Leptin Receptor–Deficient MMTV-TGF-α/*Lepr*^{db}*Lepr*^{db} Female Mice Do Not Develop Oncogene-Induced Mammary Tumors

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Being overweight is a risk factor for postmenopausal breast cancer and is associated with an increased incidence and shortened latency of spontaneous and chemically induced mammary tumors in rodents. However, leptin-deficient obese *Lep*^{ob}*Lep*^{ob} female mice have reduced incidences of spontaneous and oncogene-induced mammary tumors. Of interest, leptin enhances the proliferation of human breast cancer cell lines in which leptin receptors are expressed, which suggests that leptin signaling plays a role in tumor development. We evaluated oncogene-induced mammary tumor development in obese MMTV-TGF-α/*Lepr*^{db}*Lepr*^{db} mice that exhibit a defect in OB-Rb, which is considered to be the major signaling isoform of the leptin receptor. *Lepr* and MMTV-TGF-α mice were crossed, and the offspring were genotyped for oncogene expression and the determination of *Lepr* status. Lean MMTV-TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ (homozygous) and MMTV-TGF-α/*Lepr*$^{+}$*Lepr*^{db} (heterozygous) mice and obese MMTV-TGF-α/*Lepr*^{db}*Lepr*^{db} mice were monitored until age 104 weeks. Body weights of MMTV-TGF-α/*Lepr*^{db}*Lepr*^{db} mice were significantly heavier than those of the lean groups. No mammary tumors were detected in MMTV-TGF-α/*Lepr*^{db}*Lepr*^{db} mice, whereas the incidence of mammary tumors in MMTV-TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ and MMTV-TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice was 69% and 82%, respectively. Examination of mammary tissue whole mounts indicated an absence of duct formation and branching for MMTV-TGF-α/*Lepr*^{db}*Lepr*^{db} mice. Both age at mammary tumor detection and tumor burden (tumors/mouse and tumor weights) were similar for the lean genotypes. Serum leptin levels of MMTV-TGF-α/*Lepr*^{db}*Lepr*^{db} mice were 12–20-fold higher than levels of lean mice. Thus, despite elevated serum leptin levels, leptin receptor–deficient MMTV-TGF-α/*Lepr*^{db}*Lepr*^{db} mice do not develop mammary tumors. This study provides additional evidence that leptin and its cognate receptor may be involved in mammary tumorigenesis. Exp Biol Med 229:182–193, 2004

Key words: leptin; leptin receptor; breast cancer; mice; obesity; OB-Rb

Elevated body weight and obesity are risk factors for postmenopausal breast cancer (1–9). Furthermore, higher body weight is associated with an increased incidence and shortened latency of spontaneous and chemically induced mammary tumors (MTs) in rodents (10–15). However, although genetically obese female *Lep*^{ob}*Lep*^{ob} mice exhibited a shortened latency for development of spontaneous MTs compared with lean mice, they had a significantly lower tumor incidence (16). In addition, we recently reported that obese female MMTV-TGF-α/*Lep*^{ob}*Lep*^{ob} mice did not develop oncogene-induced MTs, whereas homozygous MMTV–TGF-α/*Lep*$^{+}$*Lep*$^{+}$ and heterozygous MMTV–TGF-α/*Lep*$^{+}$*Lep*^{ob} lean mice had incidence rates of 50% and 67%, respectively, at age 104 weeks (17). The genetic defect responsible for obesity in *Lep*^{ob}*Lep*^{ob} mice is a deficiency of the cytokine-like protein, leptin (18). The results of recent *in vitro* studies have indicated that the addition of leptin enhances the cellular proliferation of breast epithelial and malignant cell lines and that these cell lines express the leptin receptor (19–22).

The integration of these *in vivo* and *in vitro* study results led us to hypothesize that the absence of MT development in obese MMTV–TGF-α/*Lep*^{ob}*Lep*^{ob} mice could potentially be attributed to their leptin deficiency. To more conclusively establish a role for leptin action and

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signaling in the development of both normal and malignant breast and mammary tissues, we have determined the effect of leptin receptor deficiency on oncogene-induced MT development. The leptin receptor-deficient $Lepr^{db}Lepr^{db}$ mouse provides a model to undertake such an evaluation. $Lepr^{db}Lepr^{db}$ mice have a mutation in the gene that encodes the long isoform of the leptin receptor designated as OB-Rb (or OB-Rl) (23). OB-Rb is considered to be the major signaling form of this protein (24). Here we present the results of cross-breeding *Lepr* strain mice with mice that overexpress human transforming growth factor (TGF)–α that has been placed under the control of the mouse mammary tumor virus promoter/enhancer (MMTV–TGF-α mice). These mice were originally described by Matsui *et al.* (25). Homozygous MMTV–TGF-α/$Lepr^{+}Lepr^{+}$ and heterozygous MMTV–TGF-α/$Lepr^{+}Lepr^{db}$ lean mice and homozygous MMTV–TGF-α/$Lepr^{db}Lepr^{db}$ obese mice were monitored until age 104 weeks, to determine MT latency and incidence rates.

Materials and Methods

Mice. Breeding pairs of MMTV–TGF-α mice were originally obtained from Jackson Laboratory (Bar Harbor, ME) and are maintained at the Hormel Institute on the C57BL6 background. *Lepr* strain mice were obtained from Pennington Biomedical Research Center (Baton Rouge, LA). These *Lepr* mice are also maintained on the C57BL6 background but do not carry the misty gene mutation that affects body weight, length, and body fat (26). Male MMTV–TGF-α/$Lepr^{+}Lepr^{db}$ mice were mated with nontransgenic $Lepr^{+}Lepr^{db}$ female mice. This was necessary because MMTV–TGF-α female mice, although fertile, are unable to lactate (27, 28). This breeding strategy resulted in the production of all three *Lepr* genotypes. Offspring were maintained with their mothers until age 4 weeks and then were removed and housed with others of the same sex prior to genotyping.

DNA Isolation and Genotyping. Tail biopsy samples were obtained at age 5–7 weeks for genotype analysis. For genomic DNA extraction, tissues were digested as described elsewhere (17). Mice were genotyped to identify the presence of TGF-α using oligonucleotide primer 199, which was designed by Jackson Laboratory (5′GATCTTTTCTATGGAATAAGGAATGGA) and corresponds to a region of the MMTV vector just upstream of the TGF-α insert; the complementary primer 200 (5′GATCCAGTGTGACCTAGAGAAGAAAT) corresponds to a region within the TGF-α insert. The genotypes of *Lepr* mice were determined by modifications of an assay procedure originally developed by Dr. Gary Truett. The primers used were $Lepr^{db}$40R (5′-GTTATTTCTTAGTCATTCAAACCATAGTTTAGGTTTGGTT-3′) and $Lepr^{db}$30F (5′-CGGACACTCTTTGAAGTCTCTCATGACCAC-3′). In brief, genomic DNA was added to a mixture of deoinized H_2O, polymerase chain reaction (PCR) buffer, 25 m*M* $MgCl_2$, 2.5 m*M* DNTPs, 2 μ*M* each primer, and *Taq* DNA polymerase. The PCR protocol included 40 cycles at 95°C for 40 secs and 66°C for 60 secs. The restriction digest of the PCR products included a mixture of deionized H_2O, PCR buffer (Mg free; Promega), loading buffer (60% sucrose/1 m*M* cresol red), and 10 U/μl Bst E II (New England Biolabs) for 1 hr at 60°C. After digestion, the sample was subjected to agarose gel electrophoresis, which resulted in three distinct patterns that distinguished homozygous ($Lepr^{+}Lepr^{+}$) lean, heterozygous ($Lepr^{+}Lepr^{db}$) lean, and homozygous ($Lepr^{db}Lepr^{db}$) obese mice from one another.

Animal Housing. Mice were enrolled in the study at age 6 weeks. Forty mice were included in the MMTV–TGF-α/$Lepr^{+}Lepr^{+}$ group, 41 in the MMTV–TGF-α/$Lepr^{+}Lepr^{db}$ group, and 43 in the MMTV–TGF-α/$Lepr^{db}Lepr^{db}$ group. Mice usually were housed in pairs and provided commercial rodent food (Rodent Chow Diet 5001; PMI Nutrition International, Inc., St. Louis, MO) and water *ad libitum*. Twelve-hour light (0700–1900) and 12-hr dark (1900–0700) cycles were maintained, as well as a constant humidity level of 50%. The Hormel Institute Animal Facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The University of Minnesota Institutional Animal Care and Use Committee approved the study and procedures.

Characterization of Tumors. Body weights were determined weekly, at which time the mice also were palpated for the presence of MTs. Once MTs were detected, their growth was monitored with calipers. Mice were sacrificed for the following reasons: excessive weight loss, MT growth >20 mm in length, lethargy, unhealed sores, or age 104 weeks. At necropsy, a blood sample was obtained by heart puncture. Retroperitoneal and parametrial fat pads were removed and weighed. In addition, liver, kidneys, heart, spleen, lungs, and ovaries were removed and weighed. Samples from organs and tissues (except adipose) were fixed in 10% neutral buffered formalin for 24–48 hrs and then embedded in paraffin. Five-micron sections were prepared and deparaffinized by multiple rinsing with xylene and then rehydrated with ethanol solutions. Sections were stained with hematoxylin and eosin. Histopathological analysis was conducted in a blinded fashion, without prior knowledge of *Lepr* genotype. Whole mammary fat pads were excised, fixed in acetone, stained with hematoxylin, destained, and placed in Permount (Sigma Chemical) on a glass slide with a glass cover slip.

Serum Leptin. Serum leptin was determined using commercially available radioimmunoassay kits specific for mice (Linco Research, Inc., St. Charles, MO). Serum samples from MMTV–TGF-α/$Lepr^{db}Lepr^{db}$ were diluted fourfold, to be in the range of the standards.

Statistics. Data are presented as means ± SEM. Survival curves were compared by chi-square analysis. Body-weight curve comparisons among the three genotypes were made by one-way ANOVA with repeated measures, followed by a Neuman-Keul's test to determine which

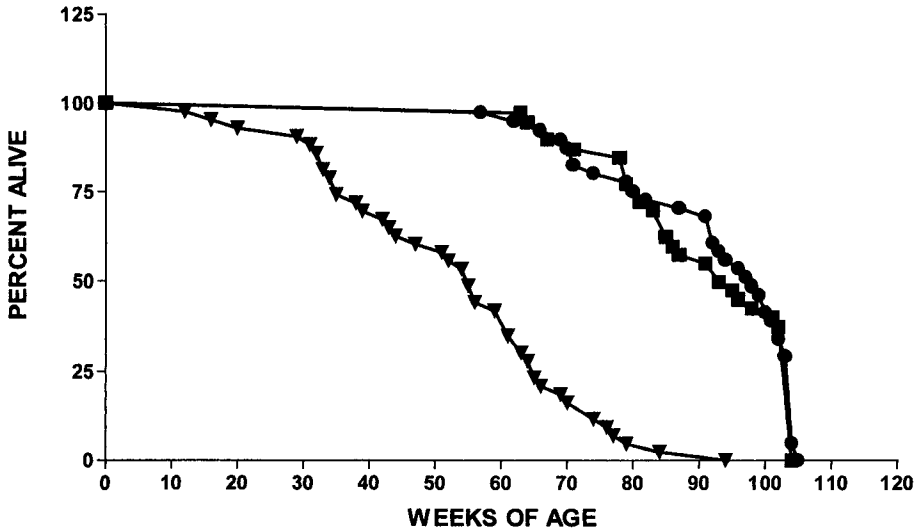

Figure 1. Survival curves of transforming growth factor (TGF)-α/*Lepr* female mice. ■, TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ (n = 40). ●, TGF-α/*Lepr*$^{+}$*Lepr*^{db} (n = 41). ▼, TGF-α/*Lepr*^{db}*Lepr*^{db} (n = 43) mice. Chi-square analysis, $P < 0.0001$.

groups were significantly different from each other. Other comparisons among the three groups were made by one-way ANOVA also followed by a Neuman-Keul's test. Comparisons between the two lean groups with and without MTs were done using two-way ANOVA followed by planned Student's t test. Comparisons between homozygous and heterozygous mice were made using Student's t test.

Results

Obese MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice died at a significantly younger age than did lean MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ and MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice (Fig. 1). The average age at death was 53 weeks for MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice, 92 weeks for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} lean mice, and 90 weeks for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ lean mice (Table 1). MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice died between ages 12 and 94 weeks, compared with between ages 57 and 104 weeks for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice and between ages 63 and 104 weeks for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ lean mice. Mice that were alive at age 104 weeks were sacrificed at that point; thus, the age of death for lean mice does not reflect true mortality, but, because of experimental design considerations, this was a necessary termination point. Forty percent (16/40) of MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice lived to age ≥100 weeks, as did 46% (19/41) of MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice.

As shown in Figure 2, body weights of MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice were significantly heavier than those of lean mice. When body weights of the two lean groups were directly compared, MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice consistently weighed significantly more than MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice (not shown). As expected, the final body weight of obese MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice was significantly heavier than those of the lean mice (Table 1). Combined fat pad weights of the obese mice also were greater than those of lean mice (Table 1). Although they were not significantly different from each other when obese mice were included in the statistical comparison, when only the two lean

Table 1. Age at Death, Final Body Weight, Combined[a] Fat Pad Weights, Fat Pad:Carcass Ratio, and Serum Leptin for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ Lean, *Lepr*$^{+}$*Lepr*^{db} Lean, and *Lepr*^{db}*Lepr*^{db} Obese Female Mice

	Age at death (weeks)	Final body weight (g)	Combined fat pad weight (g)	Fat pad:carcass[b] ratio	Serum leptin (ng/ml)
TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ lean	90.5 ± 2.2[c]* (n = 40)	29.5 ± 0.59* (n = 40)	0.436 ± 0.044* (n = 35)	0.017 ± 0.002* (n = 34)	1.44 ± 0.19* (n = 14)
TGF-α/*Lepr*$^{+}$*Lepr*^{db} lean	92.0 ± 2.2* (n = 41)	32.7 ± 0.62* (n = 41)	0.745 ± 0.073* (n = 37)	0.027 ± 0.027* (n = 36)	2.42 ± 0.61* (n = 14)
TGF-α/*Lepr*^{db}*Lepr*^{db} obese	53.6 ± 2.8* (n = 43)	63.8 ± 2.23* (n = 43)	6.245 ± 0.500* (n = 25)	0.099 ± 0.005* (n = 25)	29.88 ± 2.39* (n = 14)

[a] Left and right retroperitoneal and parametrial fat depots.
[b] Body weight minus organ (liver, kidneys, spleen, lungs, and heart) and MT and other tumor or growths weights.
[c] Values are means ± SEM.
* Significantly different from each other by one-way ANOVA followed by Neuman-Keul's test.

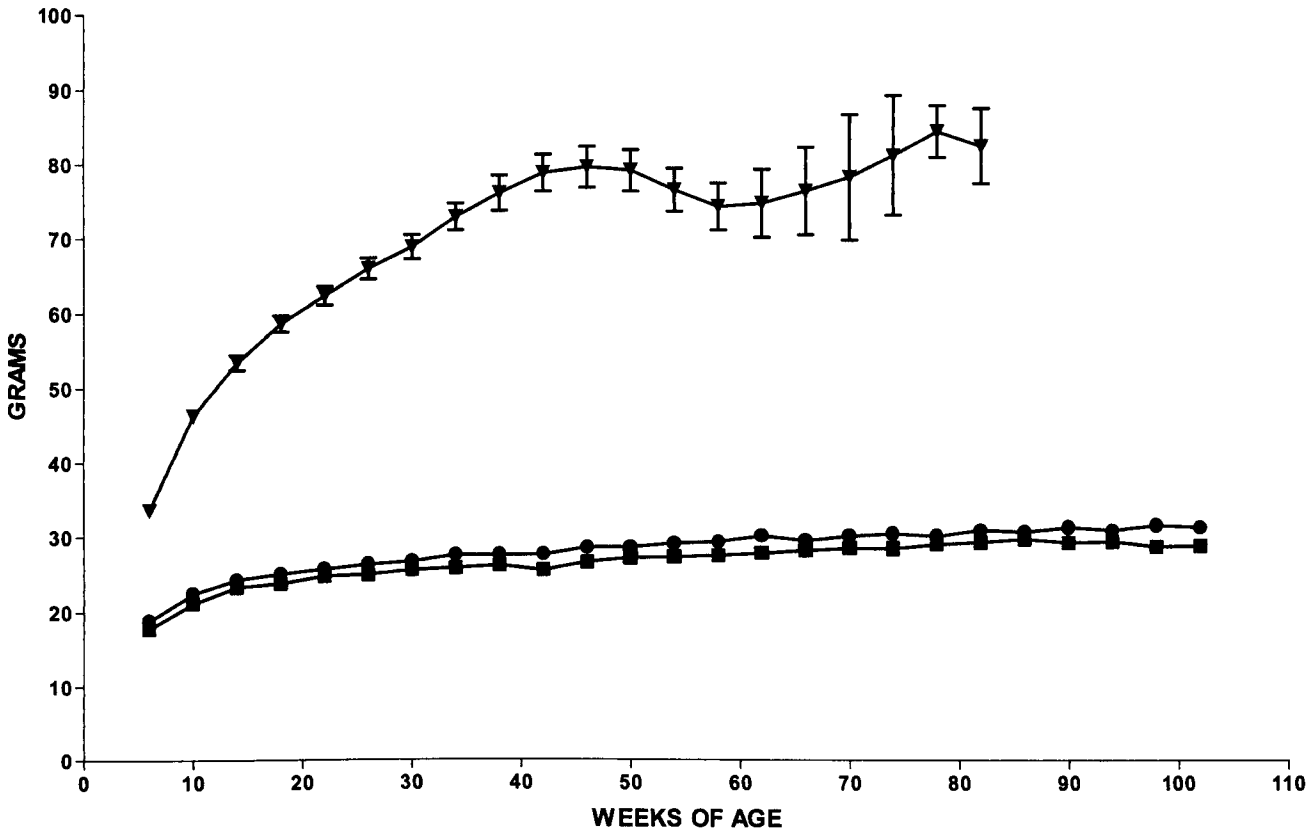

Figure 2. Body weight curves of transforming growth factor (TGF)–α/*Lepr* female mice. ■, TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ (n = 16–40, depending on age). ●, TGF-α/*Lepr*$^{+}$*Lepr*^{db} (n = 16–41, depending on age). ▼, TGF-α/*Lepr*^{db}*Lepr*^{db} (n = 2–43, depending on age) mice. ANOVA, $P < 0.0001$. The weight curve of TGF-α/*Lepr*^{db}*Lepr*^{db} mice was significantly different from that of TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ and TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice, $P < 0.001$ by Newman-Keuls multiple comparison test.

groups were compared, body and fat pad weights were significantly heavier for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} than for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice. There was a significant difference among all groups for combined fat pad to carcass ratio (MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} > MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} > MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$; Table 1). Serum leptin levels were significantly higher in MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice than in lean mice (Table 1). There was a trend for leptin levels of the heterozygous MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} lean mice to be higher than those of the homozygous MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice, but this did not reach statistical significance.

Over the course of the 2 years that the mice were monitored, 25 (69%) of 36 of the MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice that were necropsied had palpable MTs and/or MTs detected at death, compared with 31 (82%) of 38 of MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} lean mice (Table 1). The remaining lean mice were found dead, and it was not possible to determine their MT status. The average age of MT detection for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice was 80 weeks, and that for MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice was 77 weeks. No MTs were detected in obese MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice (Table 2). Thirty-seven percent (16/43) of the MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice were found dead, and the remaining mice were sacrificed prior to age 104 weeks as a consequence of excessive weight loss, open sores, lethargy, and/or breathing difficulties.

As summarized in Table 2, of the 94 and 93 MTs obtained from MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ and MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice, respectively, the majority were classified as low-grade adenocarcinomas or atypical hyperplasia. The remaining two MTs found in the MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ group were high-grade adenocarcinomas, as were the four other MTs found in MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice. Three putative MTs were removed from MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice, but these were identified as necrotic adipose tissue on histopathological analysis. Additional pathological findings in lean mice included malignancies of the eye, uterus/ovaries and intestine, as well as lymphoma, angiosarcoma, and lymphoproliferative and myeloproliferative disorders. These were not associated with the presence or absence of MTs. Despite the many spontaneous deaths and those necessitated by weight loss and body sores, MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice had few pathological findings to explain their enhanced morbidity and mortality. One obese mouse did have a hepatocellular carcinoma, and two others had signs of pneumonia.

Comparisons were made between MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ and MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice with and without MTs (Tables 3 and 4). Age at death was similar

Table 2. Summary of Histopathology Results of Transgenic MMTV–TGF-α/*Lepr* Female Mice

	MT pathology				
	Total	Low-grade	High-grade	Other	Other notable pathologies
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ lean	94	92	2	0	No MT: 1 malignant eye tumor, 1 myeloproliferative disorder, 1 lymphoma, 1 angioscarcoma; MT: 1 lymphoma, 1 granulocytic sarcoma or lymphoma, 1 leimyosarcoma
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} lean	93	89	4		No MT: 1 sarcoma, 1 ovarian granulosa cell tumor; MT: 1 lymphoproliferative disorder, 1 high-grade intestinal neoplasm, 1 uterine/ovarian tumor (mesenchymal and leiomyosarcoma)
MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} obese	3	0	0	3[a]	No MT: 2 with signs of pneumonia, 1 lung infection, 1 hepatocellular carcinoma, 1 pancreatic cyst (not malignant), 2 glomeruloscleroses

[a] These growths were all found to be necrotic adipose tissue (sometimes with calcification and inflammation) but with no signs of malignancy.

for both lean genotypes, regardless of whether MTs were present (Table 3). MTs were also detected at similar ages. Tumor burden, as reflected by MT weight, was 44% greater in MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} than in MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice ($P < 0.08$), but the number of MTs per mouse was similar (Table 3). Forty percent of MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice had their MTs detected at death, compared with only 20% of MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice. As shown in Table 4, there was a significant effect of the presence of MTs on final body weights, but when this was corrected for tumor and organ weights (i.e., carcass weight), there were no significant differences with respect to genotype or the presence of MTs (Table 4). There was a significant effect of genotype on fat pad weights, with MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} mice having heavier fat pads than those excised from MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ lean mice. Similar results were found for fat pad–to–carcass weight calculation (Table 4).

We examined mammary tissue morphology using whole mammary tissue mounts. Representative whole mounts from MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mice showed abundant ductal branching and the presence of areas of atypical hyperplasia and apparent early MT development (Fig. 3A and B). A whole mount from a MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ mouse without MTs indicated abundant ductal branching without areas of tumor involvement (Fig. 3C). Abundant ductal branching also characterized a whole mount from a nontransgenic *Lepr*$^{+}$*Lepr*$^{+}$ mouse (Fig. 3D). Similar results were seen for comparable MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db} and nontransgenic *Lepr*$^{+}$*Lepr*^{db} mice (Fig. 3E–H). In contrast, mammary tissue from MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice demonstrated essentially no branching and no sites of atypical hyperplasia or early MT formation (Fig. 3I–K). Mammary tissue from a representative nontransgenic *Lepr*^{db}*Lepr*^{db} mouse (Fig. 3L) displayed a morphology similar to that of MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice.

Discussion

We report that MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice that are genetically deficient in the long isoform, OB-Rb, of the leptin receptor do not develop oncogene-induced MTs. These findings complement the findings of our earlier study, which indicated that leptin-deficient mice also do not develop oncogene-induced MTs (17). Although MMTV–TGF-α/*Lepr*^{db}*Lepr*^{db} mice had elevated serum leptin levels, MT development did not occur. In contrast, both lean genotypes developed MTs at incidence rates comparable to our earlier results for MMTV–TGF-α/*Lep* strain lean mice (17, 29) and for the originally described MMTV–TGF-α mice (27). While the present study was under way, it was reported that obese Zucker rats that also have a mutation of the leptin receptor OB-Rb had a decreased incidence of mammary carcinomas after the administration of *N*-methyl-*N*-nitrosourea (30). In contrast to the results of most other studies, in which obesity was associated with shortened MT latency, there was no difference in MT latency between obese and lean rats.

One could argue that obesity, in general, and, more specifically, leptin and/or the leptin receptor do not play a role in the development of MMTV–TGF-α–mediated oncogene-induced tumors. However, leptin receptor–intact MMTV–TGF-α mice that develop obesity as a result of dietary intervention have significantly shortened MT latency compared with MMTV–TGF-α mice that remain lean. Furthermore, MTs obtained from MMTV–TGF-α mice with diet-induced obesity express leptin receptors.

[1] Clearly MP, Grande JP, and Maihle NJ, submitted manuscript.

[2] Hu X, McCleary-Wheeler AL, Juneja SC, Greenwood TM, Grande JP, Maihle NJ, and Cleary MP, manuscript in preparation.

Table 3. Average Ages of Death and MT Detection and Tumor Number and Weight for *Lepr*$^{+}$*Lepr*$^{+}$ Versus *Lepr*$^{+}$*Lepr*^{db} Lean Female MMTV–TGF-α Mice

	Age at death (weeks)	Age at MT detection (weeks)	No. of MT/mouse	'Tumor burden (g/mouse)
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$, no MT (n = 11)	93.5. ± 4.4[a]			
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$, MT (n = 25)	91.4 ± 2.7	80.2 ± 4.3	4.2 ± 0.5	1.483 ± 0.242*
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db}, no MT (n = 7)	97.7 ± 3.7			
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db}, MT (n = 31)	92.1 ± 2.5	77.5 ± 4.4	4.8 ± 0.4	2.130 ± 0.260*

[a] Values are means ± SEM.
* $P < 0.08$.

Several epidemiological studies have measured serum leptin levels in women with breast cancer. To date, results directly establishing a role for leptin in human breast carcinogenesis have been inconclusive. One study indicated no association of serum leptin with premenopausal breast cancer (31). However, because obesity is not considered to be a risk factor for premenopausal breast cancer, this result is not surprising. In another study that included both premenopausal and postmenopausal subjects, there also was no relationship shown of leptin with development of breast cancer (32). In a third report, serum leptin levels were significantly higher in women with breast cancer than in control subjects even when body mass index (BMI) was similar (33). In addition, higher serum leptin levels were associated with more advanced disease. Given the relatively newness of the identification of leptin, it is understandable that limited epidemiological data are available.

There are interesting results from *in vitro* studies that support a role for leptin and leptin receptors in the development of breast cancer. For example, the presence of leptin receptors has been established in several different breast cancer cell lines, and the addition of leptin enhanced cellular proliferation (19–22). Also, Hu *et al.* (19) reported that, although leptin did not stimulate colony formation in a nonmalignant breast epithelial cell line HBL100, T-47D breast cancer cells formed twice as many colonies in the presence of leptin as without. This type of *in vitro* anchorage-independent growth assay reflects the ability of a cell line to develop tumors when inoculated into athymic mice (34, 35).

It is interesting to note that leptin enhances cell invasion (36, 37) and the migration (38) of other cell types. Furthermore, leptin receptors have been identified in other malignant cell lines and tumors. For example, leptin receptors are expressed in the lung cancer cell line SQ-5 and leptin stimulated proliferation and increased mitogen-activated protein kinase (MAPK) activity in these cells (39). Leptin receptors have been identified in human adrenal tumors (40), pituitary adenomas (41), and stomach tumors (42). In addition, leptin receptors are found in several human leukemia cell lines and in leukemia cells from 50% of patients newly diagnosed with acute myeloid leukemia (43). Of interest, an increased BMI is associated with the risk of leukemia (44). Thus, although leptin receptors were initially identified to be present in the hypothalamus and thought to be part of a feedback mechanism monitoring body fat stores,

Table 4. Final Body and Carcass[a] Weights, Fat Pad Weight,[b] and Fat Pad:Carcass Ratio for *Lepr*$^{+}$*Lepr*$^{+}$ and *Lepr*$^{+}$*Lepr*^{db} Lean MMTV–TGF-α Female Mice with and Without MTs

	Final body weight (g)	Carcass weight (g)	Fat pad weight (g)	Fat pad weight:carcass
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$, no MT (n = 11)	29.2 ± 0.86	25.9 ± 0.7	0.386 ± 0.082**	0.014 ± 0.003**
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$, MT (n = 25)	30.5 ± 1.15*	25.9 ± 0.5	0.459 ± 0.083**	0.018 ± 0.001**
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db}, no MT (n = 7)	28.1 ± 0.77	26.2 ± 1.0	0.772 ± 0.125	0.029 ± 0.005
MMTV–TGF-α/*Lepr*$^{+}$*Lepr*^{db}, MT (n = 31)	33.6 ± 0.80*	28.1 ± 0.5	0.751 ± 0.112	0.026 ± 0.002

[a] Body weight minus organ (liver, kidneys, spleen, lungs, and heart) and MT and other tumor or growth weights.
[b] Left and right retroperitoneal and parametrial fat depots.
* Significant tumor effect.
** Significant genotype effect.

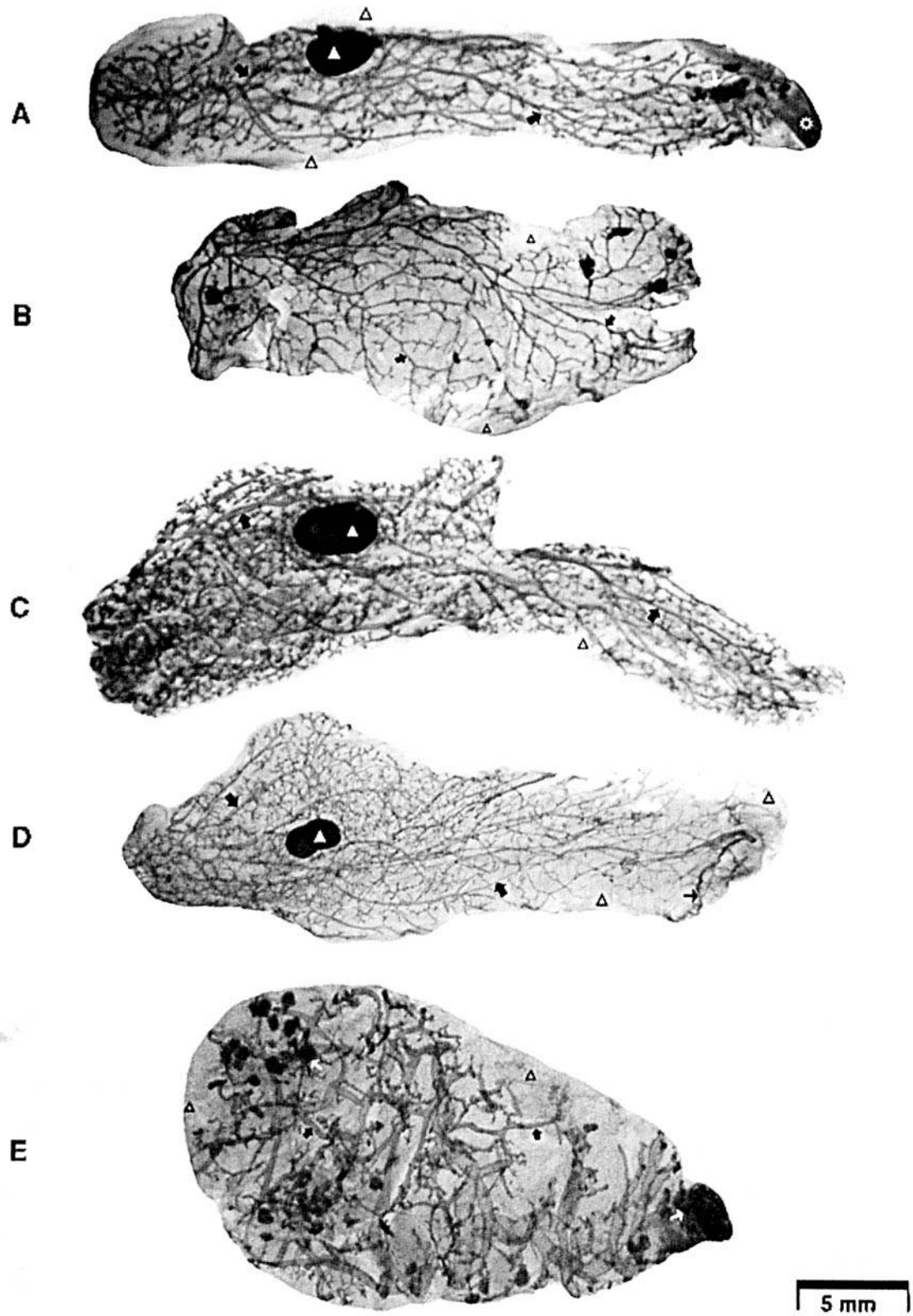

Figure 3. Mammary-tissue whole mounts from MMTV–transforming growth factor (TGF)–α/*Lepr* and nontransgenic *Lepr* female mice. Thick black arrows, mammary ducts. Open triangle, stromal tissue. White triangle, lymph gland. Thin black triangle nerve fibers. White arrow, tumor tissue. White open star, skeletal muscle/fibers. (A) TGF-α/$Lepr^{+}Lepr^{+}$ mouse, age 104 weeks. (B) TGF-α/$Lepr^{+}Lepr^{+}$ mouse, age 104 weeks. (C) TGF-α/$Lepr^{+}Lepr^{+}$ mouse, age 104 weeks. (D) Nontransgenic $Lepr^{+}Lepr^{+}$ mouse, age 83 weeks. (E) TGF-α/$Lepr^{+}Lepr^{db}$ mouse, age 92 weeks. (F) TGF-α/$Lepr^{+}Lepr^{db}$ mouse, age 93 weeks. (G) TGF-α/$Lepr^{+}Lepr^{db}$ mouse, age 104 weeks. (H) Nontransgenic $Lepr^{+}Lepr^{db}$ mice, age 73 weeks. (I) TGF-α/$Lepr^{db}Lepr^{db}$ mouse, age 84 weeks. (J) TGF-α/$Lepr^{db}Lepr^{db}$ mouse, age 61 weeks. (K) TGF-α/$Lepr^{db}Lepr^{db}$ mouse. (L) Nontransgenic $Lepr^{db}Lepr^{db}$ mouse, age 86 weeks. Scale bar, 5 mm.

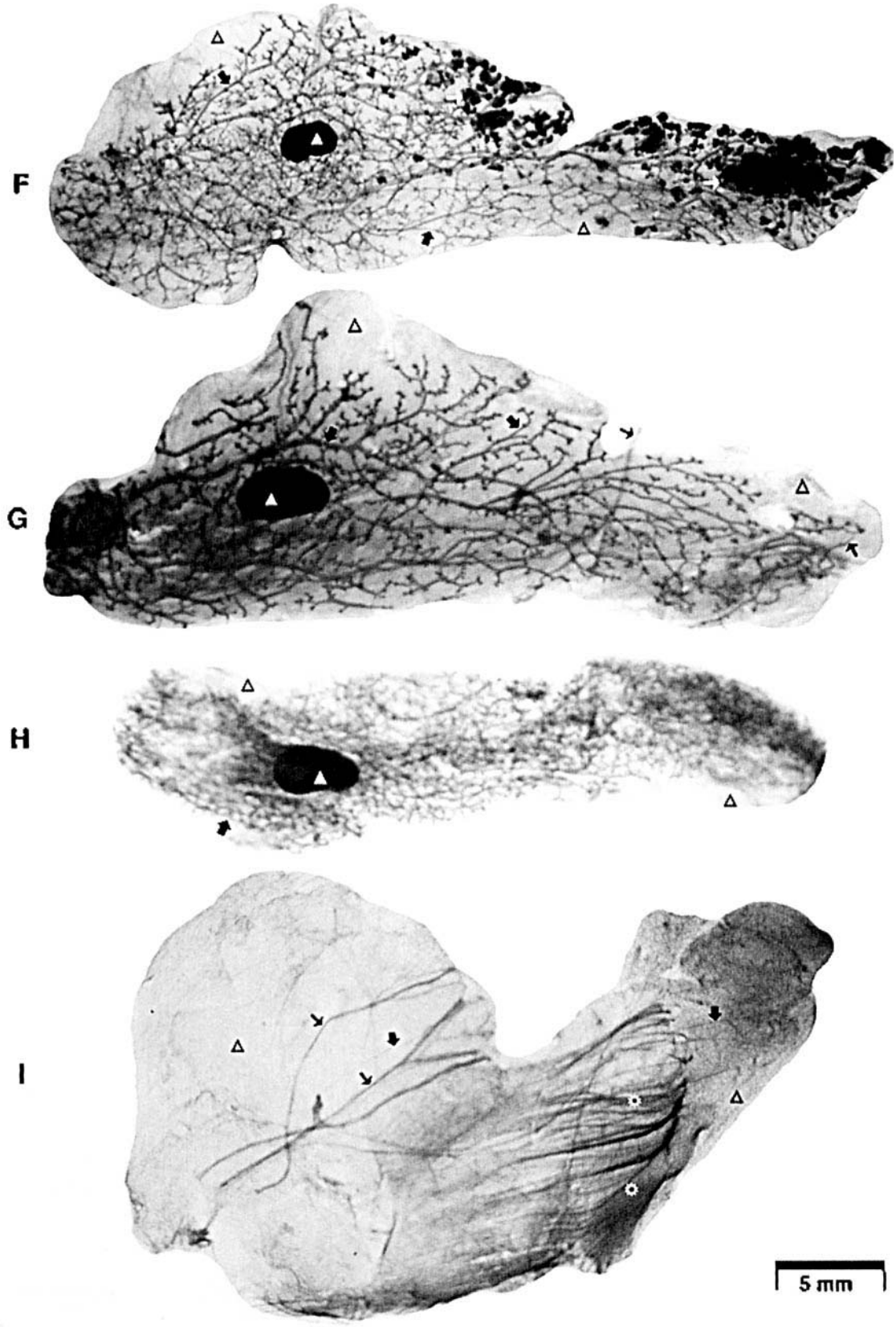

Figure 3. *continued*

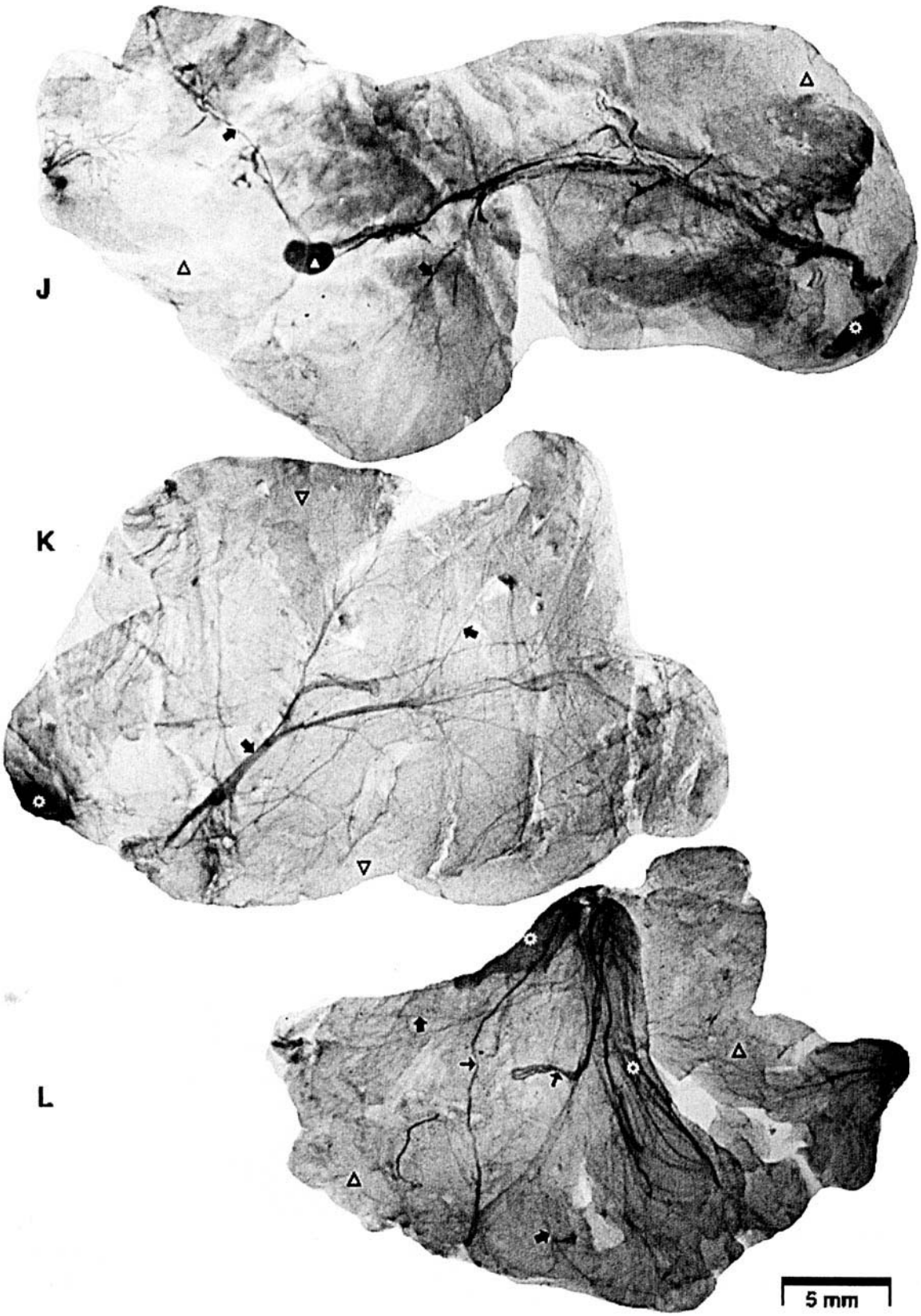

Figure 3. *continued*

continued research suggests a more diverse role for this receptor and its ligand.

In many of the studies cited above, it was not specified that the receptor identified was OB-Rb. In our study (19), we used an antibody specific for OB-Rb. The presence of both the long and short isoforms, OB-Rb and Ob-Ra, of the leptin receptor in human breast tumors has been reported, although no association of the isoforms with other tumor characteristics or disease outcome has been shown (20). The relationship of the two isoforms may be of interest, because it has been reported that the coexpression of these two isoforms can generate a mild dominant negative repression of the long (signaling) form of the receptor (45).

The leptin receptor defect in $Lepr^{db}Lepr^{db}$ mice is a mutation that results in defective leptin signaling caused by the absence of a cytoplasmic region of OB-Rb (23, 46, 47). In general, OB-Rb is considered to be the signaling form of the receptor-activating pathways, including JAK/STAT (Janus kinase/signal transducer activation of transcript), MAPK, PI3'K (phosphoinositol 3' kinase), and SOCS-3 (suppressor of cytokine signaling; Refs. 48, 49). The short isoform, OB-Ra, is present in the tissues of $Lepr^{db}Lepr^{db}$ mice (50), but decreased binding of leptin is reported for OB-Ra (51). There has been speculation that signal transduction may occur through OB-Ra (52, 53), but, in general, OB-Rb signaling is considered to be of primary physiological relevance. Evidence for the importance of OB-Rb in signal transduction is supported by the results of a recent gene-transfer study (54). There was no effect of leptin on cell proliferation or signaling in endothelial cells from obese Zucker rats with transfer of the LacZ gene, but, when the OB-Rb gene was transferred, the cells exhibited cell proliferation reaching levels similar to those of lean rats with or without the added gene. In addition, the phosphorylation of STAT3 and JAK2 was detected in cells from obese rats with OB-Rb gene transfection.

With the identification of *Lepr* and *Lep* mutations, there was speculation that human obesity could be explained and perhaps treated. However, it appears that very few cases of human obesity result from these defects (55, 56), although genetic variants of the leptin receptor have been identified (57–59). In particular, elevated BMI and serum leptin levels have been reported to accompany the Gln223Arg variant (60–62). It also has been reported that overfeeding resulted in a greater effect on serum insulin and leptin levels in relation to the Gln223Arg variant in subjects with GlnGln genotype compared with those with GlnArg and ArgArg genotypes (63).

Initially, we chose the *Lepr* and *Lep* obesity models because molecular explanations for the genetic defects had been identified and the use of dietary intervention could be avoided. In retrospect, these mice were perhaps not good models of human obesity. However, it is interesting to note that we confirmed a heterozygous effect of the *Lepr* gene on body fat level (64) and found that heterozygous lean mice weigh more than homozygous lean mice. In addition, we noted that tumor weight was greater in MMTV–TGF-α/$Lepr^{+}Lepr^{db}$ than in MMTV–TGF-α/$Lepr^{+}Lepr^{+}$ mice and that their incidence of palpable MTs was higher. Although, given the numbers of mice used, these latter differences were not statistically significant, the findings suggest that small changes in body weight and body fat may affect MT development. Specifically, in MMTV-$Lepr^{+}Lepr^{db}$ mice, higher serum leptin levels appear to compensate for the decrease in leptin receptors.

In general, the use the *Lepr* mouse strain in the present study and in our earlier study using the *Lep* mouse strain provide important insights in establishing leptin as a potential link between obesity and mammary/breast tumor development. These obese mice that are either leptin deficient or have a defect in the leptin receptor do not develop MTs. In contrast, other rodent models of obesity have shortened tumor latency and/or elevated tumor incidence. Although the general hypothesis of leptin as a growth factor for mammary tumor/breast cancer is still at an early stage, evidence continues to mount that it could be an important determinant factor. We speculate that normal serum leptin levels and a functioning leptin receptor are necessary for normal mammary tissue development. However, elevated serum leptin levels in the presence of functioning receptors or perhaps at a particular ratio of the long to short isoforms may enhance cell signaling and trigger proliferation. Recently, a knock-in mouse model was described whereby tyrosine 1138 of OB-Rb was replaced with serine (65). This change specifically disrupts the STAT3 signaling mediated through OB-Rb. Mice homozygous for this change, *s/s*, developed obesity and had elevated serum leptin levels, as do $Lepr^{db}Lepr^{db}$, however, in contrast to $Lepr^{db}Lepr^{db}$ mice, *s/s* mice are fertile. although they do not lactate. Thus, STAT3 appears to be important in body weight regulation, and possibly for lactation, but not for fertility *per se*. Continued investigations will delineate the role of leptin and its receptors and signaling pathways in mammary tumorigenesis.

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