The anti-angiogenic herbal composition Ob-X inhibits adipose tissue growth in obese mice

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Running title: Suppression of obesity by angiogenesis inhibitor

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Abstract

Objective: The growth and development of adipose tissue are thought to be associated with angiogenesis and extracellular matrix remodeling. Since the composition of the herbal extract called Ob-X has been shown to have both anti-angiogenic and matrix metalloproteinase (MMP)-inhibiting activities, we hypothesized that growth of adipose tissue can be regulated by Ob-X.

Materials and Methods: The effects of Ob-X on angiogenesis and extracellular matrix remodeling were measured using *in vitro* and *ex vivo* assays. The effects of Ob-X on adipose tissue growth were investigated with nutritionally obese mice.

Results: Ob-X inhibited angiogenesis in a dose-dependent manner in the human umbilical vein endothelial cell (HUVEC) tube formation assay *in vitro* and the rat aortic ring assay *ex vivo*. Ob-X also suppressed MMP activity *in vitro*. Administration of Ob-X to high fat diet-induced obese mice produced significant reductions in body weight gain and adipose tissue mass, compared to control. The mass of both subcutaneous (SC) and visceral (VSC) fat was reduced in Ob-X-treated mice. The size of adipocytes in SC and VSC adipose tissues was also significantly reduced in Ob-X-treated mice. Ob-X treatment decreased the blood vessel density and MMP activity in VSC adipose tissues of nutritionally obese mice. Ob-X reduced mRNA levels of angiogenic factors (VEGF-A and FGF-2) and MMPs (MMP-2 and MMP-9), whereas it increased mRNA levels of angiogenesis inhibitors

(TSP-1 and TIMP-2) in SC and VSC adipose tissues of nutritionally obese mice.

Conclusion: Ob-X, which has anti-angiogenic and MMP-inhibitory activities, reduces adipose tissue mass in nutritionally induced obese mice, providing evidence that adipose tissue growth and development may be prevented by inhibiting angiogenesis. In addition, these data suggest that regulation of adipose tissue growth by inhibiting angiogenesis may alter the expression of genes involved in angiogenesis and the MMP system.

Keywords: obesity; adipose tissue growth; angiogenesis; MMP; inhibition

Introduction

Obesity is the result of an energy imbalance caused by an increased ratio of caloric intake to energy expenditure. In conjunction with obesity, related metabolic disorders such as dyslipidemia, atherosclerosis, and type 2 diabetes have become global health problems. Obesity is characterized by increased adipose tissue mass that results from both increased fat cell number (hyperplasia) and increased fat cell size (hypertrophy).[1] Development of obesity is associated with extensive modifications in adipose tissue involving adipogenesis, angiogenesis, and remodeling of extracellular matrix (ECM).[2]

Similar to neoplastic tissues, angiogenesis—the formation of new blood vessels from pre-existing vessels—occurs in the growing adipose tissue of adults.[2] Most tissues normally do not grow throughout adulthood, and the supporting vasculature is quiescent,[3] whereas adipose tissue can grow and regress throughout life. Adipose tissue is highly vascularized, and each adipocyte is nourished by an extensive capillary network.[2,4,5] It is suggested, therefore, that adipose tissue growth is angiogenesis-dependent and may be inhibited by angiogenesis inhibitors. This is supported by reports that treatment with angiogenesis inhibitors resulted in weight reduction and adipose tissue loss, showing that adipose tissue mass is sensitive to angiogenesis inhibitors and can be regulated by its vasculature.[6-8]

Extensive changes in ECM remodeling have also been shown to occur during adipose tissue growth. Two types of proteolytic systems, the plasminogen/plasmin (fibrolytic) and matrix metalloproteinase (MMP) systems, have been implicated in tissue remodeling, via degradation of ECM and basement membrane components, or activation of adipocyte growth factors.[9,10] The MMP system plays important roles in the development of adipose tissue and microvessel maturation by modulating ECM.[10-12] In most cases, MMPs are expressed at very low levels, but expression is rapidly induced at times of active tissue remodeling associated with adipogenesis. Several lines of evidence suggest that endogenous and exogenous MMPs regulate adipogenesis.[12-14] During obesity, MMP expression is modulated in adipose tissue, and MMPs (e.g., MMP-2 and MMP-9) potentially affect adipocyte differentiation.[12,15,16] High expression of MMP-2 was observed in the adipose tissue of mice with nutritionally induced obesity, as well as in genetically obese mice.[17] More clearly, MMP inhibition impairs development of adipose tissue in mice.[18] Thus, the adipocyte-derived MMPs may be new targets for the inhibition of adipose tissue growth.

Adipose tissue produces angiogenic factors, such as vascular endothelial growth factor (VEGF)-A and fibroblast growth factor (FGF)-2, contributing to the formation of new blood vessels inside the fat pad.[19-21] VEGF-A and FGF-2 stimulate proliferation and migration of endothelial cells (ECs) and enhance adipocyte differentiation.[22-24] Adipose tissue also secretes several MMPs, including MMP-2 and MMP-9.[12] Indeed, it is well established that degradation of ECM represents the first step in the angiogenic process, and that MMP-2 and MMP-9 have been shown to be necessary for this event,[25]

indicating the synergistic actions of angiogenesis and the MMP system on the regulation of adipose tissue growth. Accordingly, we thought it plausible that growth and development of adipose tissue can be effectively regulated by Ob-X, with both anti-angiogenic and MMP-2-inhibiting properties.

We treated high fat diet-induced obese mice with Ob-X. Body and adipose tissue mass, as well as adipocyte size, were significantly reduced in Ob-X-treated mice compared to controls. Blood vessel density and MMP activity were also decreased in adipose tissue of treated mice. The expression of angiogenic factors, MMPs, and their inhibitors in adipose tissue was markedly modulated by Ob-X. These studies suggest that Ob-X can reduce the adipose tissue mass by targeting adipose tissue.

Materials and methods

Preparation of Ob-X

Ob-X was prepared from the food grade of aqueous extracts of the three herbs *Melissa officinalis* L.(Frutarom, Wadenswil, Switzerland), *Morus alba* L.(Segae FL, Buyeo, Korea) and *Artemisia capillaries* Thunb. (Segae, FL) as previously described.[26] The quality of each herbal extract in Ob-X was controlled by standardization with reference compounds by high-pressure liquid chromatography (HPLC). The corresponding reference compounds are rosmarinic acid (*Melissa officinalis* L.), 1-deoxynojirimycin (*Morus alba* L.), and 6,7-dimethylesculetin (*Artemisia capillaries* Thunb.).

In vitro HUVEC tube formation assay

To perform tube formation assay, human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Basel, Switzerland) and cultured in M199 medium supplemented with 10% fetal bovine serum and endothelial cell growth supplement (ECGS , Sigma-Aldrich, St. Louis, MO,USA) at 50 μg/ml in a 37°C incubator with humidified atmosphere containing 5% CO_2.. 200 μl of Matrigel (BD Biosciences, Bedford, MA, USA) was pipetted into the wells of a 48-well plate and allowed to solidify for 1 h at 37°C. HUVEC were plated on Matrigel-coated wells at a density of 4×10^4 cells/well and incubated for 16 h at 37°C with medium in the absence or presence of 25, 50, and

100ug/ml of Ob-X dissolved in distilled water. The formation of capillary-like tubular networks was observed with an inverted microscope and photographed. The percentage of tubule area was quantified by image analysis using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA).

Rat aortic ring assay

The effect of Ob-X on angiogenesis was studied by culturing aortic explants in three-dimensional matrix gels, using a slightly modified version of the procedure described by Kruger and Figg.[27] Forty eight-well tissue culture plates were coated with 100 μl of Matrigel and allowed to set for 30 min at 37°C in an atmosphere of 5% CO_2. Thoracic aortas were excised from 8- to 10-week-old male Sprague-Dawley rats (SLC, Shizuoka, Japan), and the fibroadipose tissue was removed. The aortas were sectioned into 1-mm long cross sections, rinsed several times with cold endothelial cell basal medium-2 (EBM-2, Lonza), placed on the Matrigel-coated wells, and covered with an additional 50 μl of Matrigel. Aortic rings were allowed to gel for 30 min at 37°C in 5% CO_2 and cultured for 24 h with 200 μl of endothelial cell growth medium-2 (EGM-2, Lonza). After the 24-h incubation the medium was removed and replaced with EBM-2, which was either supplemented with 40 ng/ml of VEGF alone or 40 ng/ml of VEGF plus various concentrations of Ob-X. The cultures were incubated for 10 days, and the medium was replaced every 2 days. At the end of 10^{th} day, aortic rings were photographed and their images were captured for processing. The area of microvessels

was quantified by image analysis using Image-Pro Plus.

MMP assay

MMP activities were measured on a spectrofluorometer LS50B (Perkin-Elmer, Waltham, MA, USA) using 2,4-dinitrophenyl-Pro-Leu-Gly-Met-Trp-Ser-Arg (Calbiochem, San Diego, CA, USA) as a substrate for MMP-2 and MMP-9. Recombinant human MMP-2 and MMP-9 were purchased from R&D Systems (Minneapolis, MN,USA) and used after activation with 1 mM APMA before the assay. MMP (10 nM) and substrate (1 μM) were mixed in 2 ml of reaction buffer (50 mM Tricine, pH 7.5, 10 mM $CaCl_2$, 200 mM NaCl) with or without Ob-X. Fluorescence intensity was measured at room temperature using a 280-nm excitation wavelength and a 360-nm emission wavelength.

Animal study

Eight-week-old male wild-type C57BL/6J mice (n=8/group) were purchased from SLC (Shizuoka, Japan) and randomly divided into two groups. Mice were fed for 12 weeks with a high fat diet (45% kcal fat, Research Diets, New Brunswick, NJ), or the same high fat diet supplemented with Ob-X (0.2%, w/w). Body weights were measured daily by a person blinded to each treatment group. Food intake was determined by estimating the amount of food consumed by the mice throughout the treatment period. Cages were inspected for food spillage, but only a little spillage was noticed and

collected to measure the food intake. After a 12-h fast on the last day of the study, the animals were sacrificed by cervical dislocation. Blood was collected from the retroorbital sinus into tubes, and serum was separated and stored at –80°C until analysis. Visceral (VSC) and inguinal subcutaneous (SC) fat pads were removed, weighed, snap frozen in liquid nitrogen and stored at –80°C until use. Portions were prepared for histology. All animal experiments were approved by the Institutional Animal Care and Use Committees of Mokwon University, and followed National Research Council Guidelines.

Histological analysis

For hematoxylin and eosin (H&E) staining, adipose tissues were fixed in 10% phosphate-buffered formalin for 1 day and processed in a routine manner for paraffin sections. Tissue sections (5 μm) were cut and stained with H&E for microscopic examination. To quantify adipocyte number and size, the H&E-stained sections were analyzed using the Image-Pro Plus analysis system

Blood vessel staining was performed using a blood vessel staining kit (Chemicon, Billerica, MA, USA). VSC adipose tissues were fixed in 10% phosphate-buffered formalin for 1 day and processed in a routine manner for paraffin sections. Sections of 3-μm thickness were cut and irradiated in a microwave oven for the epitope retrieval. Sections were incubated with a rabbit anti-von Willebrand Factor (vWF) antibody as a primary antibody, goat anti-rabbit antibody as a secondary antibody, and

streptavidin-alkaline phosphatase solution. A freshly prepared chromogen reagent was added to sections for the visualization of blood vessel.

Zymography

MMP activity in the adipose tissue extract was determined by gelatin zymography. Adipose tissues were weighed and extracted with 10 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% NaN_3 (250 mg wet weight tissue per 1 ml of buffer) at 4°C. Protein concentrations in the adipose tissue extracts were quantified using the bicinchonic acid (BCA) method. Adipose tissue extracts were mixed with zymography sample buffer (63 mM Tris HCl, 10% Glycerol, 2% SDS, 0.0025% Bromophenol blue, pH 6.8) without heat denaturation. Culture medium of HT1080 cells was used for the molecular weight markers for MMP. Electrophoresis was performed on 10% SDS polyacrylamide gels containing 0.1% gelatin at 125 V. After electrophoresis, the gels were incubated in renaturing buffer of 0.25% Triton X-100 for 30 min at room temperature, and equilibrated in developing buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, 0.2% Brij-35) for 30 min at room temperature. The gels were then incubated in developing buffer overnight at 37ºC. The gels were stained with 0.1% Coomassie Blue R-250 and destained with 10% acetic acid in 40% methanol.

RT-PCR

Total cellular RNA was prepared using the Trizol reagent (Gibco-Brl, Grand Island, NY, USA). After 2 μg total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and an antisense primer, cDNA was generated. The RNA was denatured for 5 min at 72°C and then immediately placed on ice for 5 min. Denatured RNA was mixed with MMLV-RT, MMLV-RT buffer, and a dNTP mixture, and incubated for 1 h at 42°C. Synthesized cDNA fragments were amplified by PCR in an MJ Research Thermocycler (Waltham, MA, USA). The PCR primers used for gene expression analysis are shown in Table 1. The cDNA was mixed with PCR primers, *Taq* DNA polymerase (Solgent, Daejon, Korea), and a dNTP mixture. The reaction consisted of 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and elongation for 1 min at 72°C. The PCR products were analyzed by electrophoresis on a 1% agarose gel. Relative expression levels were presented as a ratio of target gene cDNA versus β-actin cDNA. PCR products were quantified from agarose gels using the GeneGenius (Syngene, Cambridge, UK).

Statistics

Unless otherwise indicated, all values are expressed as mean ± SD. All data were analyzed by the unpaired Student *t*-test for statistically significant differences between groups.

Results

Effect of Ob-X on HUVEC tube formation

The effect of Ob-X on HUVEC tube formation *in vitro* was examined. Figure 1 shows the capillary-like tubular network structure formed by HUVEC in the control. When Ob-X was added to the culture, the capillary-like tube formation of HUVEC was inhibited in a dose-dependent manner compared with control. The percentage of tubule area by Ob-X treatment at the concentrations of 25, 50, and 100 μg/ml decreased by 23%, 31%, and 43%, respectively.

Effect of Ob-X on rat aortic explant

The effect of Ob-X on angiogenesis was further studied using an ex vivo rat aortic ring assay, which has been shown to correlate well with in vivo events of neovascularization. In this assay, the rat aortic endothelium exposed to a three-dimensional matrix containing angiogenic factors switches to a microvascular phenotype generating branching networks of microvessels.[28]. As shown in Figure 2, microvessels grew out from the rat aorta in the control when cultured in the medium containing VEGF only. The anti-angiogenic activity was quantified by measuring the cell-sprouting area around the aortas exposed to the various concentrations of Ob-X and the control. The anti-angiogenesis effect of Ob-X on the sprouting of microvessels from rat aorta was dose-dependent, and microvessel

growth was almost completely inhibited in the presence of 200 μg/ml Ob-X.

Effect of Ob-X on MMP Activity

The effect of Ob-X on MMP activity was examined with a spectrofluorometric method using human recombinant MMPs and a quenched fluorescent peptide as a substrate. Ob-X inhibited MMP-2 and MMP-9 activities with IC_{50} value of 31.3 ± 1.02 μg/ml and 49.1 ± 0.96 μg/ml, respectively (Figure 3). This result suggests that the inhibition of MMP activities by Ob-X may be one of the major mechanisms for its anti-angiogenic activity.

Effects of Ob-X on body weight, adipose tissue mass, and adipocyte size in obese mice

Because Ob-X showed anti-angiogenic and MMP-inhibitory activities, it was undertaken to determine whether Ob-X reduces adipose tissue mass in nutritionally obese mice. Wild-type C57BL/6J mice were fed either a high fat diet or the same high fat diet supplemented with 0.2% Ob-X for 12 weeks. Body weight gain for Ob-X-treated mice was 24% lower than that for untreated control mice (7.29 ± 1.14 g versus 9.60 ± 0.54 g, respectively) (Table 2). SC fat mass and VSC fat mass were also decreased by 41% and 27% lower in treated mice compared with control mice, respectively. Compared with control mice, treated mice had lower SC fat mass (1.21 ± 0.29 versus 0.72 ± 0.11 g, respectively) and VSC fat mass (2.15 ± 0.44 g versus 1.58 ± 0.28 g, respectively).

However, control and treated mice were found to have similar food consumption profiles through the study (data not shown).

Analysis of hematoxylin and eosin-stained adipose tissue sections from diet-induced obese mice revealed that the size of adipocytes in adipose tissue of Ob-X-treated mice was markedly smaller than that in control mice (Figure 4). The size of VSC adipocytes in Ob-X-treated mice (2,505 ± 308 μm^2) was 63% smaller than in control mice without Ob-X (6,817 ± 1509 μm^2). Similarly, the size of SC adipocytes in Ob-X-treated mice (2,779 ± 143 μm^2) was decreased by 32% relative to control mice (4,107 ± 734 μm^2).

Effects of Ob-X on vascularization in VSC adipose tissue of obese mice

To determine whether the decrease of adipose tissue mass by Ob-X is due to the inhibition of angiogenesis, we studied the effects of Ob-X on blood vessel density in VSC adipose tissue. Staining of VSC adipose tissue sections with an antibody against vWF, an endothelial cell marker, showed that blood vessel density of Ob-X-treated mice was lower than that of controls (Figure 5).

Effects of Ob-X on MMP activity in VSC adipose tissue of obese mice

MMP activity in adipose tissue extracts was examined using zymography on gelatin-containing gels. Gelatin zymography revealed the activity of proMMP-2 was significantly reduced in the

adipose tissue from Ob-X-treated mice compared to the control group, whereas proMMP-9 levels were not detectable (Figure 6). The proMMP-2 activities in VSC and SC adipose tissues of Ob-X-treated mice were reduced by 33% and 27%, respectively.

mRNA Expression of angiogenic factors, MMPs, and their inhibitors in VSC and SC adipose tissues of obese mice treated with Ob-X

The expression patterns of genes involved in angiogenesis were investigated in VSC and SC adipose tissue from C57BL/6J mice fed high fat diet. The mRNA expression of angiogenic and anti-angiogenic factors was down-regulated and up-regulated, respectively, in Ob-X-treated mice compared with controls. After Ob-X administration the mRNA levels of angiogenic factors VEGF-A and FGF-2 were reduced by 14% and 48%, respectively, in VSC adipose tissue (Figure 7A), and by 75% and 14%, respectively, in SC adipose tissue (Figure 7B). In contrast, the mRNA level of anti-angiogenic molecule thrombospondin-1 (TSP-1) was elevated by 23% in VSC and by 60% in SC fat pads.

Similarly, MMP mRNA expression was inhibited significantly by Ob-X treatment. MMP-2 and MMP-9 were decreased by 42% and 79%, respectively, in VSC adipose tissue, and by 44% and 44%, respectively, in SC adipose tissue. In contrast, tissue inhibitor of metalloproteinase-2 (TIMP-2) levels in SC and VSC tissue from Ob-X-treated mice were 201% and 51% higher, respectively, than

in control mice.

Discussion

Obesity is characterized by excess accumulation of adipose tissue, the growth of which has recently been reported to require angiogenesis and ECM remodeling. In this study, we prepared Ob-X, an herbal composition with anti-angiogenic and MMP-inhibiting properties, and observed its effects in obese mice. Our results demonstrate that Ob-X reduced body weight gain and adipose tissue mass, and markedly modulated the expression of genes involved in angiogenesis and the MMP system in adipose tissue.

Adipose tissue consists of adipocytes and vascular endothelial cells, which provide oxygen and nutrients to the growing mass. Close examination of developing adipose tissue microvasculature revealed that angiogenesis often precedes adipogenesis.[2] The interaction between adipocytes and endothelium is therefore presumed to be involved in the development and maintenance of adipose tissue. Newly formed adipose tissue depends on continued angiogenesis for further growth.[29] It was shown that different angiogenesis inhibitors significantly reduced body weight and adipose tissue mass,[6] strongly indicating a role of angiogenesis in adipose tissue growth. Based on these reports, we primarily examined anti-angiogenic activity of Ob-X, using HUVEC tube formation and rat aorta ring assays. Ob-X inhibited HUVEC tube formation *in vitro* in a dose-dependent manner. Ob-X also produced dose-dependent inhibition of VEGF-induced microvessel outgrowth from aortic tissue in the *ex vivo* rat aortic ring assay. These results show that Ob-X has the ability to inhibit angiogenesis.

Two major MMP activities (MMP-2 and MMP-9) were markedly inhibited *in vitro* by Ob-X. MMPs play major roles in the extracellular matrix remodeling occurring in a variety of physiological and pathological conditions, such as embryonic growth and development, wound healing, atherosclerosis, and tumor invasion and metastasis. Moreover, adipocytes are surrounded by a basement membrane that has to be extensively remodeled in order to allow the hypertrophic development of adipocytes observed in obesity.[30] Recent studies suggest that MMPs play a role in the tissue remodeling events associated with adipogenesis. MMP-2 and MMP-9 can remodel the ECM of murine and human adipogenic cells to facilitate the adipogenic process,[12,17] and regulate the bio-availability of adipocyte growth factors sequestered as inactive molecules in the matrix, or blocked by interaction with their binding proteins.[31] These results strongly suggest that Ob-X, which has the ability to inhibit MMP activity as well as angiogenesis, can regulate adipose tissue growth.

Body weight gain and adipose tissue mass of Ob-X-treated mice were significantly less than those of controls, supporting the hypothesis that Ob-X can reduce adipose tissue growth due to its inhibition of angiogenesis and MMP activity. Ob-X treatment for 12 weeks in high fat diet-fed mice decreased adipose tissue mass by 32%, and this effect was similar to the results showing a 38% reduction after a 12-week administration of galadin, a broad spectrum inhibitor of MMP.[10] Our data are also supported by other results indicating that body and adipose tissue mass of obese animals were reduced significantly by several kinds of angiogenesis inhibitors.[6,32] During Ob-X-induced adipose tissue loss, food intake was not changed. Control and treated mice were found to have similar food

consumption profiles. Appetite suppression is a common mechanism of weight loss, which may have many adverse effects. The weight loss induced by anti-angiogenic Ob-X came specifically from loss of adipose tissue mass. Moreover, Ob-X did not have any toxic effects on organs by targeting only the growing adipose tissue, thus indicating that Ob-X can be used for a long period with little toxicity. In agreement with earlier studies,[10] Ob-X differentially regulated SC and VSC fat mass in obese mice. SC fat mass was decreased much greater than VSC fat mass. Such differences in regional responses to Ob-X may be due to differences in cellular composition, pattern of MMP expression, and secretion of adipokines, which can affect MMP expression.[10,16,33,34] This suggests a role of Ob-X in adipose tissue growth and a differential effect of Ob-X between SC and VSC adipose tissue in animals. However, it may be different in humans, since 1.5 g of Ob-X per day for a 12- week treatment reduced VSC fat area by 9.5% (from 81.5 ± 4.40 cm^2 to 73.8 ± 4.72 cm^2; $p < 0.01$), which is much higher than the SC fat area reduction in a preliminary human study of Ob-X with 25 people by Computed Tomography analysis before and after intervention without changing their life style (unpublished data). In addition to weight reduction, Ob-X inhibited adipocyte hypertrophy in high fat diet-fed obese mice. The size of adipocytes was considerably smaller in Ob-X-treated mice than in controls. Morphometric analysis of SC and VSC adipose tissue histology showed that Ob-X substantially reduced the size of adipocytes in both SC and VSC adipose tissues, eventually resulting in the decreased body weight gain and adipose tissue mass. Thus, it is likely that Ob-X may be used to prevent and treat obesity and obesity-related disorders. Since there is a strong association

between visceral adiposity and insulin resistance, Ob-X may have an important role in alleviating of both insulin resistance and diabetes.[35-37]

Consistent with the *in vitro* inhibitory effects of Ob-X on HUVEC tube formation and microvessel outgrowth from rat aorta, blood vessel density of VSC adipose tissue sections from nutritionally obese mice treated with Ob-X was much lower than in untreated control mice. In contrast to the data presented in this study, the *in vivo* administration of galardin results in a higher blood vessel density in adipose tissue of mice than in untreated control mice.[10] These inconsistent findings can be explained by normalizing blood vessel density with the number of adipocytes.[14,38] Our present results indicate that the reduction of adipose tissue mass by Ob-X is due to its anti-angiogenic action.

Zymographic analysis revealed that administration of Ob-X suppressed gelatinolytic activity, especially MMP-2 activity, since proMMP-2 activity was markedly reduced in both VSC and SC adipose tissues, even though MMP-9 activity was not detectable. It has also been reported that *in situ* zymography with gelatin-containing gels on cryosections of SC or VSC adipose tissue confirmed a lower MMP activity in tissues of galardin-treated animals.[10] The roles of MMP in adipose tissue growth have been demonstrated in several studies. MMP inhibitors block the adipocyte differentiation process,[12,16,39] and treatment with MMP inhibitors impairs adipose tissue development in mice fed a high fat diet.[10,40] Furthermore, the secretion of MMP-2 and MMP-9 increases during adipocyte differentiation in both human adipocytes and mouse preadipocyte cell lines.[12,15,16] Thus,

our data suggest that the reduction of MMP-2 activity by Ob-X may contribute to the reduced adipose tissue mass.

Diversity of cell populations in the adipose tissue, including preadipocytes, adipocytes, adipose stromal cells, and inflammatory cells contributes to production of multiple angiogenic factors and inhibitors that regulate adipose angiogenesis. Angiogenic factors, such as VEGF-A and FGF-2, promote the proliferation and differentiation of endothelial cells within fat, [22-24] whereas TSP-1 inhibits angiogenesis *in vivo* and impairs migration and proliferation of cultured microvascular endothelial cells.[41] Adipocytes also produce MMPs and MMP inhibitors that are differentially expressed in adipose tissue during obesity in murine obesity models.[15,16,19] Interplay between different factors is presumed to be involved in the development and maintenance of adipose tissue. Ob-X administration to high fat diet-induced obese mice decreased the mRNA expression of VEGF-A and FGF-2 responsible for angiogenesis, whereas it increased mRNA level of the anti-angiogenic TSP-1 in both VSC and SC adipose tissues. Similarly, Ob-X decreased MMP-2 and MMP-9 mRNA levels, but increased TIMP-2. Our data indicate that Ob-X exerts a specific regulatory effect on genes involved in angiogenesis and the MMP system in adipose tissues. Our observations further show that inhibition of adipose tissue growth by Ob-X may alter the expression of genes responsible for angiogenesis and the MMP system. These expressions were different between SC and VSC adipose tissues. VEGF-A, TIMP-2, and TSP-1 mRNA levels were markedly modulated by Ob-X in SC adipose tissue compared with VSC adipose tissue, whereas FGF-2 and MMP-9 expression was

highly modulated in VSC fat. This differential expression between SC and VSC fat pads seems to influence differential growth between two adipose tissues. In addition, we cannot exclude the possibility that Ob-X may also affect adipose tissue growth by changing the expression of other factors.

MMPs also play important roles in angiogenesis. MMP inhibitors, both synthetic and endogenous, inhibit angiogenic responses both *in vivo* and *in vitro*.[42-45] Moreover, MMP-deficient mice exhibit delayed or diminished angiogenic responses during development or in response to tumor xenograft.[46] These previous reports strongly suggest that adipose tissue growth is efficiently prevented by Ob-X, with both anti-angiogenic and MMP inhibitory activity, and the mechanism of anti-angiogenic activity of Ob-X is partly due to the inhibition of MMP.

In conclusion, these studies demonstrate that Ob-X, which inhibits angiogenesis and MMP activity, regulates adipose tissue growth of nutritionally induced obesity in mice. These events may influence changes in the expression of genes involved in angiogenesis and the MMP system. Thus anti-angiogenic Ob-X, by reducing adipose tissue, provides a possible therapeutic approach for the prevention and treatment of human obesity and its related disorders.

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Figure legends

Figure 1. Inhibitory effects of Ob-X on tube formation of human endothelial cells. HUVECs were plated in Matrigel-coated wells with varying amounts of Ob-X (25, 50, and 100 ug/ml). After incubation capillary-like tube formation was photographed (original magnification 100×).

Figure 2. Inhibitory effects of Ob-X on microvessel outgrowth arising from rat aortic ring. (A) Aortic rings were embedded in Matrigel, then fed with medium containing various concentrations of Ob-X for 10 days, and photographed (original magnification 12.5×) (B) The area of microvessels was quantified by image analysis program. * $p < 0.05$ versus control group.

Figure 3. Inhibitory effects of Ob-X on MMP activity. Inhibition of MMP-2 and MMP-9 activities by Ob-X was measured by spectrofluorometry, and the IC_{50} value was determined. * $p < 0.05$ versus control group.

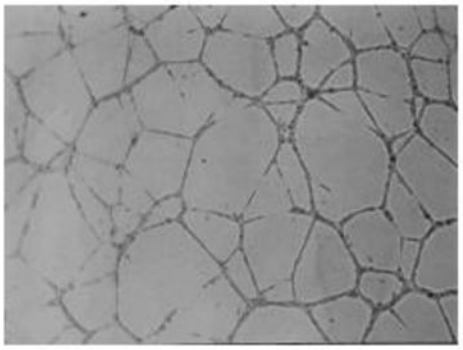
Figure 4. Light microscopic analysis of the size of adipocytes in adipose tissue. (A) VSC and SC adipose tissues were derived from mice treated with vehicle or 0.2% Ob-X for 12 weeks. All

values are expressed as the mean ± SD. * $p < 0.05$ versus control group. (B) Representative photographs of hematoxylin and eosin-stained sections of VSC and SC adipose tissues (original magnification 100×).

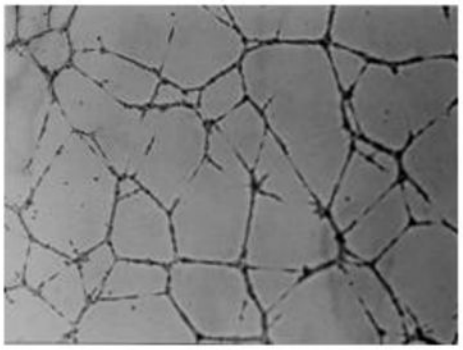
Figure 5. Histological analysis of the blood vessels in visceral adipose tissue with an antibody against von Willebrand Factor. The blood vessels of visceral adipose tissue derived from mice treated with vehicle or 0.2% Ob-X for 12 weeks were stained and analyzed (original magnification 100×).

Figure 6. Zymographic analysis of visceral adipose tissue. Extracts from (A) VSC and (B) SC adipose tissues obtained from mice kept on high fat diet for 12 weeks with or without Ob-X were applied to a gelatin-containing gel. Gelatinolytic activity was measured by zymography. All values are expressed as the mean ± SD. * $p < 0.05$ versus control group.

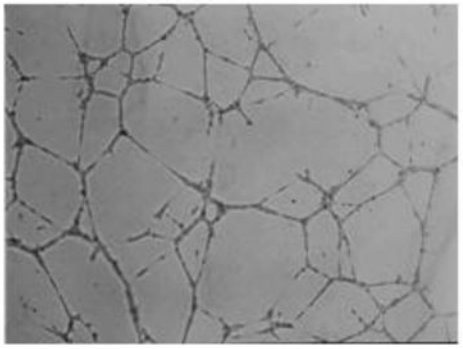
Figure 7. Effects of Ob-X on mRNA expression of angiogenic factors, MMPs, and their inhibitors in (A) VSC and (B) SC adipose tissues of diet-induced obese mice. * $p < 0.05$ versus control group.



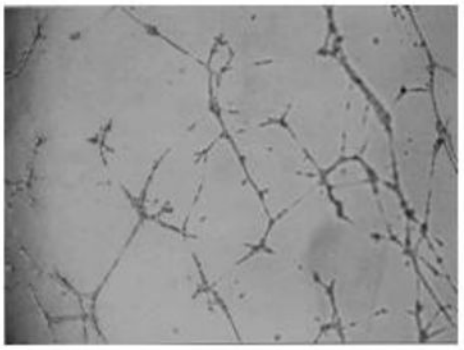
Control



Ob-X 25µg/ml



Ob-X 50 µg/ml



Ob-X 100 µg/ml

Figure 1

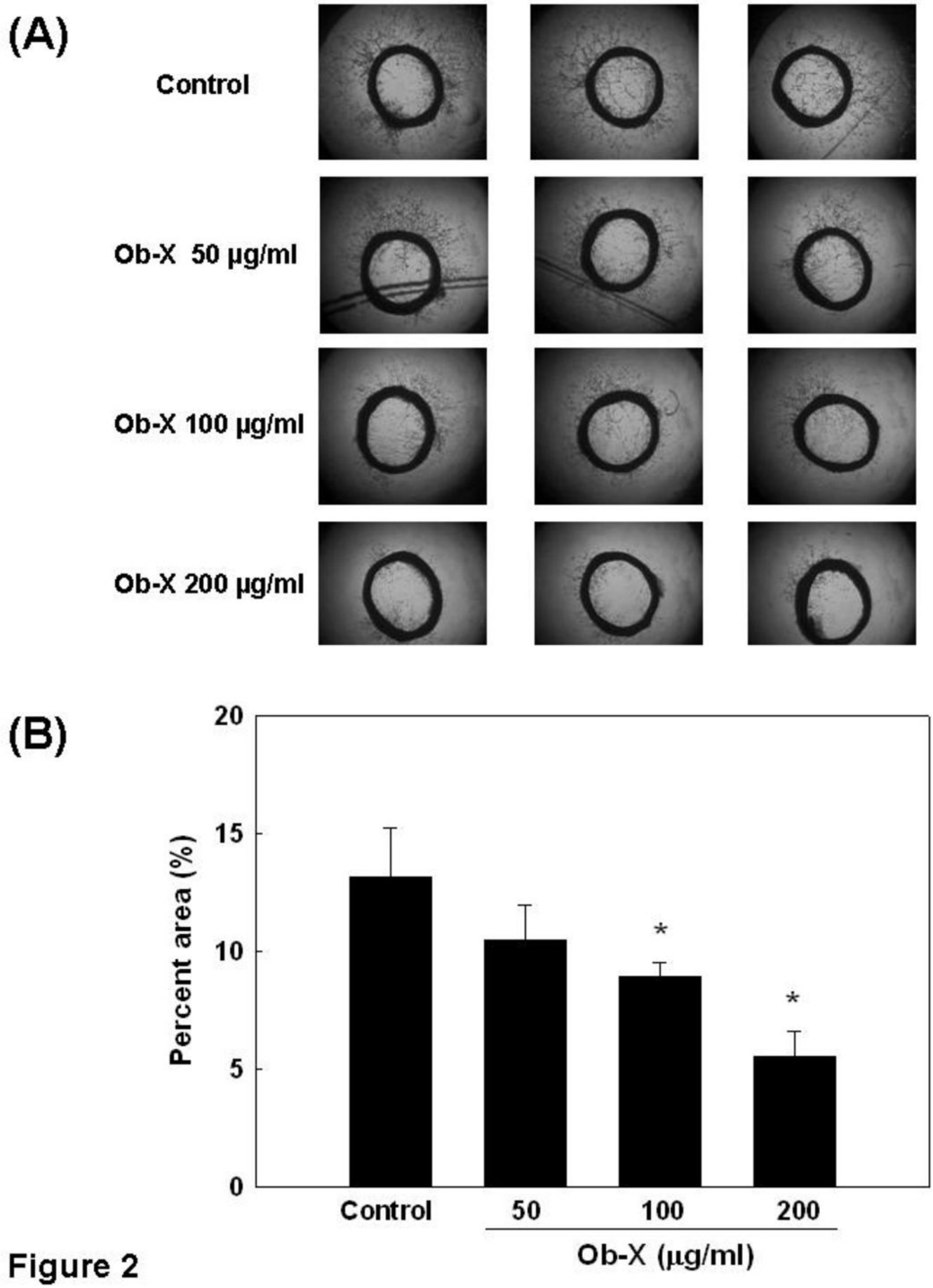
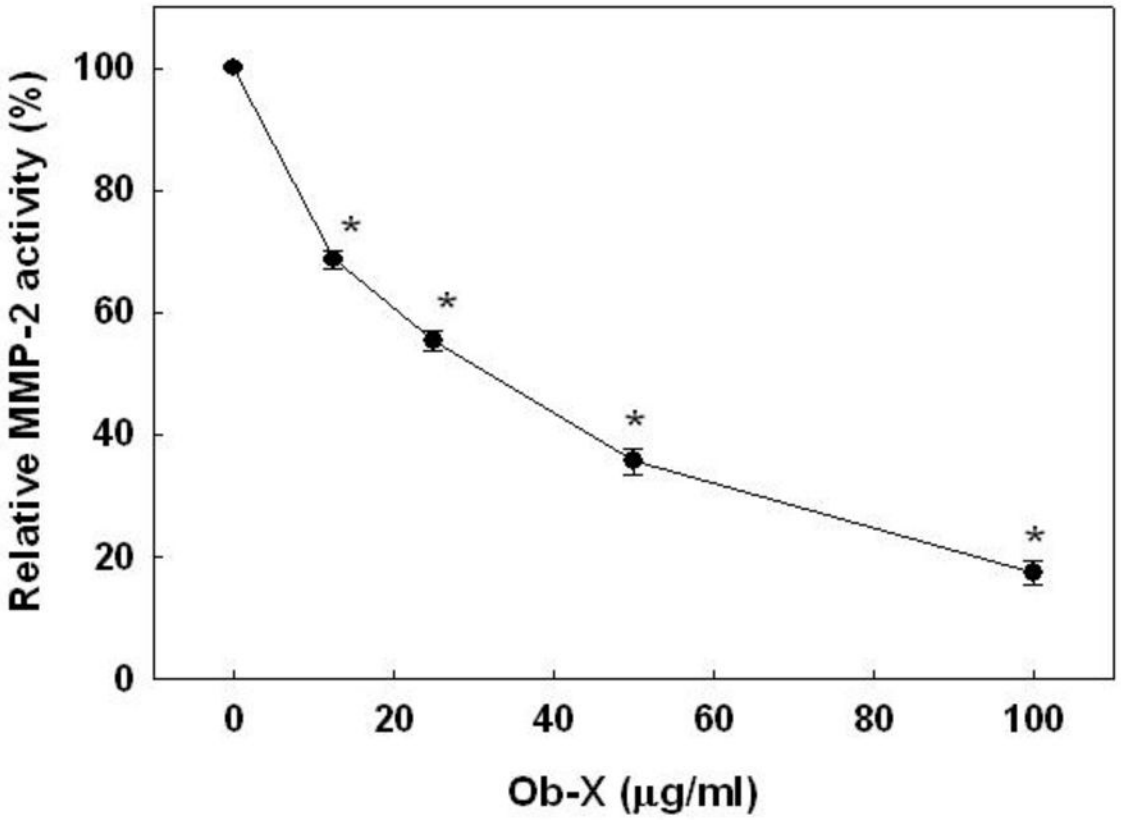

(A)
Control
Ob-X 50 μg/ml
Ob-X 100 μg/ml
Ob-X 200 μg/ml
(B)
Percent area (%)
20
15
10
5
0
*
*
Control
50
100
200
Ob-X (μg/ml)

Figure 2


Relative MMP-2 activity (%)
100
80
60
40
20
0
0
20
40
60
80
100
Ob-X (μg/ml)
*
*
*
*

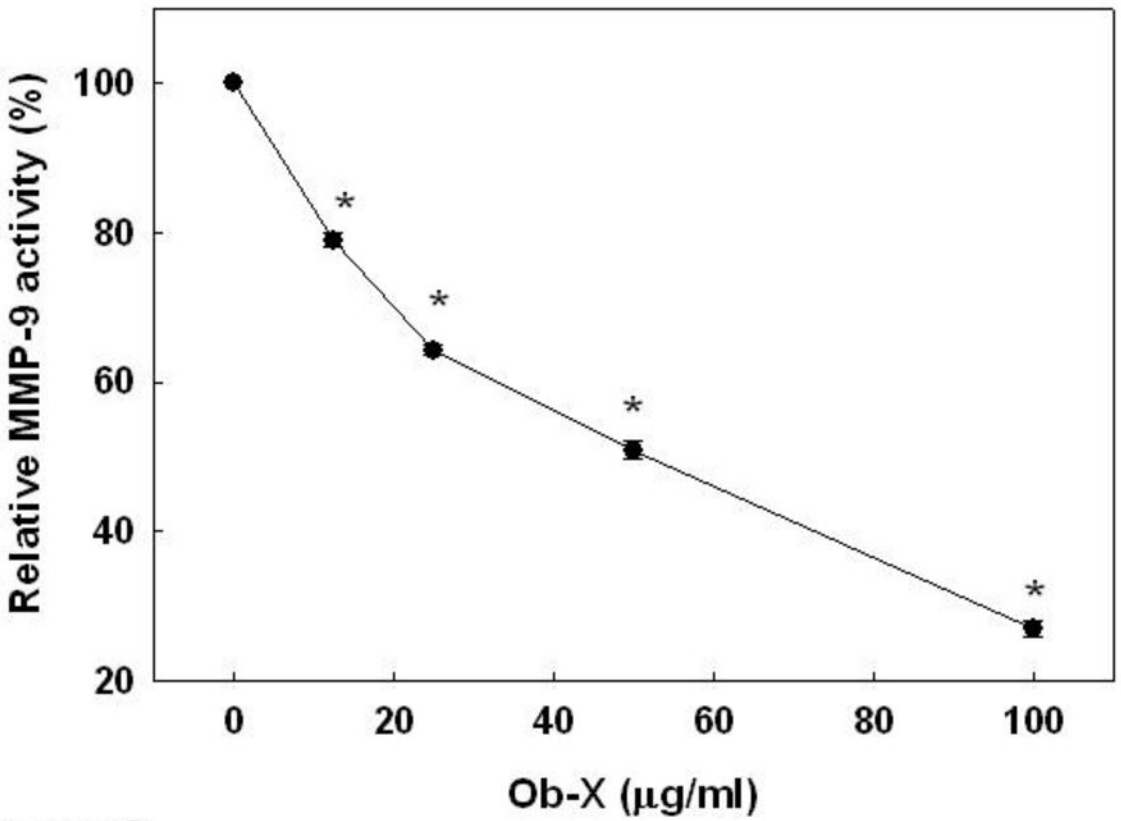

Relative MMP-9 activity (%)
100
80
60
40
20
0
20
40
60
80
100
Ob-X (μg/ml)
*
*
*
*

Figure 3

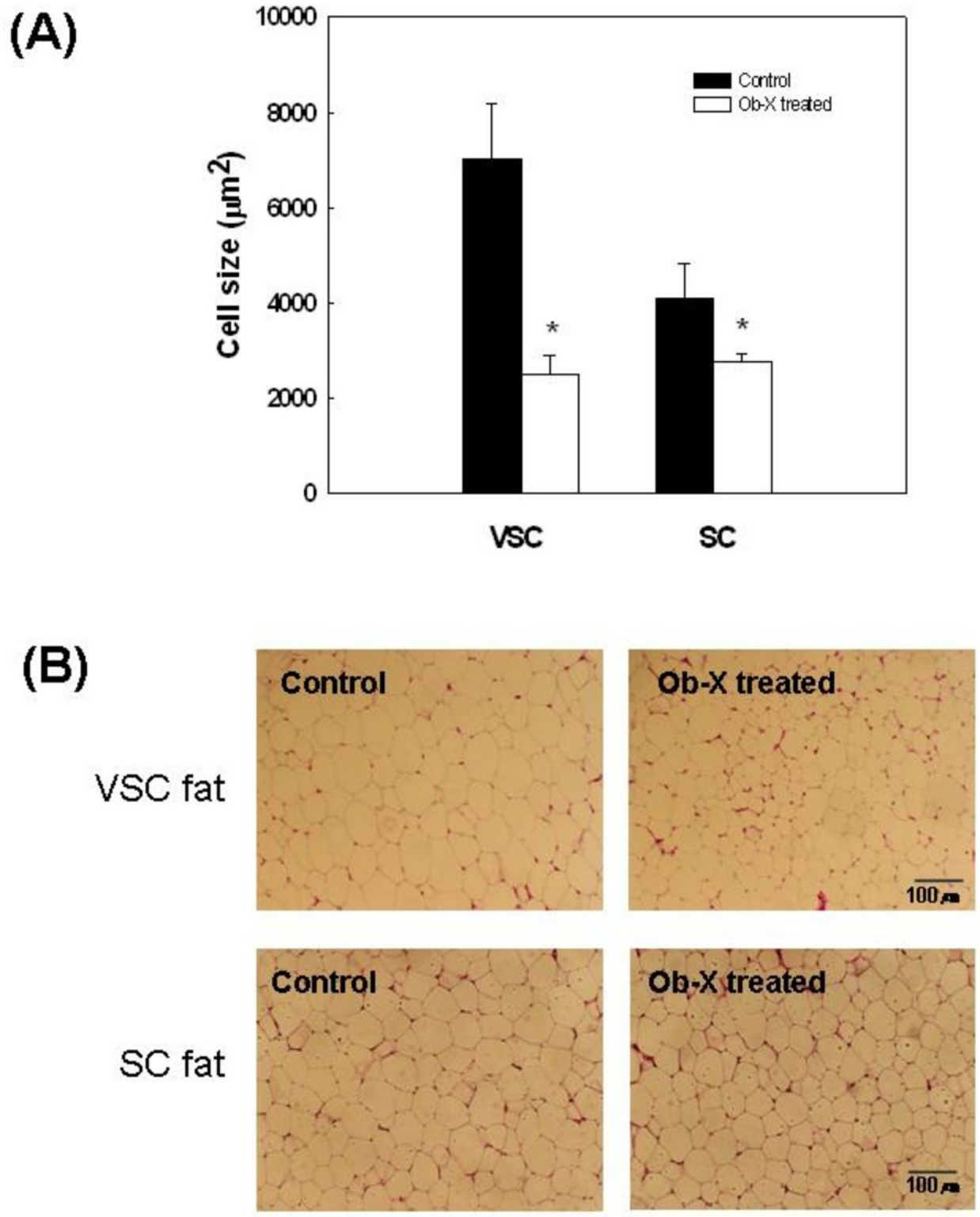

(A)
10000
8000
6000
4000
2000
0
Cell size (μm2)
Control
Ob-X treated
*
*
VSC
SC
(B)
VSC fat
SC fat
Control
Ob-X treated
Control
Ob-X treated
100 μm
100 μm

Figure 4

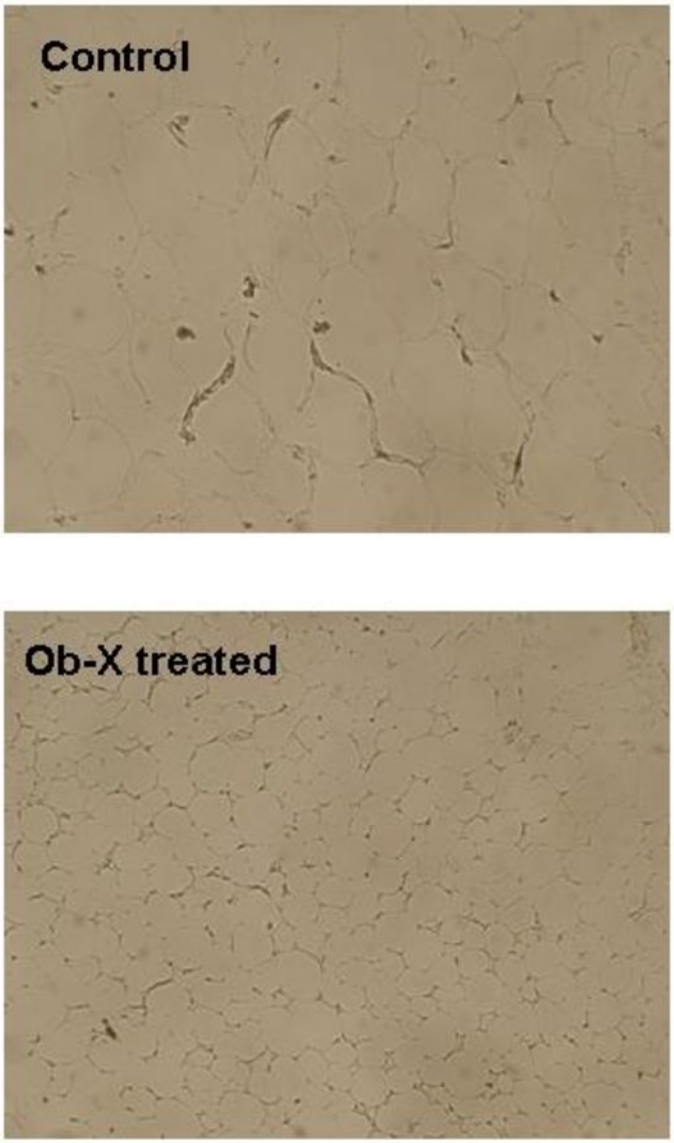


Figure 5

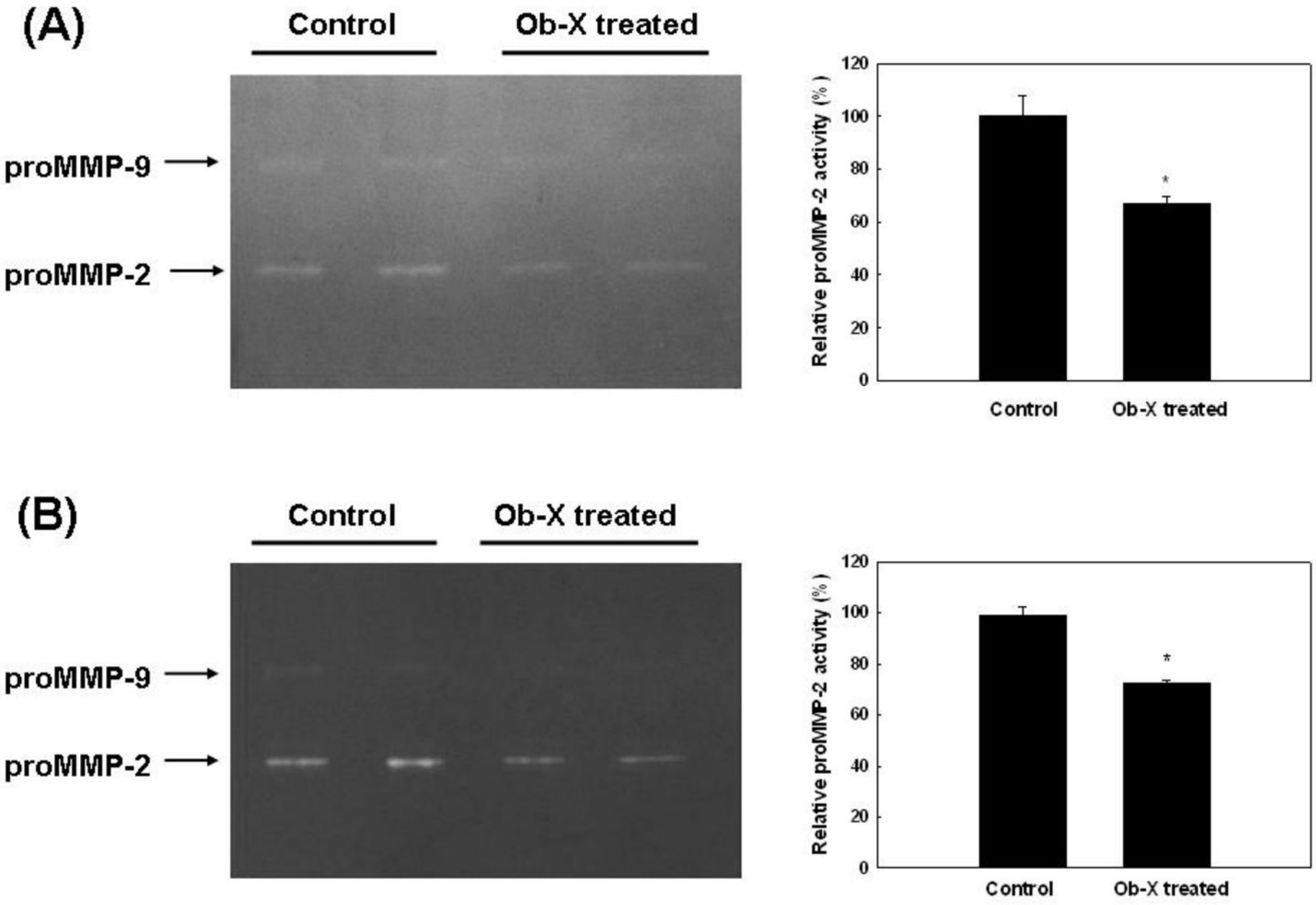
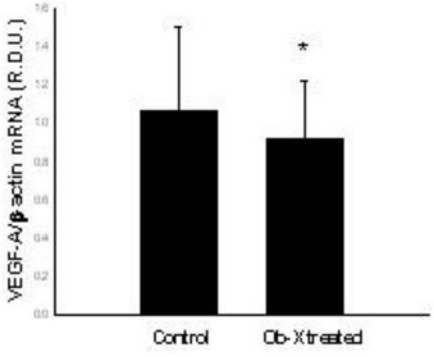
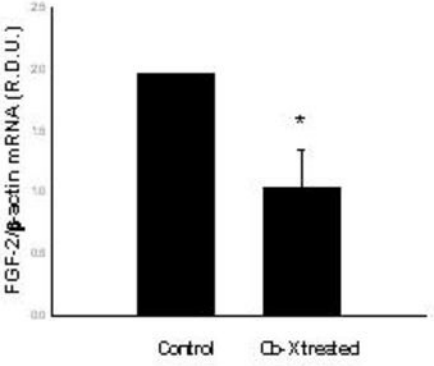
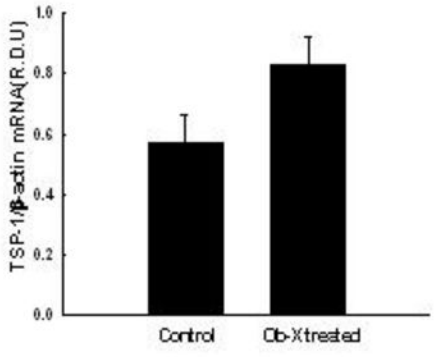

(A)
Control
Ob-X treated
proMMP-9
proMMP-2
Relative proMMP-2 activity (%)
120
100
80
60
40
20
0
*
Control
Ob-X treated
(B)
Control
Ob-X treated
proMMP-9
proMMP-2
Relative proMMP-2 activity (%)
120
100
80
60
40
20
0
*
Control
Ob-X treated

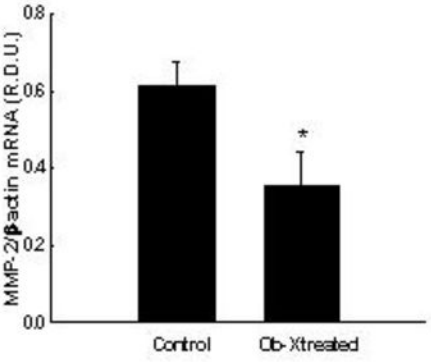
Figure 6

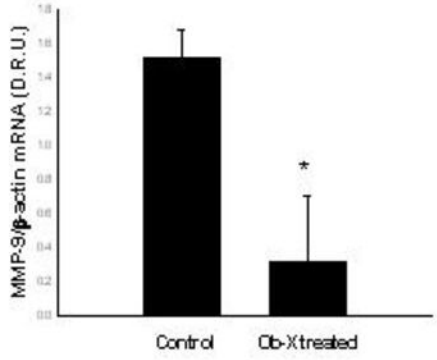
(A)

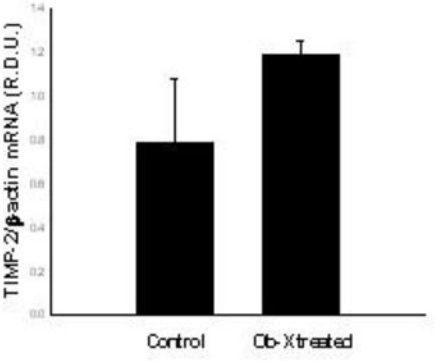

VEGF-A/β-actin mRNA (R.D.U.)
*
Control
Ob-X treated


FGF-2/β-actin mRNA (R.D.U.)
*
Control
Ob-X treated


TSP-1/β-actin mRNA(R.D.U.)
Control
Ob-X treated


MMP-2/β-actin mRNA (R.D.U.)
*
Control
Ob-X treated


MMP-9/β-actin mRNA (D.R.U.)
*
Control
Ob-X treated


TIMP-2/β-actin mRNA (R.D.U.)
Control
Ob-X treated

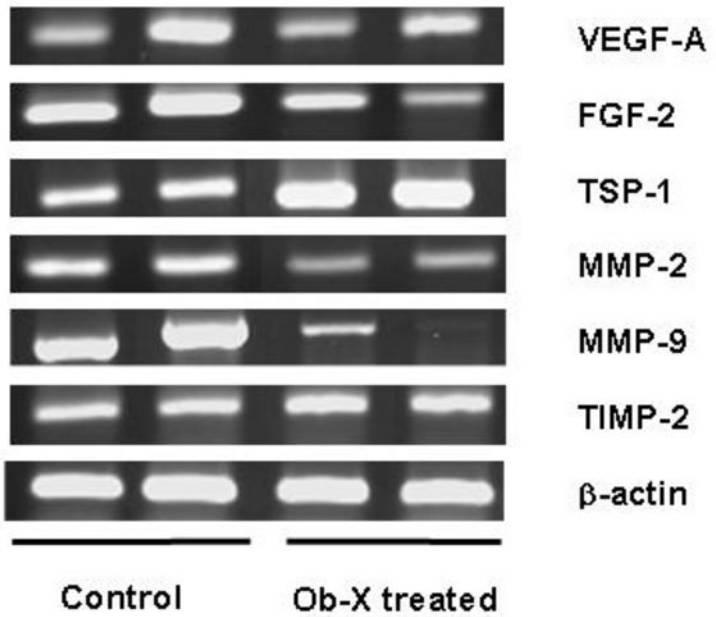
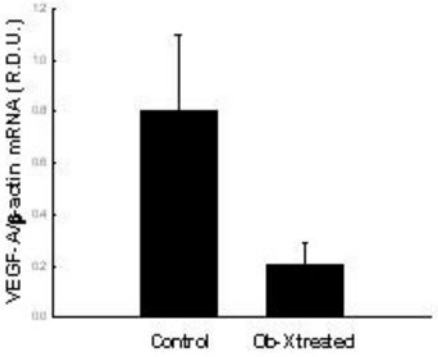
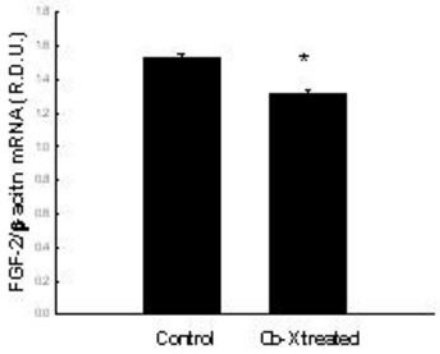
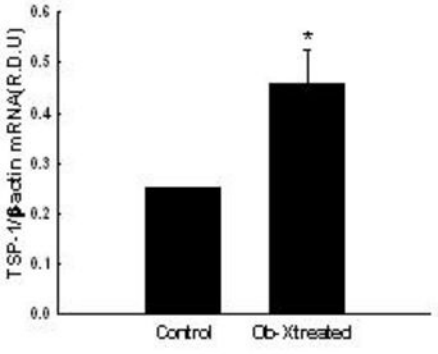

VEGF-A
FGF-2
TSP-1
MMP-2
MMP-9
TIMP-2
β-actin
Control
Ob-X treated

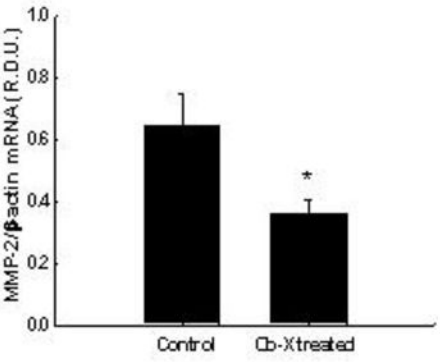
Figure 7A

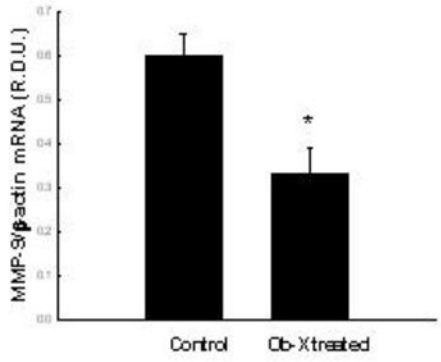
(B)

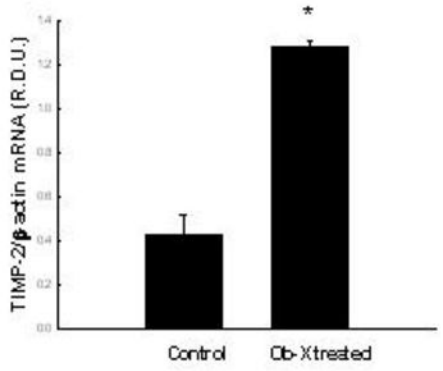

VEGF-A/βactin mRNA (R.D.U.)
Control
Ob-X treated


FGF-2/βactin mRNA (R.D.U.)
*
Control
Ob-X treated


TSP-1/βactin mRNA(R.D.U.)
*
Control
Ob-X treated


MMP-2/βactin mRNA (R.D.U.)
*
Control
Ob-X treated


MMP-9/βactin mRNA (R.D.U.)
*
Control
Ob-X treated


TIMP-2/βactin mRNA (R.D.U.)
*
Control
Ob-X treated

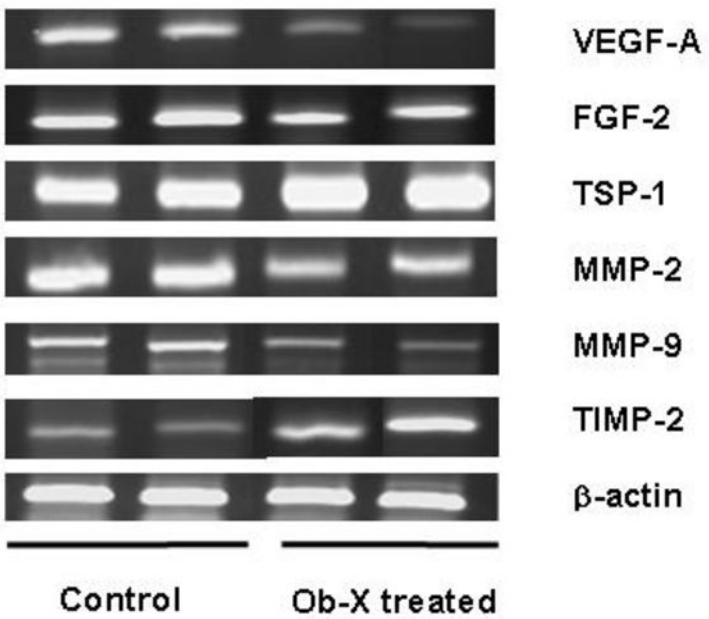

VEGF-A
FGF-2
TSP-1
MMP-2
MMP-9
TIMP-2
β-actin
Control
Ob-X treated

Figure 7B

Table 1. Sequences of primers used for the RT-PCR assays

Genes	GeneBank	Primer sequences	Size (bp)
VEGF-A	NM_009505	Forward: 5’ -gctctcttgggtgcactgga- 3’ Reverse: 5’ -caccgccttggcttgtcaca- 3’	561
FGF-2	NM 174056	Forward: 5’ -aactacaacttcaagcagaagagaga- 3’ Reverse: 5’ -atgtctgctaagagctgatcttaa- 3’	293
TSP-1	M62470	Forward: 5’ -cctcatttgttgtgtgactgagtaa- 3’ Reverse: 5’ -gtttcttatgtacaaggaacaacaa- 3’	566
MMP-2	U65656	Forward: 5’ -atctggtgtctcccttacgg- 3’ Reverse: 5’ -gttgtcggacatcactgcac- 3’	704
MMP-9	AK161176	Forward: 5’ -tgcgaccacatcgaacttcg- 3’ Reverse: 5’ -ccaagagggttttcttcttctctgg- 3’	687
TIMP-2	NM011594	Forward: 5’ -gagatcaagcagataaagatg- 3’ Reverse: 5’ -gcctctggatggactgggtc- 3’	320
β-acitn	J00691	Forward: 5´-tggaatcctgtggcatccatgaaa-3´ Reverse: 5´-taaaacgcagctcagtaacagtcc-3´	350

Table 2. Effects of Ob-X on body weight gain and adipose tissue weight in nutritionally obese mice.

	Control	Ob-X treated
Body weight (g)	30.7 ± 0.71	28.4 ± 0.62*
Body weight gain (g)	9.60 ± 0.54	7.29 ± 1.14*
VSC fat (g)	2.15 ± 0.44	1.58 ± 0.28*
SC fat (g)	1.21 ± 0.29	0.72 ± 0.11*

Values are mean ± SD of 8 animals in control or Ob-X treated groups.

* $p<0.05$ compared with control group.