Targeting the IKKβ/mTOR/VEGF signaling pathway as a potential therapeutic strategy for obesity-related breast cancer

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Abstract

Clinical correlation studies have clearly demonstrated that obesity is associated with breast cancer risk and patient survival. Although several potential mechanisms linking obesity and cancers have been proposed, the detailed molecular mechanism of obesity-mediated breast tumorigenesis has not yet been critically evaluated. In the present study, we evaluated the effects of obesity on mammary tumor initiation and progression using mice with genetic and diet-induced obesity bearing mammary tumor xenografts and mouse mammary tumor virus-neu transgenic mice that were fed a high-fat diet. We demonstrate that obesity promoted mammary tumor growth and development in these animal models. Moreover, the expressions of tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF), inhibitor of nuclear factor kappa-B beta (IKKβ), and mammalian target of rapamycin (mTOR) are upregulated in mammary tumors of obese mice, suggesting that the IKKβ/mTOR/VEGF signaling pathway is activated by TNFα in the tumors of obese mice. More importantly, inhibitors (rapamycin, bevacizumab, and aspirin) that target members of the pathway suppressed tumorigenesis and prolonged survival more effectively in obese mice than in non-obese mice. Here, we not only identified a specific signaling pathway that contributes to mammary tumorigenesis in obese mice but also a strategy for treating obesity-mediated breast cancer.
Introduction

Obesity is a serious health problem in the United States and is associated with increased risk of diabetes, hypertension, and cardiovascular diseases. Moreover, growing evidence has shown that obesity is a risk factor for multiple types of human cancer (1-4) and known to be correlated with breast cancer tumorigenesis and development (5, 6). Breast cancer is the second-leading cause of cancer-related deaths in women in the United States, and obese breast cancer patients have a higher risk of recurrent disease, higher metastasis and mortality rates, larger tumor masses, and a poorer prognosis than non-obese breast cancer patients (7-12).

Several mechanisms relating to breast cancer prognosis in obese patients have been proposed. For example, estrogen (13, 14), which is expressed at significantly higher levels in obese postmenopausal women than in non-obese postmenopausal women, is believed to increase the risks of developing breast cancer (6). The expression of insulin and insulin-like growth factors, which is also higher in obese patients, has also been proposed to play a role in breast cancer cell growth and survival (15). In addition, adipose tissues that secrete adipokines, such as leptin, adiponectin, growth factors, and proinflammatory cytokines, also enhance cancer cell growth (16-18). Particularly, recent studies have indicated that that expression of the inflammatory cytokine tumor necrosis factor alpha (TNFα) is increased in obese patients, thereby suggesting that this cytokine plays a role in obesity-mediated breast cancer development and
progression (19, 20). However, the specific signaling pathway linking TNFα and obesity to breast tumorigenesis and the exact mechanisms underlying the connection between obesity and breast cancer are still a matter of debate. Thus, investigating signaling pathways involved in obesity-mediated breast cancer, which could lead to the development of targeted therapies that inhibit obesity-related tumor growth, is a significant and timely goal.

Previously, we showed that TNFα induces the IKKβ/mTOR/VEGF pathway to promote breast cancer progression by inducing angiogenesis (21). The activated TNFα receptor activates IKKβ, which in turn phosphorylates tuberous sclerosis protein 1 (TSC1) and activates the downstream signaling pathway such as mTOR. Moreover, IKKβ activation, TSC1 phosphorylation, and VEGF expression are correlated with poor clinical outcome in breast cancer patients (21). Because obese patients have higher levels of adipocytes and thus can secrete more TNFα (22), we speculated that this newly identified mechanism could link TNFα-activated IKKβ/mTOR/VEGF (23) to obesity and therefore contribute to obesity-mediated breast cancer progression.

In this study, we sought to determine the effects of obesity on mammary tumor progression using two different mouse models of obesity: mice with genetic and diet-induced obesity (DIO) bearing orthotopic xenograft mammary tumors. Moreover, we also assessed the effects of diet-induced obesity on tumor initiation in a mouse mammary tumor virus (MMTV)-neu model.
Using these models, we clearly demonstrated that obesity promotes both the development and growth of mammary tumors. More importantly, we found that the IKKβ/mTOR/VEGF signaling pathway was highly activated by TNFα in the tumors of obese mice, and inhibition of this pathway with clinically used drugs reduced obesity-mediated tumorigenesis. Thus, our study may provide not only a molecular interpretation for the higher incidence of breast cancer and poor prognosis in obese women but also a new avenue from which to develop therapeutic strategies for obesity-mediated breast cancer.

Materials and Methods

Cell lines

EO771 mouse mammary cancer cells were obtained from Dr. F.M. Sirotnak (Memorial Sloan-Kettering, New York, NY). No further authentication was performed. Cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum.

Animal model

We purchased 4-week-old female C57BL/6J wild-type (WT), B6.V-Lepob/J obese (OB), and MMTV-neu mice (10 per group) from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained them under the institutional animal care protocol established by the Department of
Veterinary Medicine and Surgery at The University of Texas MD Anderson Cancer Center. The C57BL/6J and MMTV-neu mice were fed either a high-fat diet (HFD) (Research Diets, Inc. D12492) (24) or a normal chow diet (ND) (Teklad Global Diets, #2919) (Supplementary Table 1) starting at 4 weeks of age. DIO was initiated in mice via ad libitum access to high-fat food for at least 8-12 weeks as described previously (25) until the experiments were completed. ND and HFD mice were injected with mammary cancer cells were injected with $2 \times 10^5$ EO771 mammary adenocarcinoma cells at age 14 weeks. The OB mice, which spontaneously develop obesity even on ND (26, 27), were fed only the ND and injected with $2 \times 10^5$ EO771 mammary adenocarcinoma cells at age 14 weeks. Mice were removed from the experiment when tumor size reaches over 1.5 cm diameters in accordance with the Institution Animal Care and Use Community (IACUC) guidelines. The incidence of spontaneous tumor growth in MMTV-neu mice was noted when tumors growth is larger than 5 mm in diameter in the mammary gland. MMTV-neu develops mammary tumors spontaneously at about age 6-12 months (28, 29).

For the mammary tumor model, EO771 mouse mammary cancer cells were obtained from Dr. F.M. Sirotnak (Memorial Sloan-Kettering, New York, NY), and $2 \times 10^5$ cells were injected into the mammary fat pads of 14-week-old C57BL/6J or OB mice. After the tumors reached 3-5 mm in diameter, the mice were administered aspirin at 120 mg/kg/day (Sigma, St. Louis, MO) via subcutaneously injection (30); rapamycin (LC Labs, MA) at 5 mg/kg twice per week via
intraperitoneal injection (31); and bevacizumab (Avastin, purchased from the Department of Pharmacy at MD Anderson Cancer Center) at 10 mg/kg once per week via intravenous injection (32).

**Immunofluorescence assay**

Mammary tumor tissue was harvested and embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) for frozen embedded and sectioned. The tumor samples were stained separately with anti-TNFα and anti-VEGF (IHC World, Woodstock, MD); phosphorylated IKKβ (S176/180) [pIKKβ (S176/180)], phosphorylated S6 (S240/244) [pS6 (S240/244), (an indicator for mTOR activity)]; phosphorylated VEGF receptor (S996) [pIKKβ (S996)] (the previous three antibodies from Cell Signaling Technology); CD31 and CD45 antibody (BD Biosciences); Ki67 antibody as an indicator of cancer cell proliferation (Abcam, Cambridge, MA). Nuclei were stained with DAPI blue, and fluorescence intensity was analyzed using AxioVision software version 4.4 (Carl Zeiss, Thornwood, NY).

**Statistical analysis**

Data are shown as the mean ± standard deviation (SD) of 10 individual mice or treatments. Statistical significance between groups was analyzed using a Student t-test or one- or two-way analysis of variance. Survival was analyzed using a Kaplan-Meier plot, and MMTV-neu mice
tumor incidence was measured via a log-rank test (Mantel–Cox test) (33, 34). A P value of less than 0.05 was considered statistically significant and is henceforth indicated by an asterisk.

Results

**Genetic obesity and DIO promote mammary tumorigenesis and increase mortality**

Although clinical evidence indicates that obesity is one of the major risk factors for breast cancer (35), and despite the fact that researchers have proposed several mechanisms by which obesity and tumor progression are linked, limited information is available about the causal relationships between the molecular mechanisms and obesity-mediated breast cancer. To further explore the underlying mechanisms of obesity-mediated breast cancer, we first used two kinds of obesity mouse models, DIO and genetic obesity, to establish mammary tumor xenograft in these mice and further investigated the effects of obesity tumor progression.

We monitored the body weight of mice in these two models first and confirmed that these mice weighed more than control mice (Fig. 1A and 1B, upper panels). We then established mammary tumors in these mice and observed significantly higher tumor growth in obese mice (HFD/DIO and OB) than in lean mice (ND and WT) in both models (Fig. 1A and 1B, middle panels). Moreover, the survival rates of obese mice in both models were significantly lower than those of the control mice (Fig. 1A and 1B, lower panels). Similar results were observed in the
4T1 mouse mammary tumor models (Supplementary Fig. 1A and 1B). Together, these results indicate that obesity promoted mammary tumor growth \textit{in vivo} and reduced median survival and that these animal models are feasible for studying the underlying mechanisms of obesity-mediated tumor progression.

In a previous study, we demonstrated that the IKKβ/mTOR/VEGF pathway was more highly activated by TNFα in the livers of obese mice that had been fed an HFD than in the livers of normal-weight mice that had been fed an ND (23). Moreover, TNFα expression was recently shown to induce liver inflammation and promote tumorigenesis in mice with DIO and genetic obesity (36). TNFα, the first adipocytokine identified in adipose tissue, is known to contribute to inflammation-mediated tumor progression (21, 37, 38). To determine if the IKKβ/mTOR/VEGF signaling pathway is also involved in obesity-mediated mammary tumorigenesis, we harvested mammary tumors from these mice and subjected them to immunofluorescence staining. Immunofluorescence staining (Fig. 2A and 2B) showed that TNFα and VEGF protein levels in tumors were significantly higher in mice with DIO and genetic obesity than in control (lean) mice. We also determined via enzyme-linked immunosorbent assay (ELISA) that circulating TNFα (Supplementary Fig. 2A and 2B) and VEGF (Supplementary Fig. 2C and 2D) levels in the blood were higher in mice with DIO and genetic obesity than in control (lean) mice. Moreover, we observed higher levels of phosphorylated IKKβ and phosphorylated S6, an indicator for
mTOR activity, in obese mice than in control mice (Fig 2A and 2B), indicating that the IKKβ/mTOR/VEGF pathway was indeed activated in the tumor cells of obese mice. Together, these results suggest that TNFα plays an important role in obesity-mediated mammary tumorigenesis by activating the IKKβ/mTOR/VEGF pathway.

**Obesity mediates mammary tumorigenesis by increasing angiogenesis and tumor cell proliferation**

Our previous findings indicated that enhanced angiogenesis plays a central role in TNFα-induced breast tumor development by activating the IKKβ/mTOR/VEGF pathway. This activation resulted in VEGF expression in multiple tumor types and correlated with poor clinical outcome in breast cancer patients (21). To characterize the role of TNFα-mediated VEGF expression in our obesity-mediated mammary tumor models, we further examined in vivo angiogenesis phenomena or cell proliferation via immunofluorescence staining of tumor tissue using CD31 antibody (a marker for endothelial cells). The vascular density of mammary tumors in obese mice (OB and HFD/DIO) was higher than that of mammary tumors in control (lean) mice (Fig. 3A). Likewise, using Ki67 as an indicator of cancer cell proliferation, we also found that the proportion of proliferating cancer cells was higher in mammary tumors in OB and HFD/DIO mice than in the tumors of control (lean) mice fed ND (Fig. 3B). These results suggest
that obesity plays a role in promoting mammary tumorigenesis, at least in part, by enhancing tumor angiogenesis, which is in line with our previous studies that showed TNFα produced by adipose tissues increases of tumor angiogenesis (21, 36).

**Blocking the IKKβ/mTOR/VEGF pathway inhibits obesity-mediated tumorigenesis**

The data shown here suggest that the IKKβ/mTOR/VEGF pathway may play an important role in mammary tumor progression in obese mice. Thus, targeting this pathway may lead to new intervention strategies for obesity-mediated breast cancer. To determine whether inhibition of the IKKβ/mTOR/VEGF signaling pathway reduces the development of mammary tumor in DIO mice, we first established mammary tumors in obese and control mice as described above and then administered inhibitors or antagonists of this specific pathway to obese and control (lean) mice after the tumor volume reached a 3-5 mm in diameter. Interestingly, after treatment with aspirin (an IKKβ inhibitor), rapamycin (an mTOR inhibitor), or bevacizumab (a VEGF antagonist) for 2 weeks, a significant reduction in mammary tumor volume was observed in obese mice but not in control (lean) mice (left panels, Fig. 4A-C).

To validate that these drugs indeed blocked the specific pathway we proposed, tumor samples from the mice treated or untreated with the drugs were subjected to immunostaining with specific antibodies against the IKKβ/mTOR/VEGF pathway. The activity of IKKβ, mTOR,
and VEGFR, as indicated by their phosphorylation status, was reduced in tumor-bearing obese mice that were treated with aspirin, rapamycin, and bevacizumab, respectively (Fig. 4, middle and right panels). These results support our hypothesis that blocking the IKKβ/mTOR/VEGF pathway could inhibit obesity-mediated breast tumorigenesis.

TNFα-mediated signaling pathway promotes the development of mammary tumors in mice

The data above strongly support the notion that the TNFα-induced signaling pathway plays a critical role in mammary tumor cell proliferation in obese mice. Next, we sought to determine whether the TNFα-mediated signaling pathway also promotes mammary tumor initiation in obese mice. To address this question, we used MMTV-neu transgenic mice, which develop mammary tumors spontaneously at about age 6-12 months (28, 29). After the weaning stage, one group of MMTV-neu transgenic mice was fed an HFD, and the other group was fed an ND. We monitored tumor development and found that spontaneous development of mammary tumors was accelerated in the MMTV-neu transgenic mice group fed the HFD compared with the group that was fed an ND (Fig. 5A), suggesting that obesity also promotes tumor initiation. Mice exhibited tumor formation from day 146 to day 207 (median=159 days) and from day 181 to day 239 (median=212 days) in the HFD and ND groups, respectively ($P = 0.003$). The expression of TNFα and VEGF, as well as phosphorylated IKKβ and phosphorylated S6, was significantly
higher in mammary tumors of the HFD group than in the tumors of the ND group, thereby suggesting that the IKKβ/mTOR/VEGF pathway is also activated in the spontaneously developing mammary tumors of MMTV-neu transgenic mice with HFDs (Fig. 5B).

**Inhibition of the TNFa-mediated signaling pathway prevents the development of mammary tumor in mice**

In a parallel experiment, after the weaning stage, the MMTV-neu transgenic mice were fed an HFD but treated with aspirin, rapamycin, or bevacizumab. We found that these IKKβ/mTOR/VEGF pathway inhibitors delayed mammary tumors development in MMTV-neu transgenic mice that were fed an HFD. The mice in the HFD, HFD aspirin-treated, HFD rapamycin-treated, and HFD bevacizumab-treated groups developed tumors from days 146 to 207, days 185 to 199, days 185 to 227, and days 185 to 243, respectively. The median time for tumor formation in both rapamycin- and bevacizumab-treated obese mice was 199 days, and the median time for tumor formation in the HFD group without drug treatment was 159 days (Fig. 6A). In addition, immunofluorescence staining of tumor tissues also showed that phosphorylation of the IKKβ/mTOR/VEGF signaling pathway was decreased in mammary tumors of the obese MMTV-neu mice that were treated with these drugs (Fig. 6B). Taken together, these results suggest that obesity promotes breast tumor initiation, at least in part, by
the activation of the IKKβ/mTOR/VEGF pathway.

Discussion

In the present study, we demonstrated a novel molecular mechanism that provides a link between obesity and breast cancer in mouse models. We found that the expression of TNFα and VEGF, as well as the activation of IKKβ and mTOR, is specifically upregulated in tumors in obese mice. More importantly, multiple clinical drugs and preclinical agents that are already available to target the IKKβ/mTOR/VEGF signaling pathway have been shown to effectively reduce obesity-mediated mammary tumor growth and tumor development.

The epidemiology data have shown that obesity primarily increases breast cancer risk after menopause and affects ER-positive breast cancer (2). Our results suggest that the IKKβ/mTOR/VEGF signaling pathway plays a role in obesity-mediated tumor progression in both ER-positive and ER-negative mammary tumors as evident from our estrogen receptor (ER)-positive EO771 mouse model and MMTV-neu transgenic mouse model, which spontaneously develop ER-negative mammary tumors (39). Thus, the IKKβ/mTOR/VEGF pathway might contribute to the ER pathway-dependent development and progression of breast cancer and should be further studied in human patient samples in future. However, we cannot exclude the possibility that factors or pathways other than those identified in the current study...
are also involved in the obesity-mediated mammary tumors. For example, Morris and colleagues found that macrophages forming crown-like structures can release TNFα to further activate NF-κB transcription factor and increase aromatase levels in the mammary glands of obese mice and in obese breast cancer patients (40, 41). A monoclonal antibody called infliximab, which targets TNFα, is approved for several autoimmune diseases. While we have not included this in our assay to demonstrate a direct role of TNFα in our model since it has yet to be approved for breast cancer treatment, it would be of interest to determine the effect of infliximab in the future.

The identification of specific signaling pathways is important for the clinical application of therapeutic targets in obesity-mediated breast cancer development. In this study, we focused on the TNFα/IKKβ/mTOR/VEGF signaling pathway, which can be specifically targeted by multiple clinical drugs. Aspirin is an anti-inflammatory drug, and high-dose aspirin treatment can reverse obesity–induced insulin resistance through targeted inhibition of IKKβ (30). Rapamycin and its analogs such as everolimus (RAD-001) are immunosuppressant drugs used in organ transplantation but have also been used as anticancer drugs because they suppress cancer cell proliferation by blocking the mTOR signaling pathway (42). Bevacizumab, an anti-VEGF monoclonal antibody approved by the U.S. Food and Drug Administration in 2004, has shown anticancer activity when used both alone and in combination with other drugs in several cancer types (43). We examined the effects of each of these drugs on the major targets of the
IKKβ/mTOR/VEGF pathway (Supplementary Figure 3). Interestingly, we found that when obese mice with mammary tumors were treated with these drugs, all of the specific target molecules in the IKKβ/mTOR/VEGF signaling pathway were inhibited. This is somewhat expected as aspirin, a phytochemical derivative with typically broad activity, has recently been shown to also activate AMPK, which influences the mTOR activity (44, 45). Moreover, other studies have also reported the activation of IKKβ by VEGF via the Flk-1/Cbl/Akt signaling pathway. Therefore, IKKβ can be downregulated once VEGF is blocked by bevacizumab (46). While pathways other than the one we proposed may play a role, our results provide a rationale for targeting the IKKβ/mTOR/VEGF pathway in obesity-mediated mammary tumor development.

Since aspirin did not significantly inhibit obesity-mediated mammary tumor incidence compared with rapamycin and bevacizumab in the MMTV-neu mouse model (Fig. 6A), we further examined the effect of these drugs on inflammatory infiltration (CD45, leukocytes common antigen) induced by TNFα pro-inflammatory cytokine that is linked to tumor insurgence and tumor progression. We found inflammatory infiltration was more obvious in mice fed an HFD than those fed an ND and can be decreased by aspirin, rapamycin, or bevacizumab treatment (Supplementary Fig. 4A), suggesting that inflammation is one key point linking these factors. It is possible that the Her2/neu-mediated signaling pathway can overcome the inhibition of the IKKβ/mTOR/VEGF signaling pathway in the MMTV-neu model (47) and would of
interest to further investigate the detailed molecular mechanisms of each drug treatment in the future.

Recent preclinical and clinical reports have shown that aspirin can be used for both cancer therapy and cancer prevention (48, 49). Furthermore, epidemiologic evidence shows that aspirin can be used for adjuvant therapy in cancer patients (50). However, in our MMTV-neu obesity mouse model, aspirin did not show inhibitory effects of mammary tumor initiation compared to rapamycin and bevacizumab (Fig. 6A). Thus, in this particular mouse model (Her2-positive breast cancer), aspirin may not be a good cancer prevention drug but may be useful for cancer prevention for other types of breast cancers (ER-positive or triple-negative breast cancers). While clinical trials show that aspirin can reduce tumor incidence by 10% in all cancer types, we believe the benefit-to-risk ratio must be defined in different cancer types (51, 52) as side effects, including as gastrointestinal bleeding, from long-term aspirin treatment have been reported (48, 53, 54).

Regarding the effect of angiogenesis on obesity, we did not find significant body weight loss in the aspirin/rapamycin/bevacizumab treatment group compared with the obese control group. However, anti-angiogenesis approaches have been proposed as a method of limiting obesity, and treatment of obese mice with anti-angiogenesis inhibitors has been shown to reduce obesity. Although the pathophysiological connection between angiogenesis and adipogenesis is well
recognized, so far, there is limited data from preclinical and clinical studies to address the weight loss research that focused on treating obesity with angiogenesis inhibitors. In addition, some studies have shown different results with different types of antiangiogenic inhibitors. For instance, vatalanib, a VEGFR TKI, demonstrated antitumor activity and changes in body weight in a murine renal cell carcinoma model (55). In contrast, in db/db C57BL/6 mice, a 2-week treatment with an anti-VEGF monoclonal antibody did not result in a significant change in their body weight (56). In the clinic, much less is known about the effect of VEGF TKI on body weight as only severe weight loss is reported according to the Common Toxicity Criteria Adverse Event criteria. Important adverse events of angiogenesis inhibitors are anorexia, nausea, stomatitis, and diarrhea, and all these adverse events can cause weight loss. Thus, it is not clear for the actual effects of antiangiogenic inhibitors to cause weight loss. Moreover, edema and hypothyroidism, which are also side effects of angiogenesis inhibitors, can contribute to weight gain (57). Therefore, how antiangiogenesis treatment affects obesity and what kinds of antiangiogenic inhibitors work remain unclear.

TNFα-activated IKKβ/mTOR/VEGF signaling pathway has also been shown to play an important role in the development of human esophageal cancer (37). In addition, obese mice with PanO2 pancreatic cancer xenografts responded to the inhibitors targeting this pathway (unpublished data). Taken together, these findings suggest that this pathway could potentially
serve as a therapeutic target for other obesity-related human cancers.

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In Memoriam

We acknowledge Mrs. Serena Lin-Guo for her courageous fight against breast cancer.
References


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Figure Legends

Figure 1. Genetic and dietary obesity promotes mammary tumorigenesis and increases mice mortality. (A) C57BL/6J female mice that were fed a high-fat diet (HFD) or normal diet (ND). (B) Genetic obese B6.V-Lepob/J (OB) mice that were fed normal chow diet. Upper panels, the body weights of genetic obese and diet induced obese mice as well as control mice were monitored. Middle panels, in both high-fat diet–induced obese mice and genetic obese mice, 2 × 10^5 EO771 mammary adenocarcinoma cells were injected into the mammary fat pads at the time indicated by the arrow. Tumor growth was monitored twice a week. Lower panels, survival rate of C57BL/6J female mice were compared with HFD or ND; survival rate of genetic obese B6.V-Lepob/J (OB) mice or control mice. Mice were removed from the experiment when tumor size reaches over 1.5 cm diameters in accordance with the Institution Animal Care and Use Community (IACUC) guidelines. The data represent the mean ± SD (10 mice/group); *, p < 0.05.

Figure 2. Increased TNFα/IKKβ/mTOR/VEGF in mammary tumor of genetic and dietary obesity mice. Representative images of immunofluorescence staining of mammary tumors with TNFα, VEGF, phosphorylated IKKβ (S176/180) [pIKKβ (S176/180)], and phosphorylated S6 (S240/244) [pS6 (S240/244)] antibodies (right panels). (A) Mammary tumor in C57BL/6J female
mice that were fed a high-fat diet (HFD) or normal diet (ND). (B) Mammary tumor in genetic obese B6.V-Lepob/J (OB) mice that were fed normal chow diet. The relative fluorescence intensity from the images is shown on the right. The data represent the mean ± SD. *, p < 0.05.

Figure 3. Increased tumor angiogenesis and tumor cell proliferation in mammary tumors in obese mice. Representative images of immunofluorescence staining of mammary tumor tissues: CD31 antibody as an indicator of tumor vascular density (red) (A) and Ki67 antibody as an indicator of tumor cell proliferation (green) (B). The relative fluorescence intensity from the images is shown below. The data represent the mean ± SD. *, p < 0.05.

Figure 4. Inhibitors of the IKKβ/mTOR/VEGF pathway block obesity-mediated tumorigenesis. High-fat diet (HFD)-induced obesity mice and control (lean) mice with mammary tumors were prepared as described in Figure 1 and then treated with or without aspirin (A), rapamycin (B), or bevacizumab (C). Tumor-bearing mice were administered aspirin at 120 mg/kg/day by subcutaneously injection; rapamycin at 5 mg/kg twice per week by intraperitoneal injection; and bevacizumab at 10 mg/kg once per week by intravenously injection (10 mice/treatment group). Tumor volume was measured after drug treatment 2 weeks (left). Treatment with these drugs significantly reduced tumor volume in the HFD group but not in the
ND (control/lean) group. Representative images of immunofluorescence staining of tumor tissues stained with indicated antibodies (middle). The relative fluorescence intensity from the images is shown on the right. The data represent the mean ± SD (10 mice/group); *, p < 0.05.

Figure 5. TNFα-mediated signaling pathway promotes mammary tumor development. (A) Spontaneous mammary tumor development in MMTV-neu mice fed with either a high-fat diet (HFD) or normal diet (ND). Each group contained 10 mice. The incidence of spontaneous tumor growth in MMTV-neu mice was based on tumors larger than 5 mm in diameter in the mammary gland. (B) Representative images of immunofluorescence staining of tumor tissues with the indicated antibodies. The relative fluorescence intensity from the images is shown on the right. The data represent the mean ± SD, (10 mice/group); *, p < 0.05.

Figure 6. Inhibition of TNFα-mediated signaling pathway prevents mammary tumor development. (A) HFD-fed MMTV-neu mice were treated with drugs that inhibit the IKKβ/mTOR/VEGF pathway and spontaneous mammary tumor development was monitored. Mice were administered aspirin at 120 mg/kg/day by subcutaneously injection; rapamycin at 5 mg/kg twice per week by intraperitoneal injection; and bevacizumab at 10 mg/kg once per week by intravenously injection. Each group contains 10 mice. The incidence of spontaneous tumor
growth in MMTV-neu mice was based on tumors larger than 5 mm in diameter in the mammary gland. (B) Representative images of immunofluorescence staining of tumor tissues with the indicated antibodies. The relative fluorescence intensity from the images is shown on the right. The data represent the mean ± SD (10 mice/group); *, p < 0.05.
A. Diet-induced obesity (DIO)

B. Genetic obesity (OB)

*P<0.05

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A diet-induced obesity

B Genetic obesity

**Fig. 2. Chen et al.**

Fluorescent intensity (x1000 unit)

**ND**    **HFD**

**TNFα**

**pIKKβ S176/180**

**pS6 230/256**

**VEGF**

**WT**    **OB**

**TNFα**

**pIKKβ S176/180**

**pS6 230/256**

**VEGF**

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Fig. 3. Chen et al.

A  

CD31

ND  

OB  

HFD  

CD31 positive cells

0  4  8  12  16  

ND  OB  HFD  

B  

Ki67

ND  

OB  

HFD  

Intensity (x1000)

0  1  2  3  

ND  OB  HFD  

* *
Fig. 4. Chen et al.

A

B

C

Control
Aspirin

Control
Aspirin

Control
Aspirin

Control
Reapamycin

Aspirin

Reapamycin

Bevacizumab

Control
Bevacizumab

Control
Bevacizumab

HFD
ND
HFD
ND
HFD
ND
HFD
ND
HFD
ND
HFD

Tumor volume (mm³)

Tumor volume (mm³)

Tumor volume (mm³)

plκκβ
S176/180

pS6
236/238

pVEGFR
S996

Intensity (x1000)

Intensity (x1000)

Intensity (x1000)

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**A**

Figure 5. Chen et al.

**MMTV-neu**

![Graph showing tumor-free mice over age in days for HFD and ND groups with a p-value of 0.003.]

**B**

**MMTV-neu**

![Images and bar graphs showing fluorescent intensity for TNFα, pIκKβ S176/180, pS8 S235/236, VEGF, and pVEGFR S998 under ND and HFD conditions.]

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
**Fig. 6. Chen et al.**

**A** MMTV-neu HFD

![Graph showing tumor-free mice over time with different treatment groups (HFD, HFD/Aspirin, HFD/Rapa, HFD/Bev).](image)

**B** MMTV-neu HFD

![Images and bar graphs showing fluorescence intensity changes with different treatments.](image)
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