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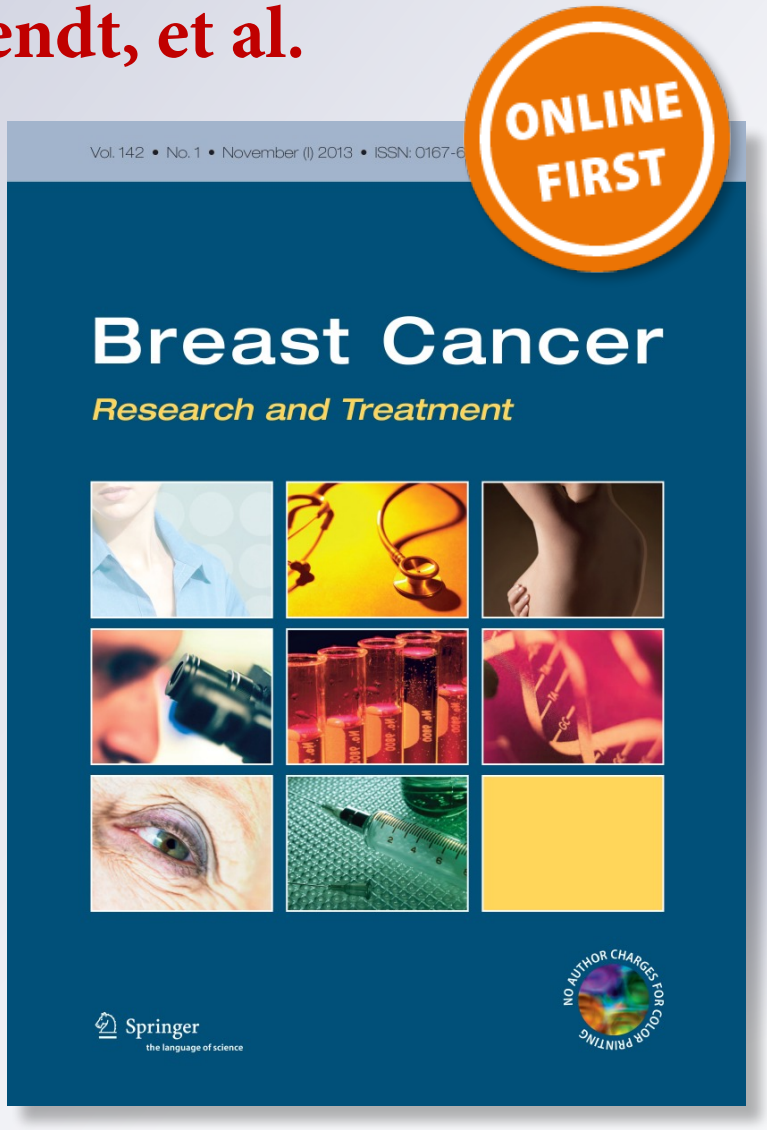

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PRECLINICAL STUDY

Role of HGF in obesity-associated tumorigenesis: C3(1)-T_{Ag} mice as a model for human basal-like breast cancer

Sneha Sundaram · Alex J. Freemerman · Amy R. Johnson · J. Justin Milner · Kirk K. McNaughton · Joseph A. Galanko · Katharine M. Bendt · David B. Darr · Charles M. Perou · Melissa A. Troester · Liza Makowski

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Abstract Obesity is associated with basal-like breast cancer (BBC), an aggressive breast cancer subtype. The objective of this study was to determine whether obesity promotes BBC onset in adulthood and to evaluate the role of stromal–epithelial interactions in obesity-associated tumorigenesis. We hypothesized that hepatocyte growth factor (HGF) plays a promoting role in BBC, which express the HGF receptor, c-Met. In C3(1)-T_{Ag} mice, a murine model of BBC, we demonstrated that obesity leads to a significant increase in HGF secretion and an associated decrease in tumor latency. By immunohistochemical analysis, normal mammary gland exhibited obesity-induced HGF, c-Met and phospho-c-Met, indicating that the activation of the cascade was obesity-driven. HGF secretion was also increased from primary mammary fibroblasts isolated from normal mammary glands and tumors of obese mice compared to lean. These results demonstrate that obesity-induced elevation of HGF expression is a stable phenotype, maintained after several passages, and after removal of dietary stimulation. Conditioned media from primary tumor fibroblasts from obese mice drove tumor cell proliferation. In co-culture, neutralization of secreted HGF blunted tumor cell migration, further linking obesity-mediated HGF-dependent effects to in vitro measures of tumor aggressiveness. In sum, these results demonstrate that HGF/c-Met plays an important role in obesity-associated carcinogenesis. Understanding the effects of obesity

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on risk and progression is important given that epidemiologic studies imply a portion of BBC could be eliminated by reducing obesity.

Keywords Basal-like breast cancer · Tumor latency · Microenvironment · Normal mammary gland · High fat diet-induced obesity · Fibroblast

Abbreviations

BBC	Basal-like breast cancer
HGF	Hepatocyte growth factor
BMI	Body mass index
ER	Estrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor-2
NAF	Normal-associated fibroblasts
CAF	Cancer-associated fibroblasts

Introduction

Obesity is associated with an increased risk of postmenopausal breast cancer in several population-based studies, but links between obesity and premenopausal breast cancer have been controversial [1, 2]. The Breast Cancer Prevention Trial suggested an elevated risk of premenopausal breast cancer among obese women, in contrast to several previous studies suggesting a protective effect of obesity in this group [3, 4]. The Breast Cancer Association Consortium reported that the triple-negative tumors are specifically associated with obesity in younger women [5, 6], which is in agreement with several smaller studies that have also linked obesity or high waist-to-hip ratio with triple-negative or basal-like breast cancer (BBC) [1, 7–9]. These studies emphasize that considering tumor subtype is essential when reporting obesity-related breast cancer risk, and that, taken together, strong associations with obesity and BBC exist.

Basal-like breast cancer is a triple-negative subtype characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and type-2 human epidermal growth factor receptor (HER2), and as such, BBCs are the only breast cancer subtype without a targeted therapy [10]. BBC disproportionately affects young (<50 years old) and African-American women [11, 12]. Given that African-Americans experience higher rates of pubertal and adult obesity [13], and retain more weight after pregnancy [14], it is important to better understand the biological mechanisms by which obesity, a modifiable risk factor, increases the risk of BBC.

Adding to the complexity of the relationship between obesity and differing breast cancer subtypes, the effects of obesity on the breast may depend upon the timing of exposure. Animal studies suggest that early life obesity is a risk factor for breast cancer, showing that high fat diet-induced obesity alters puberty onset, mammary gland development and morphology [15, 16], as well as decreased tumor latency [17]. However, there has been limited study on the effects of obesity on mammary carcinogenesis later in life. Of particular relevance to human cancer, the effect of diet-induced obesity during adulthood has not been evaluated. Adult obesity is an epidemic in the US and is increasing worldwide [18], hence understanding the role of obesity exposure during adulthood is a question with high public health impact.

Stromal–epithelial interactions are known to modulate breast cancer [19]. Stromal remodeling is important for understanding BBC specifically, because this subtype has unique interactions with surrounding stroma. Although changes to the stroma are less dramatic once maturity is reached, recent data have suggested that effects of obesity on mammary stroma are striking [20–22]. Co-culture of BBC epithelial cells with fibroblasts upregulates a wide range of cytokines, immune response pathways, and hepatic fibrosis-associated genes [23]. This is important because stroma-derived hepatocyte growth factor (HGF) can interact with the c-Met receptor, an important pathway in basal-like carcinogenesis examined herein [24, 25]. HGF levels are frequently elevated in obese patients [26] and reduced in patients with weight loss [27]. Elevations of plasma HGF are correlated with breast cancer, specifically ER-negative [28–30]. We hypothesized that, similar to results during puberty, obesity would lead to mammary tumorigenesis in adulthood, potentially through HGF/c-Met-mediated signaling. We selected a preclinical model focusing on the BBC subtype, since obesity is an important risk factor for triple-negative disease [11, 12, 31]. Our data demonstrate that in C3(1)-T_{Ag} mice associations between obesity and tumorigenesis mirrored human epidemiologic findings. Furthermore, our results indicate that HGF may play a role in mediating the onset of obesity-associated BBC and suggest that HGF/c-Met signaling is a plausible molecular target for prevention of obesity-associated breast cancer.

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Methods

Reagents and antibodies

Details are provided in the supplementary materials.

C3(1)-T_{Ag} mouse model

Animals

C3(1)-T_{Ag} mice (described previously [32]) were used to study the role of obesity on BBC, as these mice were shown to be highly representative of human BBC [33]. Studies were performed with approval and in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill (UNC-CH, NC). Female C3(1)-T_{Ag} mice were obtained through a collaboration with the UNC Lineberger Comprehensive Cancer Center (LCCC) Mouse Phase I Unit (MP1U). Nulliparous female C3(1)-T_{Ag} mice were randomly assigned to various diet groups at adulthood, 10 weeks of age, after the end of puberty (Model of study design, Supplementary (Suppl). Fig. 1). Diet details are provided in the supplementary materials and Suppl. Table 1.

Tumor latency, number, growth, and volume

Following initiation of diets at 10 weeks of age, mice were monitored for tumor development by palpating thrice weekly. Tumor latency was defined as the age at detection of the first tumor. After detection of the first tumor, tumor volumes were measured weekly over 3 weeks using ultrasound measurements with the Visualsonics 2000 (Toronto, Canada). Details are provided in the supplementary material.

Body weight and composition

Prior to starting mice on diet and weekly until sacrifice, body weight was measured. Body composition including lean mass, fat mass, free water content, and total water content of non-anesthetized mice was also measured at 0, 4, and 8 weeks on diet using the EchoMRI-100 quantitative magnetic resonance whole body composition analyzer (Echo Medical Systems, Houston, TX). Fat mass is presented as percent fat mass over total body weight. There were no significant declines in absolute lean mass in grams (data not shown).

Blood glucose

Blood glucose was measured prior to start of diet and at sacrifice following a 6 h fast using a Bayer Contour Blood Glucose Monitor (Bayer HealthCare LLC, Tarrytown, NY).

Tissue harvest

Details are provided in the supplementary materials.

Plasma and tissue cytokines panel

Details are provided in the supplementary materials.

Quantitative PCR (qPCR)

Total RNA from normal mammary gland and tumor was isolated as in Sampey et al. [34]. Real-time qPCR for HGF, c-Met, and aromatase (Cyp19a1) was performed [34].

Immunohistochemistry (IHC)/immunofluorescence (IF) of HGF, c-Met, pc-Met, α-SMA, and/or SV40-T_{Ag} in normal mammary glands and tumors

Details of IHC and IF staining and methods for scanning slides and analysis are provided in the supplementary materials.

Co-culture studies of stromal–epithelial interactions

Animals

Details are provided in the supplementary materials.

Fibroblast isolation

Normal-associated fibroblasts ("NAF") were isolated from normal inguinal mammary glands with no evidence of cancer, while cancer-associated fibroblasts ("CAF") were isolated from tumors from abdominal or inguinal mammary glands with tumors using methods from Fleming et al. [35]. Details are provided in the supplementary materials.

Co-culture

All culture experiments were completed in triplicate using NAFs and CAFs from mouse diet groups A, B, and C. 4T1 BBC-like (CRL-2539) cell line was obtained from ATCC (Manassas, VA). Details of the co-culture studies are provided in the supplementary materials. The media from these studies was centrifuged at 1,620×*g* and supernatant was assayed for HGF concentrations by ELISA (Abcam, Cambridge, MA) using a Bio-Rad Model 680 Microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). Western immunoblot analysis in 4T1 cells treated as described in supplementary materials for phospho- and total c-Met expression was performed as previously described [36].

Cell proliferation and wound migration assay

Details of the proliferation study are provided in the supplementary materials. Wound migration assay was performed as described previously by Camp et al. [23]. Percent wound closure was calculated by measuring the width of scratch at 0 h (time point of scratch) and at 12 h (end point of study) using NIH ImageJ 1.46 software and the following formula: (0 h width − 12 h width × 100)/0 h width.

Statistical analysis

Data are expressed as mean ± standard error of the mean. All analysis of variance (ANOVA) with Tukey's post hoc test for statistical differences were performed using SAS version 9.2 (SAS Institute, Cary, NC) or SPSS (version 20) software (IBM SPSS Statistic 20.0, Armonk, NY). P values < 0.05 were considered statistically significant.

Results

C3(1)-T_{Ag} basal-like tumor latency is decreased by obesity: an association with HGF

Mice were fed control or obesogenic diets in adulthood and tumor and metabolic parameters were evaluated as indicated in Suppl. Figure 1. Mice made obese by exposure to obesogenic diets (45 and 60 %) exhibited significant decreases in tumor latency compared with lean controls (10 %). The hazard ratios comparing either 45 or 60 to 10 % were 2.41 or 2.87, respectively (Fig. 1a). Median times to onset of 10, 45, and 60 %-fed mice were 19.14, 17.29, and 16.57 weeks, respectively. Obese mice displayed significantly earlier median latencies (10 vs. 45 % $P = 0.0236$ and 10 vs. 60 % $P = 0.0091$) but there was no significant difference between 45 and 60 %-fed median latencies (Fig. 1a). Using a χ^2 test with degrees of freedom of 2, 10 vs. 60 % equaled 6.801, 10 vs. 45 % equaled 5.121, and 45 vs. 60 % was 0.193. Tumor burden (Fig. 1b) or progression (Fig. 1c) were not significantly altered. We next confirmed that expression of the transgene-driving tumorigenesis in C3(1)-T_{Ag} mice was not responsible for obesity-mediated alterations in latency. Indeed, SV40 T_{Ag} staining exhibited no significant obesity-induced elevations in normal mammary gland (Suppl. Fig. 2a, b) or tumors (Suppl. Fig. 2c, d). The lack of change in tumor burden in the C3(1)-T_{Ag} mice suggested an effect of obesity on early tumor etiology, rather than on tumor progression.

Obesity strongly regulated HGF/c-Met signaling in normal mammary gland

Hepatocyte growth factor is a potential mediator of obesity-induced alterations in breast tumor onset, since it is regulated by obesity [2, 24–26] and could alter tumor etiology. The association between obesity, HGF, and tumorigenesis has not been investigated to date. Hence, we examined HGF/c-Met signaling in both normal mammary gland and tumors. Obesity significantly elevated HGF

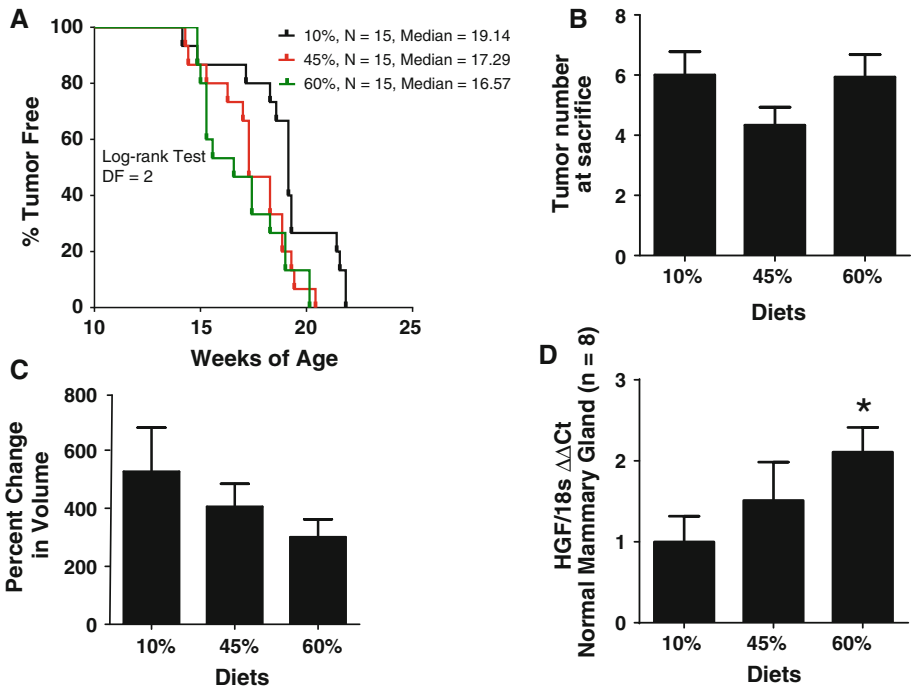


Fig. 1 Obesity-mediated decrease in latency correlates with elevated HGF expression. **a** Upon initiation of 10, 45 and 60 % kcal derived from fat diets at 10 weeks of age, mice were palpated three times weekly for tumor onset. Hazard ratios comparing either 45 or 60 % to 10 % were 2.41 or 2.87, respectively. Median latencies are indicated (10 vs. 45 % $P = 0.0236$ and 10 vs. 60 % $P = 0.0091$). **b** Number of tumors was detected at sacrifice ($n = 15$ mice). **c** Tumor volume was measured by ultrasound ($n = 15$ mice). **d** HGF mRNA expression was determined using qPCR analysis in $n = 8$ normal mammary glands. *10 versus 60 % ($P = 0.025$)

mRNA levels in the normal mammary gland (Fig. 1d, 10 vs. 60 %-fed, $P = 0.025$).

To examine protein expression and localization in tissues, IHC was undertaken. HGF expression was primarily localized to stroma with additional staining in epithelium in normal mammary glands (Fig. 2a). Quantification demonstrated that HGF protein concentrations measured in the stroma were significantly increased in obese 60 %-fed mice compared with 10 %-fed lean controls (Fig. 2b; $P = 0.011$). HGF mediates its effects via its cognate receptor c-Met, a proto-oncogenic tyrosine kinase receptor involved in BBC [24, 25, 37]. c-Met mRNA expression did not exhibit significant obesity-induced differences (Fig. 2c). Since c-Met mRNA is degraded rapidly [38], we quantified protein concentrations through IHC. Representative photomicrographs of cell membrane c-Met expression in normal mammary gland demonstrated primarily epithelial localization (Fig. 2d). Quantification revealed a strong obesity-induced upregulation of c-Met protein expression (Fig. 2e). c-Met protein was significantly increased in obese 45 % ($P = 0.04$) and 60 % ($P = 0.001$)-fed mice compared with 10 %-fed controls, and between obese 60 % ($P = 0.025$)-fed mice compared with 45 %-fed mice. To examine activation of the HGF/c-Met cascade, phospho-c-Met (pc-Met) expression was quantified. Representative photomicrographs of normal mammary gland demonstrated both membrane and cytoplasmic (internalized c-Met [39, 40]) staining (Fig. 2f). Obesity upregulated expression of pc-Met (Fig. 2g) and significant increases were observed in 45 % ($P = 0.011$) and 60 % ($P = 0.0001$) compared with 10 %-fed controls, and between obese 60 %-fed ($P = 0.0125$) mice and 45 %-fed mice. Tumors were next evaluated for obesity-mediated alterations in HGF/c-Met signaling. In tumors, HGF protein concentrations were not significantly regulated by obesity (Fig. 3a, b). Similarly, neither c-Met mRNA expression nor protein expression was significantly regulated by diet, although obesity-induced trends existed (Fig. 3c–e). Western immunoblotting of tumors demonstrated variable c-Met protein expression, with no significant differences detected between groups (Fig. 3f). In addition, no significant differences were observed in the levels of pc-Met in the tumors (Fig. 3g, h). Thus, in normal mammary glands obesity positively regulated HGF and c-Met expression and signaling in obese mice compared to lean. However, significant obesity-mediated HGF/c-Met regulation in tumors was not evident.

Analysis of metabolic parameters revealed that mice fed 45 and 60 % diets were significantly heavier compared to mice on 10 % diet (Fig. 4a, $P < 0.05$). Significant differences in weight could not be detected after week 12 on diet because the sample size was reduced as mice were sacrificed after tumor formation. Obese mice had greater fat mass compared to mice fed 10 % at 4 weeks on diet (Fig. 4b; 45 % $P = 0.009$ and 60 % $P = 0.007$ vs. 10 %), and at 8 weeks (45 % $P = 0.0001$ and 60 % $P = 0.0002$ vs. 10 %). Leptin is a marker of adiposity and increases with fat accretion [2, 41]. Mice fed 45 % ($P = 0.005$) and 60 % ($P = 0.003$) diets exhibited elevated leptin concentrations measured at sacrifice compared to mice on 10 % diet (Fig. 4c). Of note, decreased leptin concentrations of obese mice fed 60 % diets compared to 45 %-fed mice may be due to the disease status of the mice as these levels were measured at sacrifice after tumor onset, when tumor-induced cachexia may occur [42–44].

Alterations in tumor onset are likely not due to the changes in metabolic parameters since glucose, insulin, or $HOMA_{IR}$ did not vary by obesity (Fig. 4d–f). Inflammatory proteins were measured in plasma, normal mammary, and tumors. Obese mice fed 45 % ($P = 0.023$) and 60 % ($P = 0.048$) diets exhibited an increase in plasma TNF-α concentrations compared to mice on 10 % diet (Fig. 5a). However, no significant differences were observed in TNF-α concentrations in normal mammary glands or tumors. Similarly, plasma and tissue concentrations of IL-6 and MCP-1 were not significantly modified by obesity (Fig. 5b, c). In addition, plasma concentrations of hormones including estrogen and progesterone were not altered by obesity (Fig. 5d, e). Finally, aromatase (Cyp19a1) expression was not detectable by qPCR in any of the diet groups in normal mammary gland or in tumors (data not shown). Epididymal adipose tissue was used as a positive control: obese adipose expressed high levels of aromatase compared to lean controls (data not shown).

Obesity and tumor-associated fibroblast-derived HGF drove basal-like cell proliferation and migration

Hepatocyte growth factor was detected in tissue fibroblasts from normal mammary glands and tumor tissue (Fig. 6a). HGF secretion and function was next investigated in primary fibroblasts. Secretion of HGF over various time points from 4T1 cells remained low over 24 h of mono-culture (Fig. 6a). Secretion of HGF from all primary fibroblasts groups at 20 and 24 h in mono-culture was significantly elevated over earlier time points and 4T1 cells. Interestingly, fibroblasts isolated from obese mice secreted significantly greater HGF compared to fibroblasts isolated from lean mice. In addition, within a given diet, CAFs secreted greater HGF compared to NAFs. HGF secreted from 10 %-fed NAFs was significantly lower than all other groups ($P = 0.001$), and secretion from 60 %-fed CAFs was elevated compared to 10 % NAF, 60 % NAF, and 10 % CAF ($P = 0.0001$, $P = 0.005$, and $P = 0.043$, respectively). HGF secreted from fibroblasts activated the

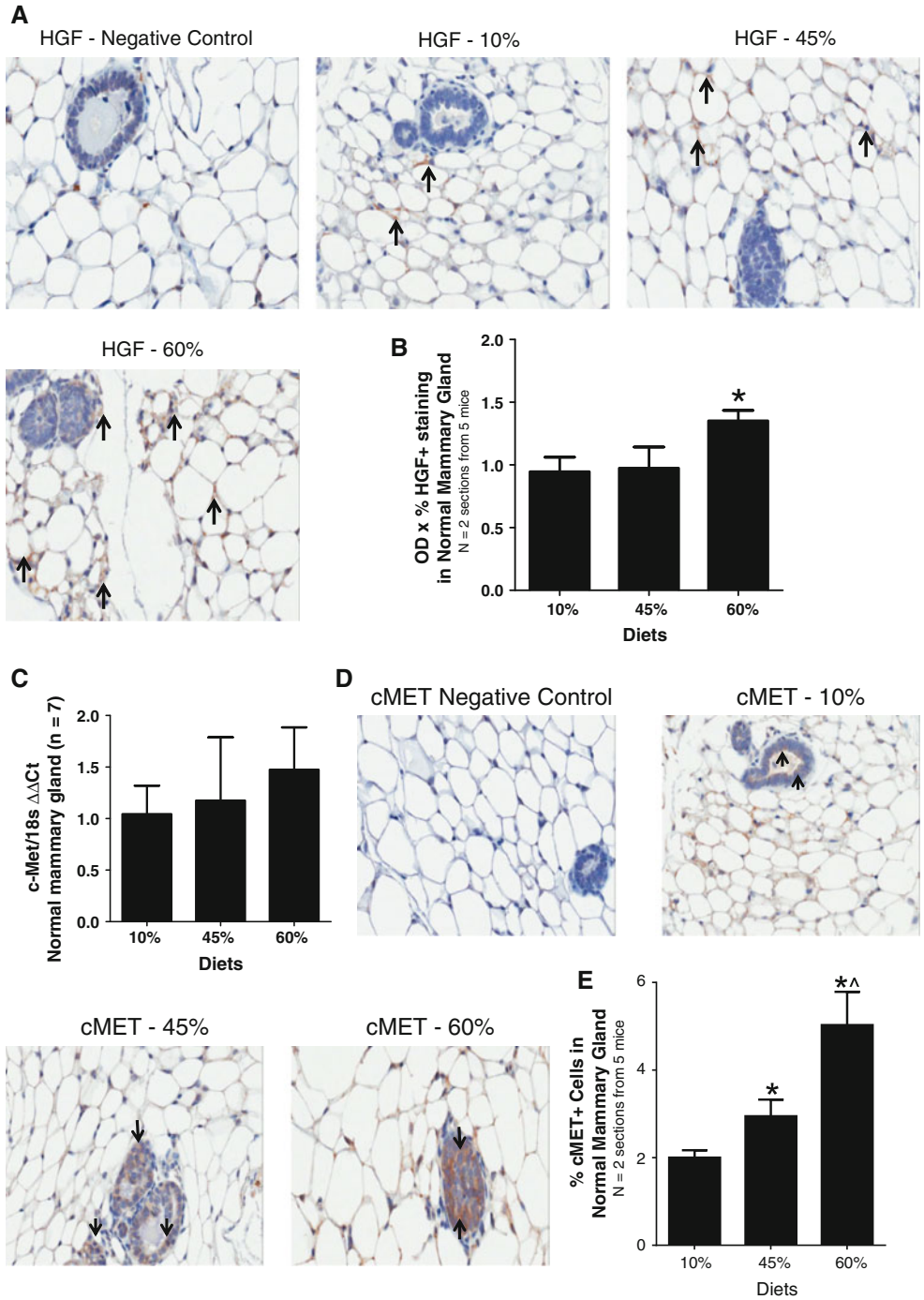


Fig. 2 Obesity upregulated HGF, c-Met, and pc-Met expression in normal mammary glands. **a** Representative photomicrographs (×40) of HGF staining in negative control (secondary only), 10 %, 45 and 60 % diet groups in normal (unaffected) mammary glands. *Arrows* indicate positive HGF staining in the stroma. **b** Total HGF protein levels of the normal mammary glands were quantified using the Aperio ImageScope color deconvolution algorithm for $n = 2$ sections from five mice per diet group. *10 versus 60 % ($P = 0.011$). **c** c-Met mRNA expression was determined using qPCR analysis in normal mammary glands for $n = 7$. **d** Representative photomicrographs (×40) of the IHC analysis of negative control and membrane localized c-Met staining in 10, 45, and 60 % diet groups (*arrows*). **e** Total c-Met protein levels in normal mammary glands were measured using the Aperio ImageScope membrane algorithm and quantified as above. *10 versus 45 % ($P = 0.04$) and 10 versus 60 % ($P = 0.001$); ^45 versus 60 % ($P = 0.025$). **f** Representative photomicrographs (×40) of the IHC analysis of negative control and pc-Met staining in 10, 45, and 60 % diet groups. **g** Total pc-Met protein levels in normal mammary glands were measured using the Aperio ImageScope color deconvolution algorithm and quantified as above. *10 versus 45 % ($P = 0.011$) and 10 versus 60 % ($P = 0.0001$); ^45 versus 60 % ($P = 0.0125$)

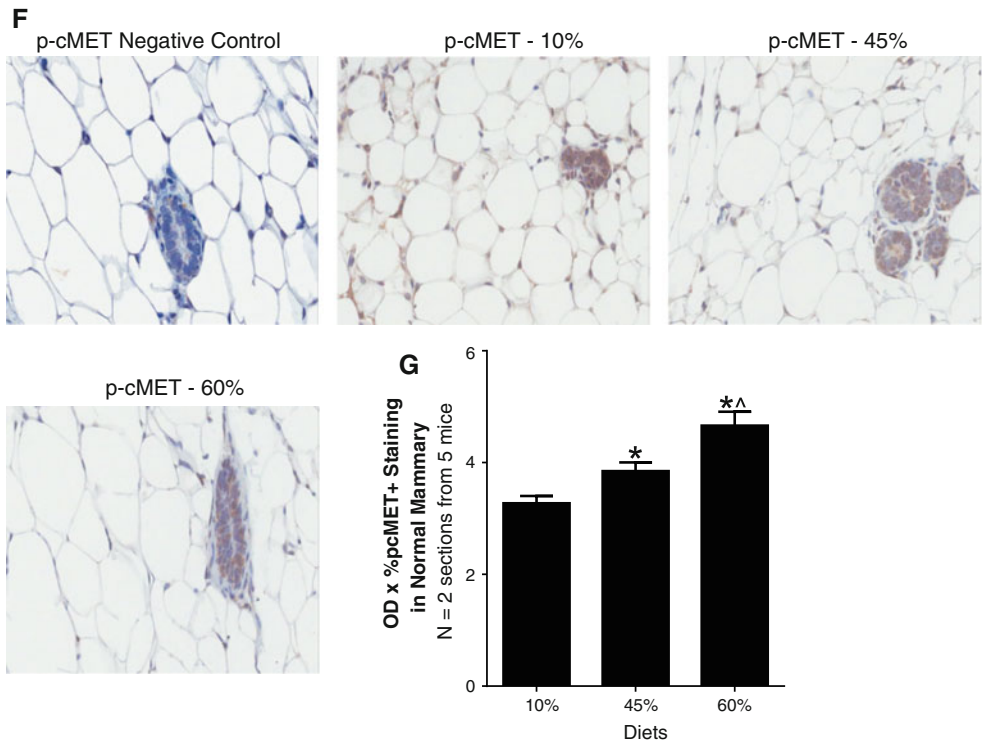


Fig. 2 continued

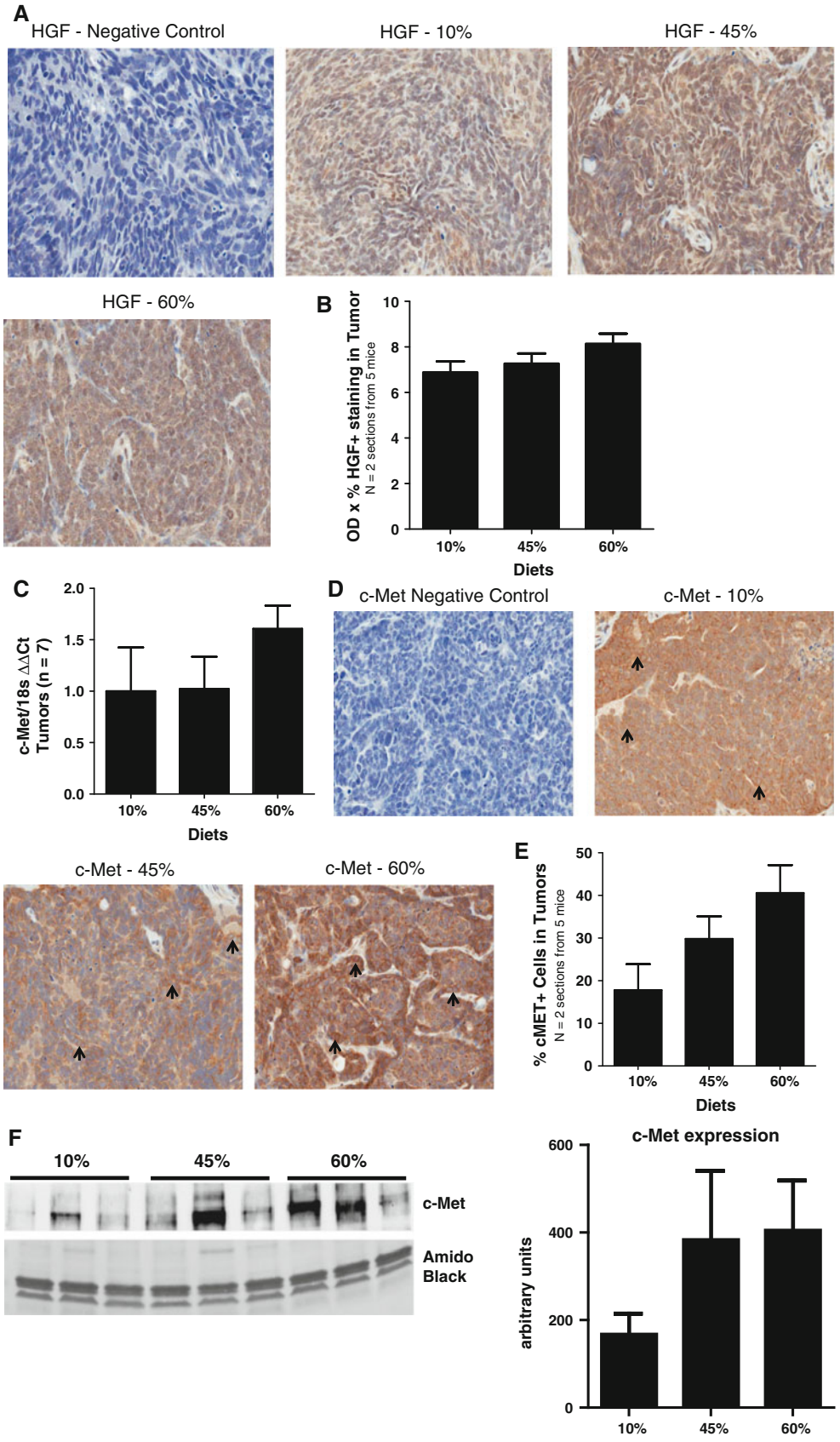
c-Met signaling cascade in 4T1 cells similar to recombinant murine HGF (Fig. 6c).

Based on an elevation of HGF with obesity in vivo and ex vivo, we assayed cellular proliferation and migration associated with fibroblast-derived HGF signaling [23]. In parallel with HGF concentrations from primary NAFs and CAFs (Fig. 6b), conditioned media from NAFs and CAFs drove 4T1 proliferation as measured by BrdU incorporation. The greatest proliferation was driven by fibroblasts isolated from tumors from 60 %-fed mice (Fig. 6d, $P = 0.003$ vs. 10 % NAFs and $P = 0.02$ vs. 60 % NAFs). The extent of proliferation was similar to 50 ng/ml recombinant HGF-stimulated 4T1 proliferation. Cell counts revealed identical proliferative capacity (data not shown).

Finally, to test the effect of stromal–epithelial interactions, primary fibroblasts were co-cultured with 4T1 cells and a scratch test "wound assay" was conducted to examine migratory capacity. In parallel with elevated HGF levels in fibroblast-conditioned media (Fig. 6b) and effects on proliferation (Fig. 6d), CAFs demonstrated a significantly greater effect on wound closure than NAFs in both cases, where fibroblasts were isolated from lean and obese mice (Fig. 6e, f). The greatest percent wound closure occurred when 4T1 cells were co-cultured with 60 % CAFs compared to all other groups (60 % CAF vs. 10 % NAF: $P = 0.0001$ and 60 % NAF: $P = 0.006$). Effects of fibroblasts–epithelial co-culture were significantly reversed in the CAF-4T1 model by an HGF neutralizing antibody (Fig. 6e, f, 10 % CAF: $P = 0.0001$ and 60 % CAF: $P = 0.000004$ vs. untreated). Anti-HGF antibody treatment demonstrated the greatest inhibition of wound closure in 4T1s co-cultured with 60 % CAFs ($P = 0.0001$ vs. all other groups).

Discussion

Genetic characteristics of BBC have been well-defined, including loss of retinoblastoma, p53 mutation, BRCA1 mutation, and signaling defects [10]. However, stromal interactions likely play a contributing role in BBC etiology, and may underlie higher rates of local recurrence for this subtype. Fibroblasts are an abundant stromal cell type in the tumor microenvironment and play a fundamental role in the etiology and progression of tumors [19, 45]. Stromal cell interactions are important in tumor cell proliferation, macrophage polarization, as well as in reorganization of the extracellular matrix which contributes to metastasis [23, 45–49]. While our previous work using ex vivo co-culture models suggest unique interactions between human fibroblasts and BBC cells [23, 49, 50], stromal–epithelial interactions early in carcinogenesis are poorly understood. Herein, we demonstrate a plausible role of obesity-modulated fibroblast-derived growth factor expression in normal mammary gland, with implications for etiology of BBC.



◀ **Fig. 3** Obesity minimally modified HGF, c-Met, and pc-Met expression in tumors. **a** Representative photomicrographs (×40) of the IHC analysis of tumors showing HGF staining in negative control and 10, 45, and 60 % diet groups. **b** HGF protein levels in tumors were quantified using the Aperio ImageScope color deconvolution algorithm for $n = 2$ sections from five mice per diet group. **c** c-Met mRNA expression was determined using qPCR analysis in tumor tissues for $n = 7$. **d** Representative photomicrographs (×40) of the IHC tumor analysis of membrane localized c-Met staining in negative controls and 10, 45, and 60 % diet groups (*arrows*). **e** Total c-Met protein levels in tumors were measured using the Aperio ImageScope membrane algorithm and quantified as above. **f** Western immunoblot expression of c-Met in $n = 3$ tumors per diet group and quantification (c-Met normalized to amido black staining loading control). **g** Representative photomicrographs (×40) of the IHC tumor analysis of pc-Met staining in negative controls and 10, 45, and 60 % diet groups. **h** Total pc-Met protein levels in tumors were measured using the Aperio ImageScope color deconvolution algorithm and quantified as above

Both in vivo and ex vivo, we demonstrated that stroma-derived HGF expression is associated with obesity in a preclinical model of BBC. Normal mammary glands exhibit obesity-induced increases in HGF and c-Met expression as well as phosphorylated c-Met, providing an optimal microenvironment for tumorigenesis. Indeed, obesity significantly decreased tumor latency. Interestingly, decreases in latency occurred in the absence of changes in either tumor burden or tumor volume. It is possible that the increased HGF and c-Met expression detected in the normal mammary glands prior to tumor onset act to mediate tumor etiology in C3(1)-T_{Ag} mice. c-Met signaling causes luminal progenitors to preferentially undergo basal lineage commitment at the expense of luminal cell-fate specification [25]. There is little work completed on basal-like murine models and to date only one prior publication from our group has indicated c-Met in basal-like murine tumors [24]. Evidence presented herein that obesity strongly regulates HGF/c-Met suggests a novel targetable pathway.

Our data suggest that the effects on BBC etiology include a role for HGF/c-Met and ruled out metabolic effects as significant mediators of this association. This is important because obesity has been hypothesized to play a role in carcinogenesis through altered metabolic homeostasis, including hyperglycemia and hyperinsulinemia secondary to insulin resistance [51]. Although we observed increased body weight and adiposity in the obese groups relative to lean controls, obese mice did not develop hyperglycemia, hyperinsulinemia, or evidence of insulin resistance. Leptin was elevated by obesity in C3(1)-T_{Ag} mice. Leptin is mitogenic, anti-apoptotic, pro-angiogenic, and pro-inflammatory, and, thus, is implicated in the stimulation, migration, and invasion of tumor cells [2, 52, 53]. Further studies to determine the role of leptin in BBC etiology need to be completed. Although significant increases in systemic TNF-α in obese mice were observed, local levels of all cytokines and chemokines measured indicated that important pro-inflammatory mediators

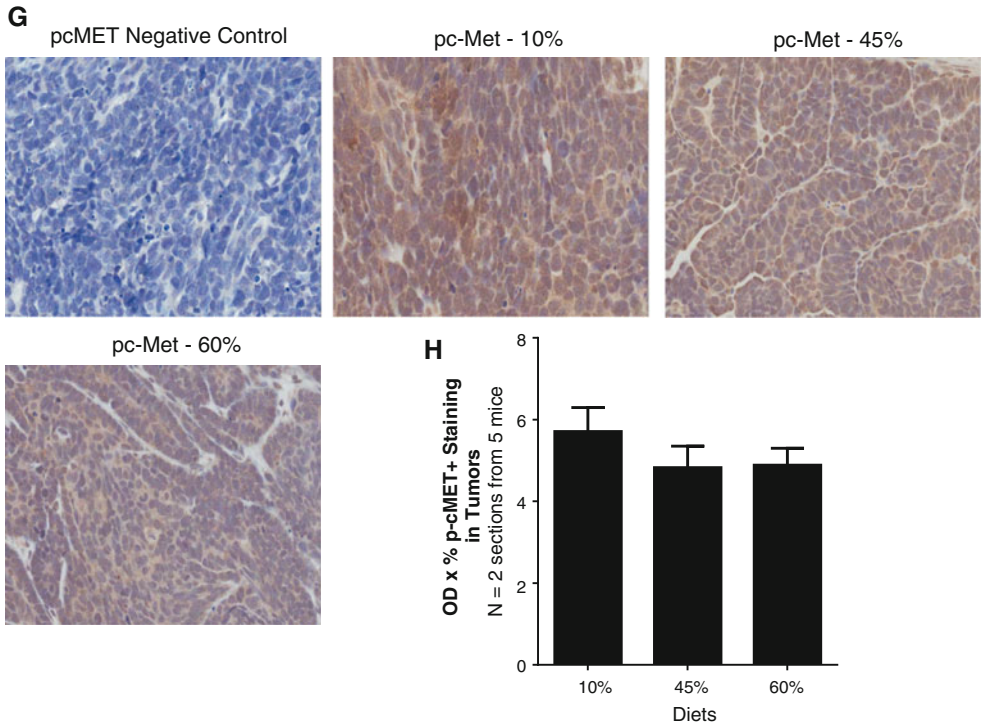


Fig. 3 continued

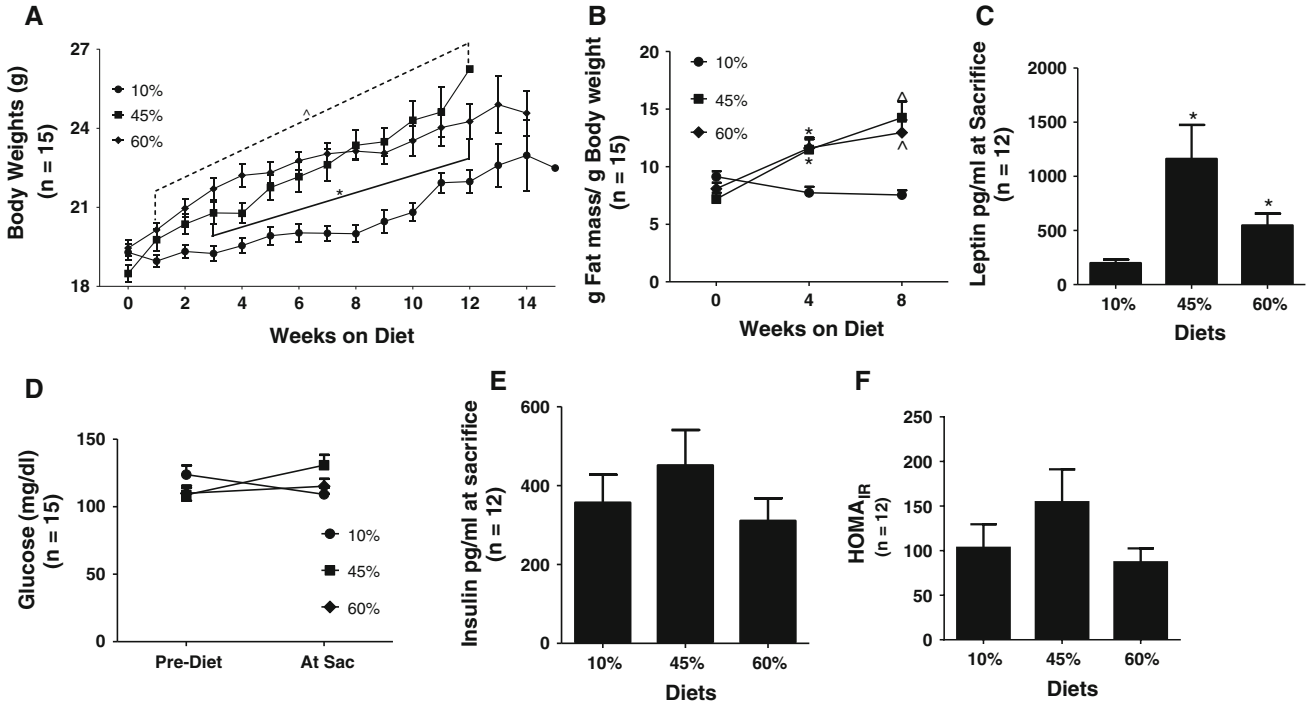


Fig. 4 Diet affects body weight, body composition, and plasma leptin concentration but not metabolic parameters. **a** Body weight increased in proportion with fat content in diet. *10 versus 45 % ($P < 0.05$) over weeks 3–12 (*solid line*) and ^10 versus 60 % ($P < 0.05$) over weeks 2–12 (*dashed line*). $N = 15$ mice per group at start of diet. **b** Percent fat content relative to total body mass is shown for $n = 15$. At 4 weeks on diet *10 versus 45 % ($P = 0.009$) and 10 versus 60 % ($P = 0.007$) and at 8 weeks on diets, ^10 versus 45 % ($P = 0.0001$) and 10 versus 60 % ($P = 0.0002$). **c** Leptin levels in plasma at sacrifice were measured ($n = 12$). *10 versus 45 % ($P = 0.005$) and 10 versus 60 % ($P = 0.003$). **d** Blood glucose concentrations prior to start of diet and at sacrifice were measured in 6 h fasted mice ($n = 15$ mice). **e** Insulin in plasma at sacrifice was measured ($n = 12$). **f** Homeostasis model assessment of insulin resistance ($HOMA_{IR}$) at sacrifice ($n = 12$)

(TNF-α, IL-6 and MCP-1) were not altered by obesity, hence, did not contribute to tumor onset in C3(1)-T_{Ag} mice in this experimental design. However, our previous work and A.J. Dannenberg's studies have demonstrated that both obese women and murine models display elevated macrophage infiltration in normal breast tissue [22, 54, 55]. Future studies examining the effects of local inflammation and macrophages on BBC are needed to clarify the time course of microenvironmental alterations and obesity-mediated tumor onset.

In epidemiologic studies that have examined obesity risk among all breast cancers or among cancers stratified by ER status, risk of breast cancer was elevated only among postmenopausal women [6], leading some to hypothesize that elevated aromatase may play a role. However, several more recent studies have shown that obesity and adiposity may increase risk for triple-negative breast cancers [4]. In the triple-negative subgroup, a prominent role for aromatase and estrogen signaling is less likely; however, we investigated systemic hormones concentrations and aromatase expression in our model to rule out any hormonal effects. The lack of obesity-mediated differences in plasma estrogen and progesterone concentrations, and absence of aromatase expression in the mammary gland or tumors, suggests that the latency was not driven by systemic or local hormone concentrations. Furthermore, it has been reported that estrogen levels in C3(1)-T_{Ag} mice do not affect the tumor growth and progression, as shown by Yoshidome et al. [56]. Similarly, we demonstrated a lack of significant elevations in SV40 T_{Ag} levels, indicating that obesity-mediated alterations in transgene expression could not explain detected reductions in tumor latency. Therefore, metabolic and hormonal action did not appear to be the primary drivers of obesity-mediated alterations in latency.

An intriguing finding was the persistent ex vivo release of HGF from fibroblasts. In vivo, HGF was localized to fibroblasts in both normal mammary glands and tumor tissues. HGF secretion was significantly increased from NAFs from obese mice compared to lean, demonstrating that in vivo obesity-induced elevation of HGF expression is a phenotype that is conserved in primary culture. In addition, CAFs derived from mice on either diet secreted significantly greater HGF than NAFs, demonstrating that the tumor microenvironment primes fibroblasts for elevated HGF release. Conditioned media from fibroblasts induced cell proliferation in direct correlation with HGF

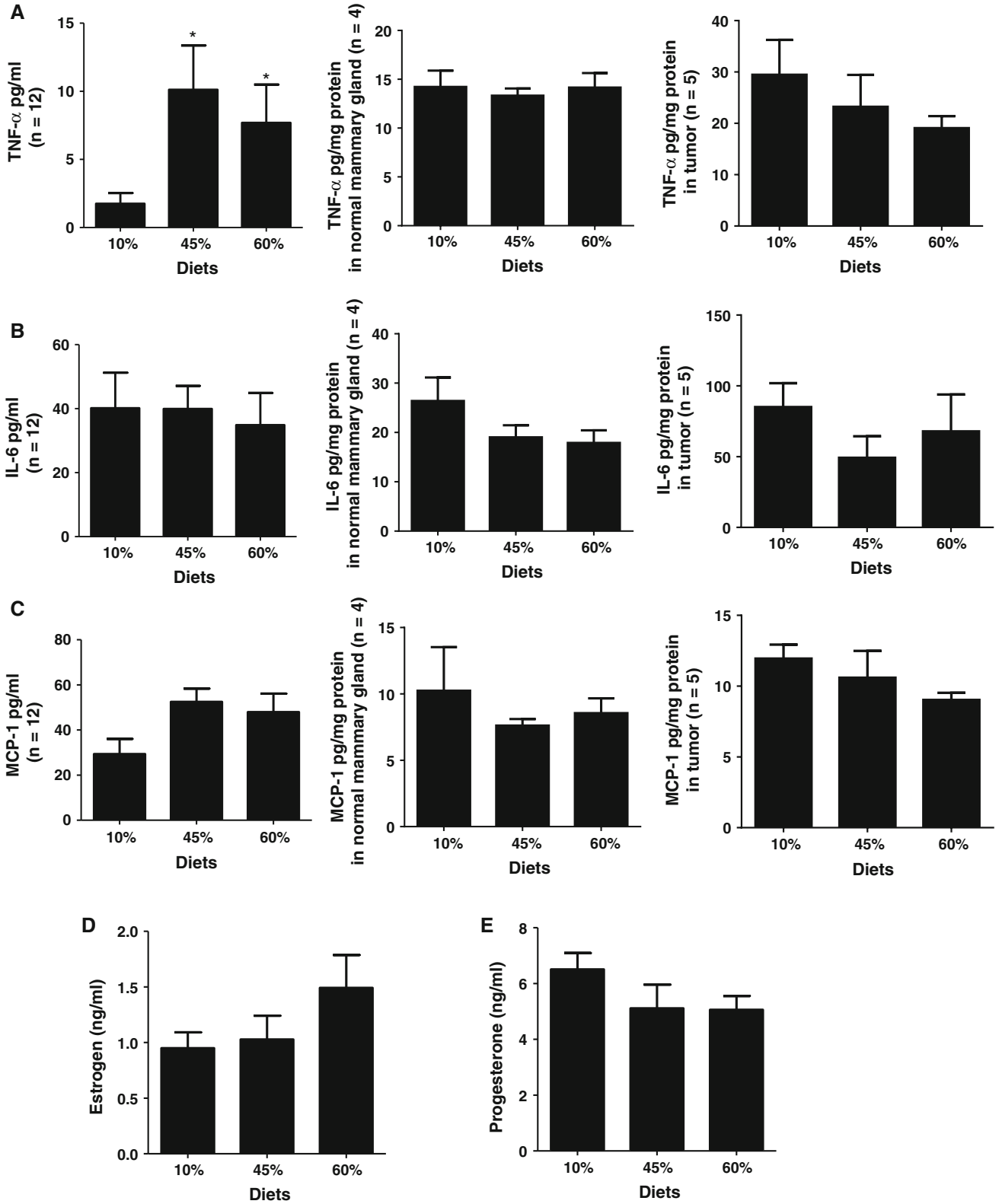


Fig. 5 Plasma levels of TNF-α, but not IL-6 or MCP-1, nor tissue levels of cytokines are regulated by obesity. Cytokine and chemokine levels of **a** TNF-α, **b** IL-6 and **c** MCP-1 in plasma, normal mammary gland, and tumor tissues; or levels of hormones **d** estrogen and **e** progesterone were measured at sacrifice ($n = 12$ for plasma samples, $n = 4$ for normal mammary glands and $n = 5$ for tumors). *TNF-α plasma levels: 10 versus 45 % ($P = 0.023$ and 10 versus 60 % ($P = 0.048$)

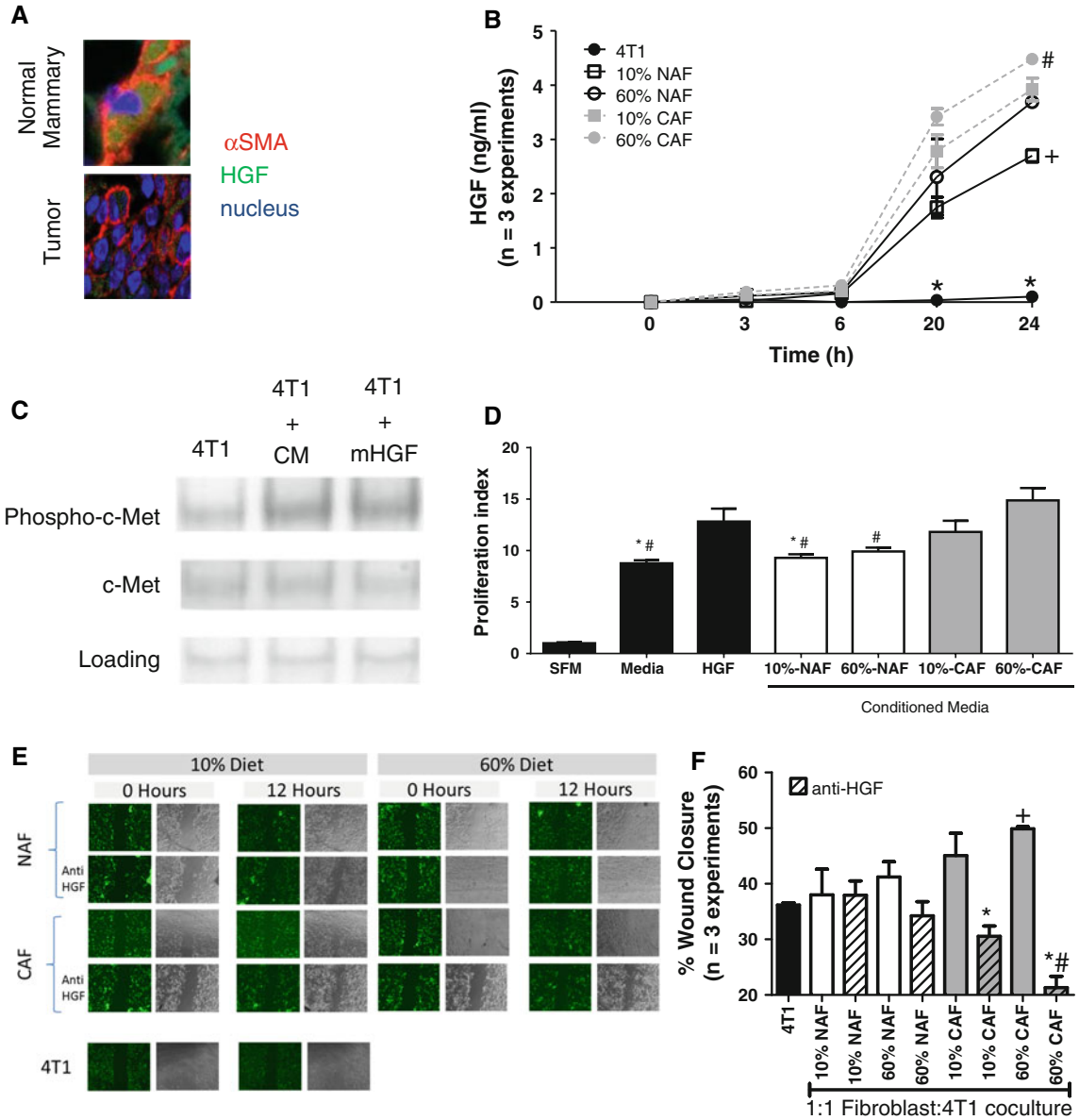


Fig. 6 HGF mediates wound closure driven by obese and/or cancer-derived fibroblasts. **a** Representative confocal photomicrographs (×63) of HGF (*green*) co-stained with fibroblast marker α-smooth muscle actin (αSMA, *red*) in normal mammary and tumor. Nucleus (*blue*) is stained with DAPI. **b** HGF was quantified in conditioned media at indicated time points from mono-cultures of 4T1 basal-like epithelial cells, as well as primary NAF and CAF isolated from lean (10 %-fed mice) and obese (60 %-fed mice) for $n = 3$ experiments. $^{*}P = 0.0001$ versus all other groups; $^{+}$versus all other groups ($P = 0.001$); $^{\#}$versus 10 % NAF ($P = 0.0001$), 60 % NAF ($P = 0.005$), and 10 % CAF ($P = 0.043$). **c** Phospho-c-Met and total c-Met expression in 4T1 cells was determined using Western immunoblot analysis after 15 min treatment with conditioned media (CM) from primary 60 % CAFs or recombinant mouse HGF (50 ng/ml). Loading control is non-specific band from c-Met immunoblot. **d** Proliferation index measured using BrdU incorporation is shown for $n = 3$ experimental groups. *versus HGF (50 ng/ml) ($P = 0.024$); $^{\#}$versus 60 %-CAF with 10 %-NAF ($P = 0.003$) and 60 % NAF ($P = 0.02$). **e** Fluorescently labeled 4T1 cells were co-cultured with NAFs or CAFs isolated from lean (10 % diet) and obese (60 % diet) mice, with or without HGF neutralizing antibody during the 24 h co-culture period and the 12 h scratch test. Representative photomicrographs of wound migration assay at 0 h (time point of scratch) and 12 h (end of study). **f** Percent wound closure is shown for $n = 3$ experiments. *versus control (no anti-HGF) for 10 % CAF ($P = 0.0001$) and 60 % CAF ($P = 0.0004$). $^{+}$versus 10 % NAF ($P = 0.0002$) and versus 60 %-NAF ($P = 0.006$) controls. $^{\#}$versus all groups ($P = 0.0001$)

concentrations secreted from the NAFs and CAFs, with obese-derived tumor fibroblast-conditioned media being as effective as recombinant HGF in driving epithelial cell proliferation. Obesity regulated HGF/c-Met-driven proliferation is one mechanism demonstrated in vitro that may be an underlying mechanism in vivo.

To probe the HGF/c-Met mechanism in a faithful model of the mammary microenvironment, we co-cultured primary fibroblasts from C3(1)-T_{Ag} diet-exposed animals with 4T1 BBC-like epithelial cancer cells [23, 46, 54] and completed a scratch test wound response assay. Earlier work by our group demonstrated a strong wound-response signature in more aggressive and BBC samples from patients, as well as in the normal mammary gland extra-tumoral microenvironment [45, 47, 48, 50]. In fibroblasts/4T1 co-culture herein, completeness of wound closure was elevated in association with HGF expression increases, such that fibroblasts isolated from obese mice and/or from tumors demonstrated the greatest extent of wound closure. Since HGF is the only known ligand of c-Met, inhibiting HGF signaling using a neutralizing antibody is effective at blunting c-Met activation [49, 50, 57]. Our results demonstrated that neutralization of HGF had the most dramatic effects in conditions where fibroblast-driven proliferation was the greatest, demonstrating specificity for HGF/c-Met signaling. The persistence of HGF secretion from primary fibroblasts ex vivo is consistent with work demonstrating that HGF is secreted in greater amounts from primary adipocytes isolated from obese versus lean subjects [27]. Our work presented here suggests that fibroblast-derived HGF may be regulated by obesity through epigenetic means [58], which could have long-lasting effects on the mammary gland stroma.

Conclusions

In sum, our data demonstrate that obesity reduces BBC latency in C3(1)-T_{Ag} mice. HGF may be a potential mediator of tumor onset: expression is elevated by obesity in normal mammary glands and persists in isolated primary fibroblasts. Indeed, in vitro modeling of the normal and tumor microenvironment demonstrate that fibroblasts derived from tumors were an important regulator of proliferation and wound response, specifically through HGF/c-Met signaling. Increased proliferation and motility induced by HGF allows for cells with already elevated proliferative rates (such as the T_{Ag}-overexpressing epithelium of this model) to locally invade and develop tumors. c-Met inhibitors are currently in clinical trials for BBC [59]. Our findings support a novel role for obesity-mediated HGF/c-Met signaling as modifiable risk factor for basal-like tumor onset, which has important public health implications with regard to BBC risk. Whether increased risk of breast cancer associated with obesity is reversible with weight loss is still an important area of uncertainty.

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Conflict of interest C. M. Perou holds a pending patent assignment for PAM50 Genomic Classifier. He also holds a position on the Board of Directors as well as Stock ownership in University Genomics and Bioclassifier. All other authors declare no conflict of interest.

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