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Postmenopausal obesity promotes tumor angiogenesis and breast cancer progression in mice

Jian-Wei Gu,[1,2,*] Emily Young,[2] Sharla G. Patterson,[1,3] Kristina L. Makey,[2] Jeremy Wells,[2] Min Huang,[2] Kevan B. Tucker[2] and Lucio Miele[1]

[1]Cancer Institute; [2]Department of Physiology and Biophysics,[3] Department of Surgery; University of Mississippi Medical Center; Jackson, MS USA

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Obese postmenopausal women have a 50% higher risk of breast cancer than non-obese women. There is not an animal model that mimics postmenopausal obesity related to breast cancer progression. Using age-relevant C57BL/6 mice, this study determined whether postmenopausal obesity increases VEGF expression, tumor angiogenesis and breast tumor growth. Ovariectomy (OVX) was performed in 12 sixty week-old female mice, then followed by a low-fat (5%, LF, n = 6) or a high-fat (60%, HF, n = 6) diet for 12 weeks. In the eighth week of the dietary program, 10^6 E0771 (mouse breast cancer) cells were injected in the left fourth mammary gland. Tumor size was monitored for 4 weeks. Body weights were monitored weekly. At the end of the experiment, blood samples, visceral fat and tumors were collected for measuring VEGF expression using ELISA and intratumoral microvessel density (IMD) using CD31 immunochemistry. Body weight was significantly increased in OVX/HF mice, compared to OVX/LF group (55.3 ± 1.7 vs. 41.5 ± 1.5 g; $p < 0.01$). There was a two-fold increase in the ratio of visceral fat/BW in OVX/HF mice, compared to those in OVX/LF group (0.062 ± 0.005 vs. 0.032 ± 0.003; $p < 0.01$). Postmenopausal obesity significantly increased breast tumor weight over the control (4.62 ± 0.63 vs. 1.98 ± 0.27 g; $p < 0.01$) and IMD (173 ± 3.7 vs. 139 ± 4.3 IM#/mm^2; $p < 0.01$). Tumor VEGF levels were higher in OVX/HF mice, compared to OVX/LF group (73.3 ± 3.8 vs. 49.5 ± 4.3 pg/mg protein; $p < 0.01$). Plasma VEGF levels (69 ± 7.1 vs. 48 ± 3.5 pg/ml) and visceral fat VEGF levels (424.4 ± 39.5 vs. 208.5 ± 22.4 pg/mg protein) were significantly increased in OVX/HF mice, compared to OVX/LF group, respectively ($n = 6$; $p < 0.01$). Interestingly, adipose tissue primary culture showed that subcutaneous fat released more VEGF, compared to visceral fat (6.77 ± 1.14 vs. 0.94 ± 0.16 pg/mg tissue; $n = 6$; $p < 0.01$). These findings support the hypothesis that postmenopausal obesity promotes tumor angiogenesis and breast cancer progression, possibly through increased adipose tissue mass and adipokines such as VEGF that could systemically and locally affect breast cancer progression.

Introduction

Epidemiological studies suggest that obesity is an established risk factor for breast cancer, particularly for estrogen receptor-positive (ER^+) tumors, in postmenopausal women.[1-5] Also, overweight/obesity is associated with a 78–91% increased risk of recurrence and a 36–56% increased risk of death in women with breast cancer regardless of menopausal status.[6] Obese postmenopausal women have a 50% higher risk of breast cancer than non-obese women.[7] The mechanisms of postmenopausal obesity-induced breast cancer are poorly understood due to the lack of an established animal model. On the other hand, many studies have found there is an inverse relationship between weight or body mass index (BMI) and breast cancer in premenopausal women.[8-11] The increased incidence of breast cancer in lean young women is found to be strongest amongst the youngest age group (≤35 years).[8] However, these relationships have not been reflected in animal studies such as ovariectomy-induced, high-fat diet-induced and monogenetic obese mice, in which the mice were relatively young and failed to mimic human postmenopausal breast cancer. For example, using relatively young female mice shows that high-fat diet-induced obesity does not affect mammary tumor development.[12] There is as yet no reliable animal model to study the effects of postmenopausal obesity on ER^+ breast cancer. Human xenograft models require immunocompromised animals, and thus lack tumor microenvironment components associated with the immune system. In addition, transgenic models carrying oncogenes are generally ER-negative. The establishment of an animal model that faithfully mimics human postmenopausal breast cancer is essential for determining the potential mechanisms underlying obesity-induced postmenopausal breast cancer. We hypothesize that ovariectomy (OVX) plus a high-fat diet with the inoculation of E0771 (mouse ER^+ breast cancer) cells in female wild-type >60 week old

*Correspondence to: Jian-Wei Gu; Email: jgu@umc.edu
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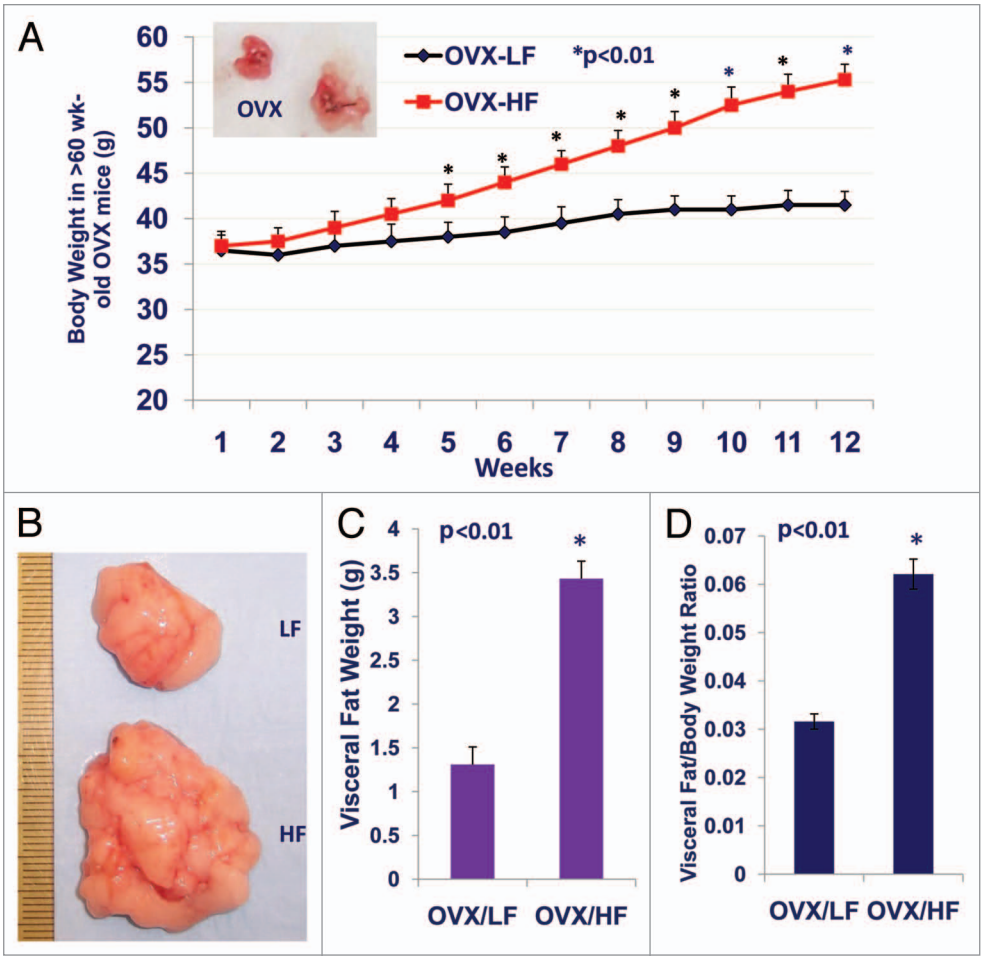

Figure 1. (A) Shows the images of both ovaries (OVX) removed from a mouse and the body weight profiles for >60 wk old OVX female C57BL/6 mice fed HF and LF for 12 weeks. High-fat (60%) diet significantly increased the body weight in >60 wk old ovariectomized (OVX) mice, compared to the OVX mice with low-fat (5%) diet, after 4 weeks of dietary program ($p < 0.05$). After 12 weeks of dietary program, body weight was significantly increased in OVX/HF mice, compared to OVX/LF group (55.3 ± 1.7 vs. 41.5 ± 1.5 g; $p < 0.01$). (B) Shows the images of visceral fat isolated from OVX female C57BL/6 mice fed with LF and HF. (C) The visceral fat weight was significantly increased in OVX/HF mice, compared to OVX/LF group (3.43 ± 0.47 vs. 1.31 ± 0.14 g; $p < 0.01$). (D) There was a two-fold increase in the ratio of visceral fat/BW in OVX/HF mice, compared to those in OVX/LF group (0.062 ± 0.005 vs. 0.032 ± 0.003; $p < 0.01$).

mice (C57BL/6; life span is about 140 weeks) can mimic human obesity-induced postmenopausal breast cancer.

The growth and expansion of a tumor is mainly dependent on angiogenesis, the formation of new capillaries from pre-existing blood vessels. Angiogenesis requires stimulation of vascular endothelial cells through the release of angiogenic factors. Of these, the vascular endothelial growth factor (VEGF) is the most critical regulator in the development of the vascular system and is commonly overexpressed in a variety of human solid tumors including breast cancer.[13] We have recently reported that VEGF and its receptors are the important biological markers for breast cancer malignancy and progression, and have demonstrated that the paracrine effects (especially angiogenesis) and the autocrine effects (proliferation and migration) of VEGF are involved in promoting breast cancer progression.[14] In addition, we have reported that obesity markedly increases melanoma tumor growth rate by mechanisms that involve the upregulation of VEGF pathways.[15]

The present study determines the following: (1) whether postmenopausal obesity increases VEGF expression, tumor angiogenesis and breast tumor growth in mice; (2) whether increased visceral fat size is correlated to increased breast tumor size; and (3) whether there is a difference in VEGF release from visceral fat compared to subcutaneous fat.

Results

High-fat diet increased body weight and visceral fat mass in >60 week-old ovariectomized female mice. In this study, 12 sixty-week old, C57BL/6J ovariectomized female mice were randomized to receive a high-fat (60%, HF, Harlan Laboratories TD.06414, n = 6) diet or a low-fat (4%, LF, Harlan Laboratories TD.02016, n = 6) diet for 12 weeks. Baseline body weight for all overiectomized female mice was 26.5 ± 1.6 grams (±SE); values among the two groups were not significantly different ($p < 0.05$). **Figure 1A** showed that high-fat (60%) diet significantly increased the body weight in >60 week old ovariectomized (OVX) mice, compared to the OVX mice with low-fat (4%) diet, after 4 weeks of dietary program ($p < 0.05$). After 12 weeks of dietary program, body weight was significantly increased by 33% in OVX/HF mice, compared to OVX/LF group (55.3 ± 1.7 vs. 41.5 ± 1.5 g; $p < 0.01$). The visceral fat from each mouse was weighed and the images of visceral fat from the mice with HF or LF diet were shown on **Figure 1B**. **Figure 1C** showed that the visceral fat weight was significantly increased in OVX/HF mice, compared to OVX/LF group (3.43 ± 0.47 vs.1.31 ± 0.14 g; $p < 0.01$). **Figure 1D** showed that there was a two-fold increase in the ratio of visceral fat/BW in OVX/HF mice, compared to those in OVX/LF group (0.062 ± 0.005 vs. 0.032 ± 0.003; $p < 0.01$).

Postmenopausal obesity promoted breast tumor growth in female mice. In this study, ovariectomy (OVX) was performed in 12 sixty week-old female mice, then followed by a low-fat (5%, LF, n = 6) or a high-fat (60%, HF, n = 6) diet for 12 weeks. In the 8 week of the dietary program, 10^6 E0771 (mouse breast cancer) cells were injected in the left fourth mammary gland. Tumor size was monitored every other day in two perpendicular

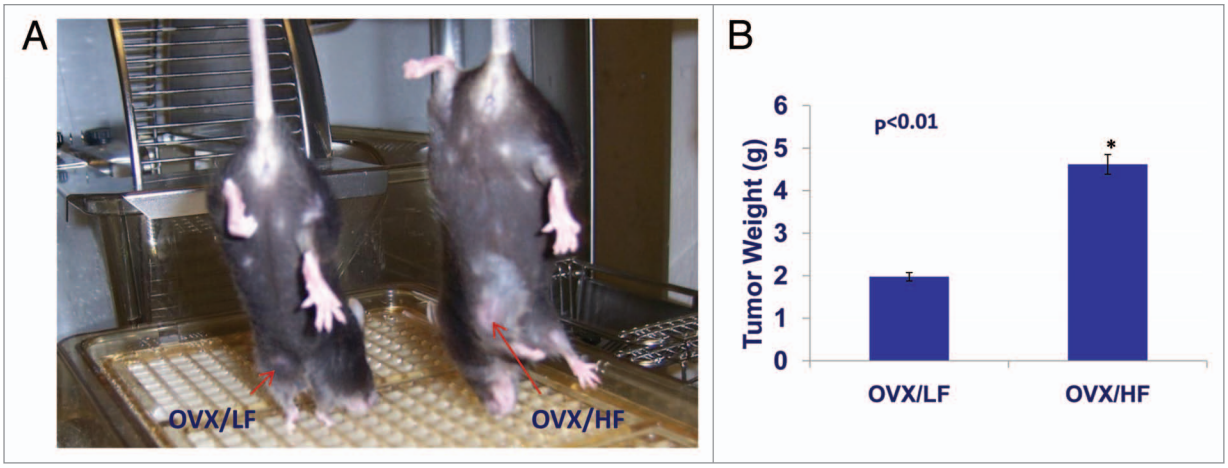

Figure 2. Twelve weeks of high-fat diet significantly increased breast tumor weight in >60 wk old ovariectomized (OVX) mice, compared to those mice with low-fat diet (4.62 ± 0.63 vs. 1.98 ± 0.27 g; N = 6; p < 0.01).

dimensions parallel with the surface of the mice using dial calipers. The tumor growth rate in obese mice was higher than those in non-obese mice since palpable tumors appeared by the fourth day after the inoculation of the cancer cells in obese mice and by the seventh day after the inoculation of the cancer cells in non-obese mice. At the conclusion of the experiment, postmenopausal obesity (OVX/HF) significantly increased breast tumor weight over the control (OVX/LF) (4.62 ± 0.63 vs. 1.98 ± 0.27 g; N = 6; p < 0.01) (**Fig. 2**). Interestingly, linear regression analysis showed there was a strong positive linear correlation between increased breast tumor weight and visceral fat weight in OVX mice (R^2 = 0.7379; N = 12; p < 0.01) (**Fig. 3**). However, there was no significant difference in heart or kidney weight/body weight ratio between postmenopausal obesity (OVX/HF) mice and the control mice (OVX/LF) as indicated in **Table 1**. These results clearly demonstrated that postmenopausal obesity-induced breast tumor growth was specifically associated with increased adipose tissue mass.

Postmenopausal obesity promoted tumor angiogenesis of breast cancer in mice. Growth and expansion of tumor mass was mainly dependent on angiogenesis because neovascularization contributes to rapid tumor growth by providing an exchange of nutrients, oxygen and paracrine stimulus of the tumor.[16] Therefore, in this study, we used a morphometric analysis of immunohistochemical staining for CD31 to determine the effect of postmenopausal obesity on tumor angiogenesis of breast cancer. Representative images of CD31 staining of the breast cancer tumors showed that the control tumor (**Fig. 4A**) had fewer microvessels than the postmenopausal obesity-induced tumor (**Fig. 4B**). Morphometric analysis (**Fig. 4C**) indicated that postmenopausal obesity (OVX/HF) caused a significant increase in average microvessel density (AMVD, the number of microvessels per mm^2 area) of breast cancer tumors when compared to the control (OVX/LF) breast cancer tumors (173 ± 3.7 vs. 139 ± 4.3 microvessels number per mm^2; n = 6; p < 0.01).

Postmenopausal obesity increased VEGF protein levels in plasma and breast tumor in mice. In **Figure 5**, ELISA assay

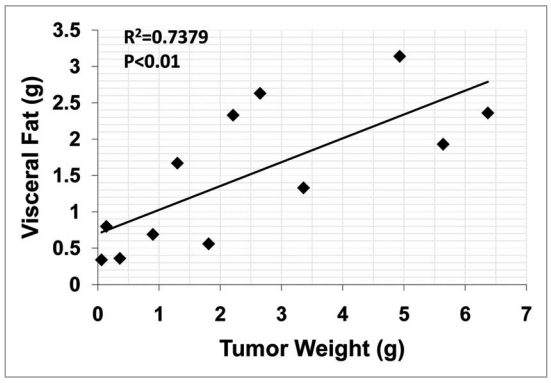

Figure 3. Linear regression analysis showed there was a strong positive linear correlation between increased breast tumor weight and visceral fat weight in OVX mice (R^2 = 0.7379; N = 12; p < 0.01).

Table 1. The ratio of visceral fat, heart and kidneys over body weight in OVX/LF and OVX/HF mice

	Visceral fat/ Body weight	Heart/Body weight	Kidneys/Body weight
OVX/LF	0.0316 ± 0.0031	0.00312 ± 0.00044	0.00910 ± 0.00017
OVX/HF	0.0621 ± 0.0052	0.00293 ± 0.00039	0.00869 ± 0.00021
t-test	p < 0.001	p = 0.3456	p = 0.2735

N = 6 in each group; significant difference if p < 0.05 between OVX/LF vs. OVX/HF group.

showed that plasma VEGF levels (69 ± 7.1 vs. 48 ± 3.5 pg/ml) and tumor VEGF levels (73.3 ± 3.8 vs. 49.5 ± 4.3 pg/mg protein; p < 0.01) were significantly increased in OVX/HF mice, compared to OVX/LF group, respectively (n = 6; p < 0.01). This clearly demonstrated that postmenopausal obesity-induced breast tumor growth and angiogenesis were associated with increased VEGF expression in circulation and breast tumors.

VEGF protein levels in visceral fat versus subcutaneous fat. VEGF was the most critical stimulator in angiogenesis and

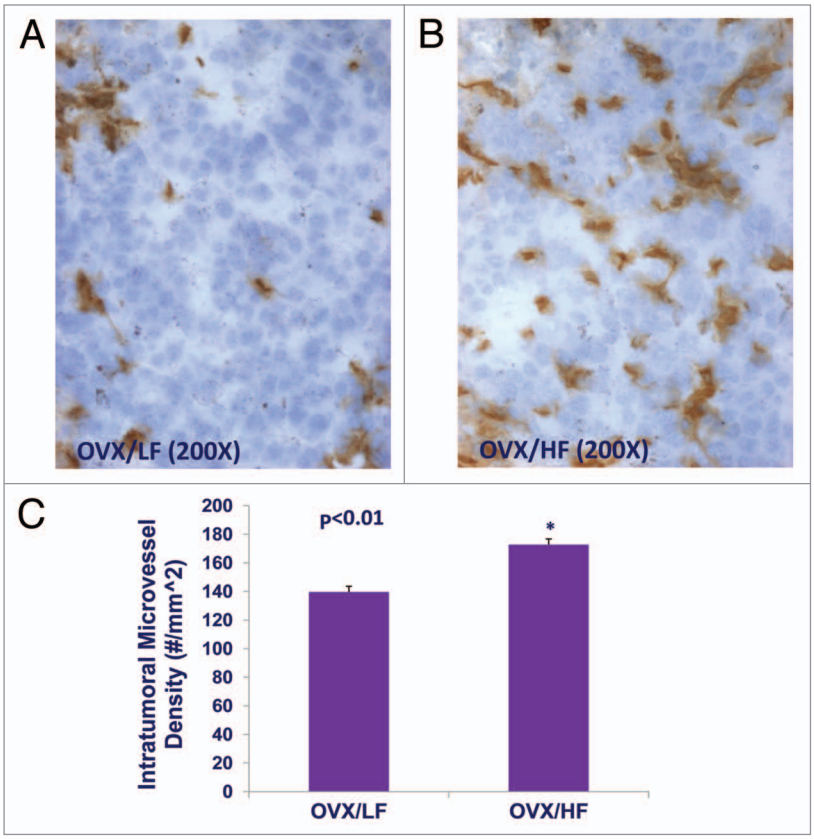

Figure 4. The digital images of CD31 immunohistochemistry staining in OCT-embedded cryosections of mouse breast cancer tumors obtained from >60 wk old OVX female C57BL/6 mice fed LF (A) and HF (B) for 12 weeks. Sections were incubated with rat anti-mouse CD31 antibody followed by mouse anti-rat IgG (Vector laboratories, Burlingame, CA), Extravadin Peroxidase (Sigma, St. Louis, MO) and peroxidase substrate (Vector laboratories, Burlingame, CA). Sections were counterstained with hemotoxylin. The brown staining indicated microvascular vessels. Morphometric analysis (C) indicated that postmenopausal obesity (OVX/HF) caused a significant increase in average microvessel density (AMVD, the number of microvessels per mm^2 area) of breast cancer tumors when compared to the control (OVX/LF) breast cancer tumors (173 ± 3.7 vs. 139 ± 4.3 microvessels number per mm^2; n = 6; p < 0.01).

considered to be one of the key adipokines.[17] As we discovered that postmenopausal obesity-induced breast tumor angiogenesis and growth were associated with increased adipose tissue mass (visceral fat), we further determined whether VEGF protein levels in visceral fat were different between posmenopausal obese (OVX/HF) mice and the control (OVX/LF) mice, and whether there was a different VEGF expression in visceral fat compared to subcutaneous fat in postmenopausal obese (OVX/HF) mice. ELISA assay showed that VEGF levels in visceral fat (424.4 ± 39.5 vs. 208.5 ± 22.4 pg/mg protein) were significantly increased in OVX/HF mice, compared to those in OVX/LF group (n = 6; p < 0.01) (**Fig. 6A**). ELISA assay showed that abdominal subcutaneous fat expressed more VEGF proteins than visceral fat in OVX/HF mice (692 ± 72 vs. 431 ± 44 pg/mg protein; n = 6; p < 0.01) (**Fig. 6B**). The adipose tissue primary culture showed that subcutaneous fat released more VEGF, compared to visceral fat (6.77 ± 1.14 vs. 0.94 ± 0.16 pg/mg tissue; n = 6; p < 0.01) (**Fig. 6C**).

Discussion

The major new findings from this study include the following: (1) A high-fat diet significantly increases body weight in >60 week-old ovariectomized female mice, in which adipose tissue mass (visceral fat) is more siginficantly increased than body weight. (2) Postmenopausal obesity (OVX/HF) significantly increases breast tumor weight over the control (OVX/LF), in which there is a strong positive linear correlation between increased breast tumor weight and visceral fat weight in OVX mice. (3) Postmenopausal obesity significantly promotes breast tumor angiogenesis, which is associated with increased VEGF protein levels in plasma and breast tumors in comparison with those in the control mice (OVX/LF). (4) There is a significant increase in adipose tissue (visceral fat) mass that is associated with increased VEGF protein levels within visceral fat in postmenopausal obese mice, compared to those in the control (OVX/LF) mice. (5) Abdominal subcutaneous fat expresses more VEGF proteins than visceral fat in postmenopausal obese mice.

Obese postmenopausal women have a 50% higher risk of breast cancer than non-obese women.[7] On the other hand, many studies have found there is an inverse relationship between weight or body mass index (BMI) and breast cancer in premenopausal women.[8-11] There is as yet no reliable animal model to study the effects of postmenopausal obesity on breast cancer progression. For example, using relatively young female mice shows that high-fat diet-induced obesity does not affect mammary tumor development.[12] However, in the present study, the results indicate that ovariectomy (OVX) plus a high-fat diet with the inoculation of E0771 (mouse ER^+ breast cancer) cells in female wild-type >60 week old mice (C57BL/6; life span is about 140 weeks) can mimic human obesity-induced postmenopausal breast cancer. All these findings as mentioned above support the hypothesis that postmenopausal obesity promotes tumor angiogenesis and breast cancer progression, possibly through increased adipose tissue mass and adipokines such as VEGF that may systemically and locally affect breast cancer progression. The establishment of an animal model that faithfully mimics human postmenopausal breast cancer is essential for determining the potential mechanisms underlying obesity-induced postmenopausal breast cancer.

The growth and expansion of a tumor is mainly dependent on angiogenesis. Angiogenesis is also required for adipose tissue expansion during weight gain and a direct relationship between obesity and angiogenesis has been demonstrated by experiments in which angiogenesis inhibition prevents obesity and causes weight loss in genetically obese mice.[18] VEGF is a key angiogenic factor. One mechanism by which obesity has been suggested to contribute to enhanced tumor growth is by

promoting angiogenesis. Our finding that plasma VEGF levels are significantly higher in postmenopausal obese mice (OVX/HF) than those in the control mice (OVX/LF), supports a role for enhanced angiogenesis in postmenopausal obese mice. Using E0771 (mouse breast cancer) cells in C57BL/6 mice, we have recently reported that VEGF and its receptors are the important biological markers for breast cancer malignancy and progression, and demonstrated that the paracrine effects (especially angiogenesis) and the autocrine effects (proliferation and migration) of VEGF are involved in promoting breast cancer progression.[14] Thus, enhanced VEGF expression and angiogenesis in obesity contribute to breast cancer progression.

Silha et al. have reported that plasma VEGF is elevated in overweight and obese humans.[19] In the present study, we have found there is a significant increase in plasma VEGF protein levels in postmenopausal obese mice and other obese mouse models, such as obese melanocortin receptor 4 knockout ($MC4R^{-/-}$) mice[15] and obese leptin-deficient ($ob^{-/-}$) mice.[15] However, the mechanisms of obesity-induced VEGF expression are not clear. Obesity is associated with chronic low-grade tissue hypoxia,[20] oxidative stress,[21] and inflammation.[22] These conditions likely enhance VEGF expression in obesity since VEGF can be upregulated by hypoxia, oxidative stress and inflammation.[13]

Epidemiological studies suggest that overweight/obesity has been causally associated with breast cancer in postmenopausal breast cancer and a poor prognosis regardless of menopausal status, which is associated with increased adipose tissue-derived adipokines such as vascular endothelial growth factor (VEGF) and leptin.[17] Interestingly, in the present study, 12 weeks of a high-fat diet more significantly increases visceral fat mass (162% increase) than body weight (33% increase) in postmenopausal obese (OVX/HF) mice, compared to the control (OVX/LF) mice. There is a two-fold increase in the ratio of visceral fat/body weight in postmenopausal obese (OVX/HF) mice, compared to those in the control (OVX/LF) mice. In addition, VEGF protein levels in visceral fat are significantly increased in OVX/HF mice, compared to those in OVX/LF group. These findings support the notion that increased visceral fat mass and increased VEGF expression in visceral fat contribute to enhanced VEGF expression and angiogenesis in postmenopausal obesity, which is consistent with the evidence of a strong positive linear correlation between increased breast tumor weight and visceral fat weight in OVX mice. All these results support the hypothesis that postmenopausal obesity promotes tumor angiogenesis and breast cancer progression, possibly through increased adipose tissue mass and adipokines such as VEGF.

It has been shown that the difference in regional body fat distribution is a critical factor in the association between obesity and related metabolic complications.[23] Visceral obesity is defined as fat accumulation around viscera and inside the intra-abdominal solid organs.[24] The amount of visceral fat is increased with age in both genders.[25] Abdominal adiposity (as measured by waist circumference) has also been found to be positively associated with risk of postmenopausal breast cancer.[26] It has been shown that visceral adipose tissue secretes more interleukin-6 and tumor necrosis factor-α, and less adiponectin compared to subcutaneous

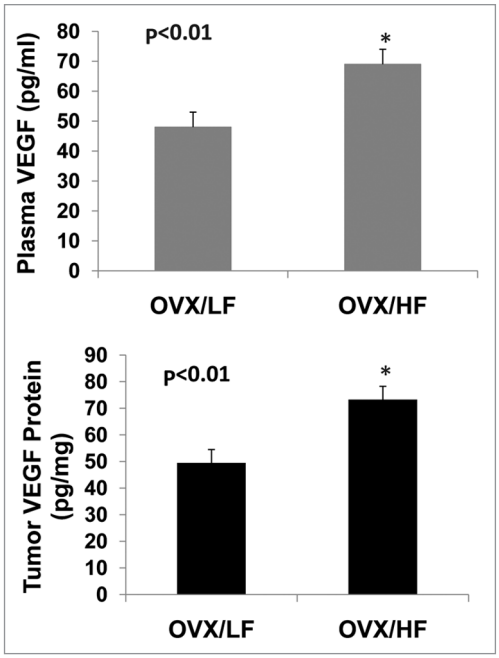

Figure 5. Effects of a high-fat diet on VEGF protein levels in plasma and breast tumors of OVX mice. ELISA assay showed that plasma VEGF levels (69 ± 7.1 vs. 48 ± 3.5 pg/ml) and tumor VEGF levels (73.3 ± 3.8 vs. 49.5 ± 4.3 pg/mg protein; $p < 0.01$) were significantly increased in OVX/HF mice, compared to OVX/LF group, respectively ($n = 6$; $p < 0.01$).

fat.[27] However, there is no previous information on whether VEGF protein levels are different between visceral and subcutaneous fat in obesity. In present study, the adipose tissue primary culture shows that subcutaneous fat releases more VEGF, compared to visceral fat in postmenopausal obese (OVX/HF) mice and abdominal subcutaneous fat expresses more VEGF proteins than visceral fat in OVX/HF mice. These findings indicate that subcutaneous fat releases more VEGF than visceral fat in postmenopausal obesity. The increased conversion of androgens to estrogens by the aromatase enzyme in peripheral adipose tissues[28] along with reduced levels of serum sex hormone binding globulin have been hypothesized to be the main link between obesity and increased risk of postmenopausal breast cancer.[29] The paracrine effects (especially angiogenesis) and the autocrine effects (proliferation and migration) of VEGF are involved in promoting breast cancer progression.[14] These findings suggest that increased VEGF expression in subcutaneous fat may also locally promote breast cancer progression in postmenopausal obesity.

Conclusions and perspectives. Our observations indicate that ovariectomy plus a high-fat diet with the inoculation of E0771 (mouse ER^+ breast cancer) cells in female wild type >60 week old mice can mimic human obesity-induced postmenopausal breast cancer. Postmenopausal obesity promotes tumor angiogenesis and breast cancer progression, which is associated with increased adipose tissue mass (visceral fat), increased plasma VEGF and increased VEGF expression in visceral fat and breast tumors. Subcutaneous fat releases more VEGF than visceral fat in postmenopausal obese mice. These findings support the hypothesis that postmenopausal obesity promotes

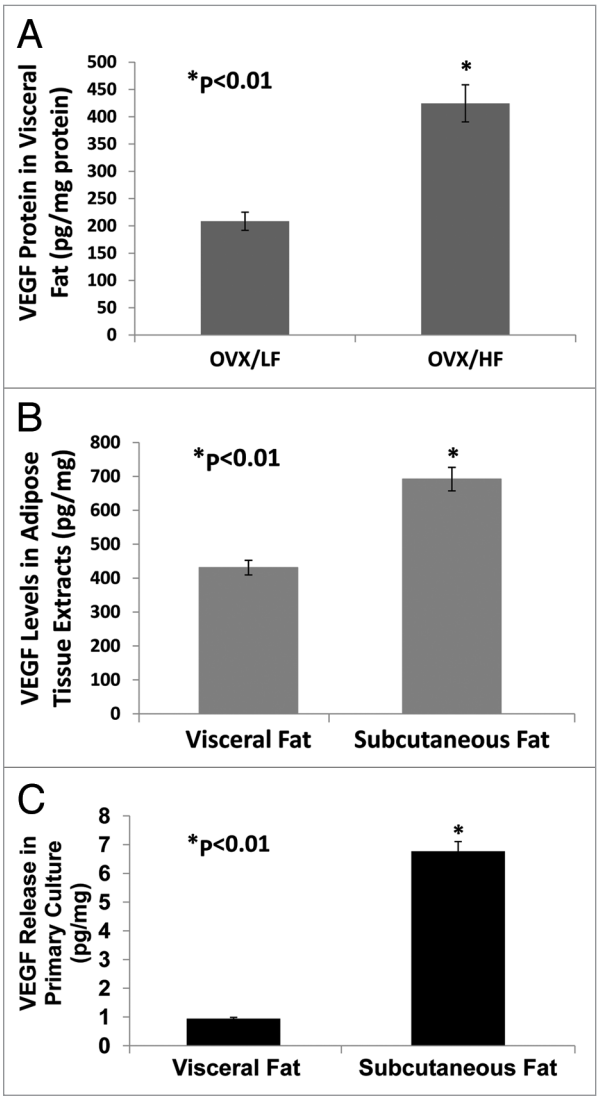

Figure 6. Differential expression of VEGF protein in visceral fat between OVX/LF and OVX/HF mice and those in visceral fat compared to subcutaneous fat in postmenopausal obese (OVX/HF) mice. (A) ELISA assay showed that VEGF levels in visceral fat (424.4 ± 39.5 vs. 208.5 ± 22.4 pg/mg protein) were significantly increased in OVX/HF mice, compared to those in OVX/LF group (n = 6; $p < 0.01$). (B) The abdominal subcutaneous fat expressed more VEGF proteins than visceral fat in OVX/HF mice (692 ± 72 vs. 431 ± 44 pg/mg protein; n = 6; $p < 0.01$). (C) The adipose tissue primary culture showed that subcutaneous fat released more VEGF, compared to visceral fat (6.77 ± 1.14 vs. 0.94 ± 0.16 pg/mg tissue; n = 6; $p < 0.01$).

tumor angiogenesis and breast cancer progression, possibly through increased adipose tissue mass and adipokines such as VEGF that could systemically and locally affect breast cancer progression. The establishment of an animal model that faithfully mimics human postmenopausal breast cancer is essential for determining the potential mechanisms underlying obesity-induced postmenopausal breast cancer. Future studies will address whether VEGF interplays with leptin and estrogen to promote breast cancer progression in postmenopausal obesity using our novel animal model. More studies are required to strengthen the mechanisms of postmenopausal obesity-induced breast cancer progression.

Materials and Methods

Animal protocols. The protocols were carried out according to the guidelines for the care and use of laboratory animals implemented by the National Institutes of Health and the Guidelines of the Animal Welfare Act and were approved by the University of Mississippi Medical Center's Institutional Animal Care and Use Committee. C57BL/6J female mice used in this study were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were allowed to acclimate for 1 week for housing individually in shoebox cages in a temperature controlled room kept on a 12/12 hr light/dark cycle and given regular mouse chow and water ad libitum. At sixty weeks of age, C57BL/6J ovariectomized female mice were randomized to receive a high-fat (60%, HF, Harlan Laboratories TD.06414, n = 6) diet or a low-fat (4%, LF, Harlan Laboratories TD.02016, n = 6) diet for 12 weeks. In the 8th wk of the dietary program, all mice were inoculated subcutaneously on the left pad of the fourth mammary gland with 100 μl of 10^6 E0771 cells suspended in phosphate-buffered saline, using a 23-gauge needle. The body weight of the mice was monitored weekly. Tumor size was monitored every other day in two perpendicular dimensions parallel with the surface of the mice using dial calipers. After 12 weeks of the dietary program, all mice were anesthetized with isoflurane and the blood samples and tumors were collected. The tumors were weighed for analysis. Then they were placed into either liquid nitrogen for total protein extraction and nuclear protein extraction or embedded in OCT compound (Sakura Finetek, Torrance, CA) for immunohistological study.

Ovariectomy. Surgical removal of the ovaries is a well-characterized approach to mimic the postmenopausal state in mice.[30] Sixty-week old C57BL/6J female mice were anesthetized with isoflurane. Hair was clipped over the surgical area and scrubbed with Betadine and ethanol swipe. A small midline incision (~1.0 cm) was made in the skin halfway between the middle of the back and the base of the tail, starting at the last rib. The skin was moved to one side and a small incision was made through the peritoneal lining on each side. The entire ovary was removed with a single cut between the fallopian tube and the uterine horn. The skin was then closed with 5-0 silk. The removed ovaries on both sides showed on the image in **Figure 1A**.

Morphometric analysis of tumor angiogenesis. The quantification of blood vessels in tumor tissues was determined by our previously reported methods.[31,32] Consecutive thin cryosections (5 μm) of OCT compound (Sakura Finetek, Torrance, CA) embedded tumor tissues were fixed in acetone at 4°C for 10 min. After washing in phosphate-buffered saline (PBS), the sections were treated with 3% H_2O_2 for 10 minutes to block endogenous peroxidase activity, and then blocked with normal rabbit serum. Then, the sections were washed in PBS and incubated with rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Pharmingen, San Diego, CA) at a 1:200 dilution overnight at 4°C. Negative controls were incubated with rat serum IgG at the same dilution. All sections were washed in PBS containing

0.05% Tween-20, and then incubated with a second antibody, mouse anti-rat IgG (Vector laboratories, Burlingame, CA), at a 1:200 dilution for 1 hour at room temperature, again followed by washing with PBS containing 0.05% Tween-20. The sections were incubated in a 1:400 dilution of Extravadin Peroxidase (Sigma, St. Louis, MO) for 30 min. After washing in PBS containing 0.05% Tween-20, the sections were incubated in peroxidase substrate (Vector laboratories, Burlingame, CA) for 5 min. The sections were washed in PBS containing 0.05% Tween-20 and were counterstained with hematoxylin. A positive reaction was indicated by brown staining. The microvascular vessels were quantified by manual counting under light microscopy. A microscopic field (0.7884 mm^2) was defined by a grid laced in the eye-piece. At least 20 microscopic fields were randomly taken from each tumor for analysis. Any endothelial cell or cell cluster showing antibody staining and clearly separated from an adjacent cluster was considered to be a single, countable microvessel. The value of average microvascular density (AMVD) was determined by calculating the mean of the vascular counts per mm^2 obtained in the microscopic fields for each tumor sample.

Visceral fat collection and adipose tissues primary culture. At the end of the experiment, the mice with HF or LF diet were anesthetized with isoflurane and the intra-abdominal fat (visceral fat) was collected from each mouse. The visceral fat from each mouse was weighed and the images of visceral fat from the mice with HF or LF diet were shown on **Figure 1B**. The abdominal subcutaneous fat was isolated with an angled blade. For the primary cultures of adipose tissues, the handling of visceral fat and abdominal subcutaneous fat were done under aseptic conditions. The abdominal subcutaneous fat or visceral fat tissue was cut with scissors into small pieces (20–30 mg); the tissue explants (100 mg/ml) were then incubated for 24 hours in suspension culture with M199 media (GIBCO) supplemented with 10% FBS (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B and incubated at 37°C in a humidified 5% CO_2/air injected atmosphere. Aliquots of the medium were taken and stored in a liquid nitrogen tank for measurement of VEGF.

Measurements of protein levels of VEGF by ELISA. Protein levels of VEGF in plasma, breast tumor, visceral fat, abdominal subcutaneous fat and the medium cultured with adipose tissue explants were determined using mouse VEGF and VEGF Flt-1 ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The total proteins of breast tumor, visceral fat, abdominal subcutaneous fat were extracted using NE-PER Cytoplasmic Extraction Reagents (Pierce, Rockford, IL), according to the manufacturer's protocol. The total protein concentration of these tissue extractions was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The protein concentrations of VEGF were normalized and expressed as pictograms per milligram of total tissue extraction protein. For adipose tissue primary culture, VEGF levels were normalized and expressed as pictograms per milligram of total tissue explants.

Statistical analysis. All determinations were performed in duplicated sets. Where indicated, data is presented as mean ± SE. Statistically significant differences in mean values between the two groups were tested by an unpaired Student's t-test. Linear regression was performed by the correlation analysis between two continuous variables. A value of $p < 0.05$ was considered statistically significant. All statistical calculations were performed using SPSS software (SPSS Inc., Chicago, IL).

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