. values ($n=6$ /group). Data were analyzed by two-way

ANOVA followed by Tukey's *post hoc* analysis. $P < 0.05$ considered statistically significant. Differences by diet (relative to control in respective genotype) indicated by an asterisk and differences by genotype denoted by a bar with an asterisk.

Figure 5

Effect of circulating IGF1 on Wnt1 murine mammary tumor growth in littermate control and liver-specific IGF1 deficient (LID) mice. Transplanted tumors were allowed to grow for 4 weeks and measured biweekly by electronic calipers ($n=10$). Data are presented as means $\pm$ s.d. values. Growth was analyzed by Student's *t*-test; statistically different when $P<0.05$ (*).

in this model) are largely IGF1 pathway-dependent. Thus, lifestyle interventions such as low-calorie diets, either alone or in combination with low-dose pharmacological approaches, may represent safe and effective strategies for controlling luminal B breast cancer, including suppressing ER α^{low} mammary tumor progression.

Our results further indicate that factors in addition to components of the classical IGF1 signaling pathway may also represent targets for controlling luminal B tumor progression. We found that tumors from LID mice, relative to LC mice, have enhanced EMT (i.e. decreased *MMP2*, *Stat3*, *twist*, *snail*, *fibronectin*, *vimentin*, and *N-cadherin*, and increased *E-cadherin*, gene expression), and increased expression of several chemokines (i.e. *Ccl1*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl6*, *Ccl17*, *Cxcl*, *Cxcl9*, and *Ccr5*), in addition to decreased expression of phosphorylated Akt and its downstream target cyclin D1. Limited evidence suggests that activation of the IGF1 pathway promotes invasiveness through upregulation of EMT in MCF7 breast cancer cells, MCF10A immortalized mammary epithelial cells, and ARCaP_M prostate cancer cells and that, in contrast, reduced activation of the IGF1 pathway attenuates invasion and EMT of MDA-MB231 breast cancer cells (Kim *et al.* 2007, Graham *et al.* 2008, Lorenzatti *et al.* 2011, Walsh & Damjanovski 2011). While elevated IGF1 has been shown to increase the expression of inflammatory cytokines such as *Il1 β* , *Il6*, and *Tnf α* (Wu *et al.* 2010, modulation of chemokine expression (other than *Ccl2*–*Ccl5* in muscle tissue, Pelosi *et al.* (2007)) by IGF1 in

mammary tumors has not, to our knowledge, previously been demonstrated.

Our finding that Met1 tumor growth is insensitive to DIO, relative to control diet, in LC mice is consistent with previous findings in MMTV-neu transgenic mice (a model of an ER $-$, Her2-overexpressing luminal breast cancer), in which spontaneous tumor development and serum IGF1 levels were not altered by a high fat, DIO diet (Cleary *et al.* 2004). These findings in two different luminal models of breast cancer are in contrast to previous reports in mouse or rat models of other mammary tumor subtypes (or with undefined subtypes) that show DIO enhances mammary tumor growth (Dogan *et al.* 2007, Nunez *et al.* 2008, Gu *et al.* 2011, Kim *et al.* 2011, Dunlap *et al.* 2012). For example, MMTV-Wnt1 mammary tumors display a molecular profile similar to many mouse and human basal-like mammary tumors, and their growth is highly responsive to changes in circulating IGF1 and is enhanced in DIO mice, and reduced in CR mice, relative to controls (Nunez *et al.* 2008, Dunlap *et al.* 2012). In that model, MMTV-Wnt1 tumor cells were injected into syngeneic C57BL/6 mice, which generally gain more weight and have higher IGF1 levels on the DIO diet than FVB mice, the background strain of the Met1 cells, and MMTV-Wnt1 brei used in the present experiments, as well as the MMTV-neu mice used by Cleary *et al.* (2004). Thus, additional studies are needed to fully discern whether the lack of effect of DIO on Met1 tumor growth relates to the biology of luminal-type vs basal-like tumors, or to the muted response of the FVB host mice to DIO. We observed a modest enhancing effect of DIO (associated with increased tumoral expression of several chemokine genes) on Met1 tumor growth, in association with increased chemokine gene expression, when IGF1 levels were genetically reduced. To our knowledge, no previous studies have suggested an interrelationship between DIO, IGF1, and chemokine gene expression in mammary cancer.

Two recent *in vitro* studies using sera from *ob/ob* mice and media from adipocyte cultures (Kushiro & Nunez 2011, Kushiro *et al.* 2011), as well as a recent report of an *in vivo* study using MMTV-Wnt1 cells (Dunlap *et al.* 2012), suggest that increased adiposity promotes EMT. This is consistent with our observation that several EMT markers are increased in tumors from LC mice in response to DIO, including *Mmp9*, *Twist*, *vimentin*, and *Tgfb*, while *E-cadherin* is decreased. Elevated markers of EMT are associated with enhanced tumor aggressiveness (Rucklidge *et al.* 1994, Thiery 2003). For example, upregulation of *Mmp9* expression in primary mammary epithelial cells results in elevated tumor proliferation rates, morphological

changes, and tissue architecture remodeling (Lee *et al.* 2000). Additionally, increased proliferation of human breast cancer cells (MDA231) is associated with increased expression of Tgf β (Tobin *et al.* 2002), while increased angiogenesis in MCF7 mammary tumor cells is associated with increased expression of Twist (Mironchik *et al.* 2005). A limitation of this study is the lack of macroscopic metastasis to lung or liver tissue which may be attributed to the short duration of tumor growth (4 weeks). Future studies are needed to further establish the links between energy balance, IGF1, and EMT in ER α ^{low} luminal mammary tumor progression and metastasis. Because tumors from LID mice display increased E-cadherin expression and decreased expression of several other EMT-related genes regardless of diet, a novel association between IGF1 and EMT in a model of ER α ^{low} luminal B breast cancer is suggested that warrants further investigation.

In conclusion, in a murine model of ER α ^{low} luminal B breast cancer, circulating IGF1, in association with tumoral Akt, EMT, and chemokine signaling, regulates tumor growth. Also while the anticancer effects of CR, at least in part, are IGF1-dependent, the procancer effects of DIO associated with enhanced chemokine expression manifest only when circulating IGF1 levels are low. Thus, components of the IGF1/Akt, EMT, and chemokine pathways represent possible mechanistic targets for inhibiting ER α ^{low} luminal breast cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N A Ford, N P Nunez, and S D Hursting contributed to study design and interpretation of data, and wrote the manuscript. N A Ford also conducted the wet lab analyses for the Met1 study. V B Holcomb conducted the MMTV-Wnt1 study.

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