Estrogen-Dependent Growth of a Rat Pituitary Tumor Involves, But Does Not Require, a High Level of Vascular Endothelial Growth Factor

DANNY CRACCHIOLO, JASON W. SWICK, LUCY MCKIERNAN, ERICA SLOAN, SUPRIYA RAINA, CHARLES SLOAN, AND DOUGLAS L. WENDELL

Department of Biological Sciences, Oakland University, Rochester, Michigan 48309-4401

Long-term (10-week) treatment of Fischer 344 (F344) rats with the synthetic estrogen diethylstilbestrol (DES) increases the level of vascular endothelial growth factor (VEGF) in the pituitary. This is concurrent with the development of a large tumor of the pituitary of F344 rats. A role for VEGF in estrogen-dependent pituitary tumor growth is also supported by the fact that pituitary VEGF level is not increased by estrogen treatment in rats of the tumor-resistant Brown Norway (BN) strain. However, VEGF is not increased by estrogen treatment in an F_1 hybrid of F344 and BN, even though F_1 hybrid rats do form pituitary tumors in response to estrogen. Quantitative trait locus (QLT) mapping reveals that control of estrogen-dependent VEGF expression is linked to the *Edpm5* QTL, which was previously identified as a QTL for estrogen-dependent pituitary tumor growth. In contrast, the QTL *Edpm2-1* and *Edpm9-2*, which have been shown to each have a significant effect on estrogen-dependent pituitary mass of a magnitude similar to *Edpm5*, do not have any effect on VEGF level. Taken together, our results support the association of VEGF expression with growth of the estrogen-induced rat pituitary tumor, as has been reported by others, but they also indicate that there is significant pathways of growth regulation that are independent of high-level VEGF expression. [Exp Biol Med Vol. 227(7):492–499, 2002]

Key words: estrogen; VEGF; pituitary; genetics; tumor

The rat pituitary provides a model system for investigating the control of growth by estrogen-responsive tissues and tumors because common laboratory strains differ greatly in their ability to control pituitary growth when stimulated by chronic estrogen treatment. When rats of the strain Fischer 344 (F344) are chronically treated with estrogen, their pituitary gland experiences uncontrolled growth to form a tumor that is 10 to 20 times normal mass in a matter of several weeks (1–4). In contrast, rats of the strains Holtzman, Sprague-Dawley, and Brown Norway (BN) maintain normal pituitary size upon chronic estrogen treatment (4–6).

In addition to uncontrolled proliferation of the lactotroph cell population, the pituitary of estrogen-treated F344 rats undergoes changes in vasculature of both normal and abnormal form. The pituitary capillaries undergo an increase in vessel diameter (7–9), and there also appears to be a modest increase in the density of capillaries in upon estrogen treatment of F344 rats (7, 8). The occurrence and significance to angiogenesis to the growth of the estrogen-induced rat pituitary tumor is supported by the fact that the angiogenesis inhibitors fumagillin and TNP470 inhibit the growth in mass and vascular area of the estrogen-induced growth of the F344 strain (9, 10). There are also two major abnormal developments. Whereas the pituitary normally receives all of its blood *via* a portal system from the hypothalamus, estrogen-treated F344 rat pituitaries are invaded by arterial blood supply (5). This does not happen in the pituitary of untreated F344 rats or in the pituitary of either treated or untreated Sprague-Dawley rats. Schechter *et al.* (11) have proposed that upon estrogen treatment, cells at the periphery of the F344 pituitary release growth factors to recruit the systemic blood vessels. The other abnormal vascular phenomenon observed in the pituitary of estrogen-treated F344 rats is loss of structural integrity and formation of hemorrhagic lakes. Many blood vessels in the pituitary of the estrogen-treated F344 rat are disrupted, and large hemorrhagic lakes form that are not lined by endothelium (11). Jacubowski *et al.* (12) found that although total pituitary blood flow does increase in the diethylstilbestrol (DES)-treated F344 rat pituitary, blood flow relative to pituitary mass actually decreased. They also noted that large vascular lakes occur in the pituitary of the Fischer rat given long-term (6–10 weeks) DES treatment (12). They concluded that the pituitary of the DES-treated F344 blood supply is com-

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[1] To whom request for reprints should be addressed at Department of Biological Sciences, Oakland University, 2200 North Squirrel Road, Rochester, MI 48309-4401. E-mail: wendell@oakland.edu

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promised by the combination of the gland outgrowing its blood supply and the formation of the vascular lakes (12).

Work by others has implicated vascular endothelial growth factor (VEGF) in the growth and vascular changes of the estrogen-induced rat pituitary tumor. Estrogen treatment of F344 rats leads to an increased level of a 29-kDa species of VEGF in both the pituitary of F344 rats and cells of the GH3 pituitary cell line (7, 13). 2-Methoxyestradiol, which inhibits the estrogen-induced growth of the F344 rat pituitary, also reduces estrogen-induced VEGF expression (8). What is lacking from much of the work is a comparison of the effect of estrogen on tumor resistant and tumor susceptible rat strains. Observation of an effect by estrogen in F344 rats alone can be interpreted either as part of the tumor growth process or simply a response to estrogen alone. Of all the recent reports on estrogen's effects on VEGF expression in the rat pituitary (7, 8, 13–15), only one utilizes both tumor-susceptible and tumor-resistant strains. Long *et al.* (14) investigated the effect of the xenoestrogen Bisphenol A on VEGF expression in pituitary and did not detect a difference in VEGF mRNA level between F344 and Sprague-Dawley.

Six genetic loci have been identified that contribute to the variation in pituitary growth control between F344 and BN, and evidence indicates that there are additional loci as well. These genetic factors were originally identified by quantitative trait locus (QTL) mapping using the trait of pituitary mass of estrogen-treated rats generated by an F_2 intercross of F344 and BN. The QTL are named *Edpm*, for estrogen-dependent pituitary mass, and for the number of the rat chromosome on which they reside. These loci combine additively to control the growth of the pituitary in rats treated chronically with estrogen (16).

To assess the contributions of the *Edpm* QTL to the various components of tissue growth and vascular development, further analysis was performed on the pituitaries of estrogen-treated rats generated by a backcross to F344. In this case, in addition to pituitary mass, biochemcial components of growth were assayed in the pituitaries and were used as quantitative traits. This work revealed differentiation of function of the *Edpm* QTL. *Edpm2* and *Edpm9-2* are strongly associated with pituitary DNA content (an indicator of cell number), but do not have any significant association with pituitary hemoglobin content (an indicator of blood volume) (17). In contrast, *Edpm5* is strongly associated with pituitary hemoglobin content (17), but not with pituitary DNA. *Edpm5* is also strongly associated with the control of the level of the matrix metalloproteinase (MMP)-9, which is upregulated by estrogen in the pituitary of F344 rats but not BN (18). These data point to *Edpm5* being involved in changes in vascular structure and/or blood volume during the course of estrogen-induced pituitary tumor growth.

In this work, we use genetic tools to investigate the regulation of VEGF level in the rat pituitary and its possible role in growth of the estrogen-induced rat pituitary tumor. Our results support a role for VEGF in this tumor, but also indicate that significant routes of growth promotion exist that do not involve VEGF.

Materials and Methods

Animals and Hormone Treatment. F344 rats, substrain F344/SsnHsd (F344), BN, substrain BN/RijHsd, and an F_1 hybrid of F344 and BN (F_1) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). For QTL mapping, a backcross was performed by mating F_1 hybrid females to F344 males. One hundred twenty-nine female progeny from this backcross, referred to as the BC_1 generation, have been described previously (17). For estrogen treatment, 21-day-old female rats were administered subcutaneous Silastic tubing implants containing 5 mg of DES, as previously described (4). Ten weeks later, rats were sacrificed by decapitation and the pituitary was dissected from the cranium. Pituitary mass (wet weight) was measured and pituitaries were immediately frozen and stored at −80°C. Untreated controls were rats of the same age that were not given a DES implant. In the BC_1 generation, all rats were given estrogen treatment because the experimental variable in the backcross is animal genotype, not hormone treatment. All procedures were approved by the Oakland University Institutional Animal Care and Use Committee.

Detection and Measurement of VEGF. Pituitaries were homogenized on ice in chilled buffer; either 40 m*M* potassium phosphate buffer, pH 7.5 (4) or in RIPA buffer (18). (The choice of buffer did not affect subsequent VEGF detection). Pituitaries were homogenized with 10 strokes of a glass-Teflon (Potter-Elveheim) homogenizer on ice. Homogenates were divided into aliquots, flash frozen in liquid nitrogen, and stored at −80°C until use. The samples of BC_1 generation rat pituitary employed in this work were aliquots of the same homogenates that were employed in previous studies (17, 18). Protein concentration of homogenates was determined by triplicate assays by the BCA method (Pierce, Rockford, IL). For each sample, the mean of the triplicate assays of protein concentration was used to calculate the volume of the given homogenate that would contain 20 μg of total protein. This amount was combined with sample buffer and loaded onto gels, as described below.

VEGF protein was detected by Western blot. Gels for Western blots were standard Laemmli gels with the exceptions that the resolving gel was 15% acrylamide, both resolving and stacking gels contained 1 m*M* dithiolthreitol (DTT), and samples, each containing 20 μg of total protein, were loaded into gels immediately after boiling to prevent oligomerization of VEGF (Murdoch F, personal communication). The SDS-PAGE sample buffer consisted of (final concentrations) 62.5 m*M* Tris-HCl, pH 6.8, 2% SDS, 10% (v/v) glycerol, 100 m*M* DTT, and 0.01% bromophenol blue (Murdoch F, personal communication). Gels were blotted onto nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Piscataway, NJ) by electrophoretic transfer using a Mini Trans-Blot apparatus according to manufacturer's directions (Bio-Rad Laboratories, Hercules,

CA). Blocking, primary and secondary antibody incubations, and washes were done in TBS (24 m*M* Tris-HCl, pH 7.4, 2 m*M* KCl, and 163 m*M* NaCl) plus 0.02% Tween 20 and 10% nonfat dry milk. The primary antibody was the anti-VEGF antibody A-20 from Santa Cruz Biotechnology (catalog # sc-152, Santa Cruz Biotechnology, Santa Cruz, CA) and was used at 1/1000 dilution. The secondary antibody was horseradish peroxidase-conjugated donkey-anti-rabbit IgG (Amersham Pharmacia Biotech). Antibody on blots was detected using the ECL Plus chemiluminescence detection reagent kit and Hyperfilm ECL film according to manufacturers instructions (Amersham Pharmacia Biotech).

To verify equivalence in sample loading, prolactin (PRL) was detected on the blots presented in Figures 1 and 2. PRL level can be used to compare individual samples of the same genotype and treatment combination and can also be used to compare samples of estrogen-treated rats of the same genotype. This is because PRL synthesis rate and PRL as a fraction of total pituitary protein are the same in tumor-resistant and tumor-susceptible rat strains after chronic estrogen treatment (19). (Although circulating PRL does in-

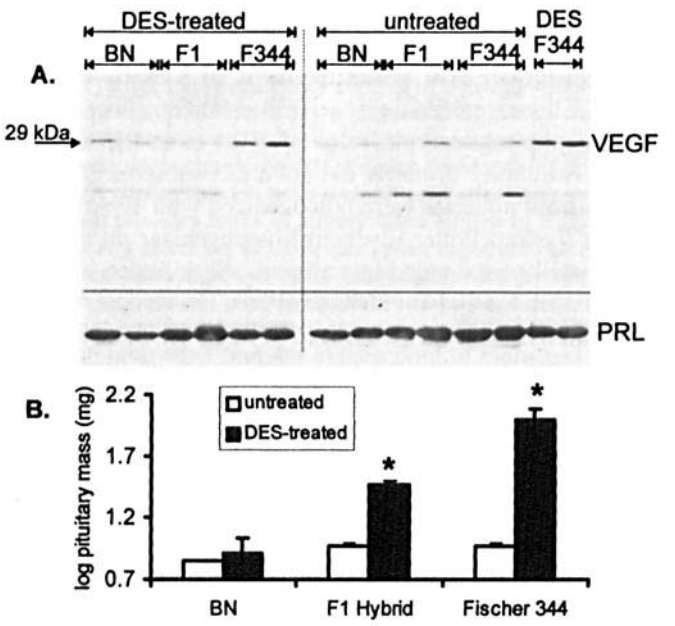

Figure 1. Effect of estrogen and genotype on VEGF level in the pituitary. Rats are F344 (forms pituitary tumors after chronic estrogen treatment), BN (does not exhibit significant pituitary growth after chronic estrogen treatment), and an F_1 hybrid of these strains (forms a tumor of intermediate size in response to chronic estrogen treatment). Estrogen-treated animals were given a 10-week chronic DES treatment, and untreated animals were of the same age but were not given implant. VEGF was detected by Western blot, with each lane containing pituitary homogenate from a different animal. PRL was subsequently detected on these same blots as a loading control. (A) Western blot of rat pituitary homogenates. (B) Mean pituitary mass of the animals used for VEGF detection in A; black bars are estrogen-treated animals and white bars are untreated animals; error bars are 1 SEM. The asterisk indicates statistically significant difference between treated and untreated animals of a given genotype ($P < 0.005$). The dotted vertical line in A indicates that there were two separate blots. The rightmost two lanes, labeled "DES F344" are the same samples as those in lanes 5 and 6 and serve as an internal positive control.

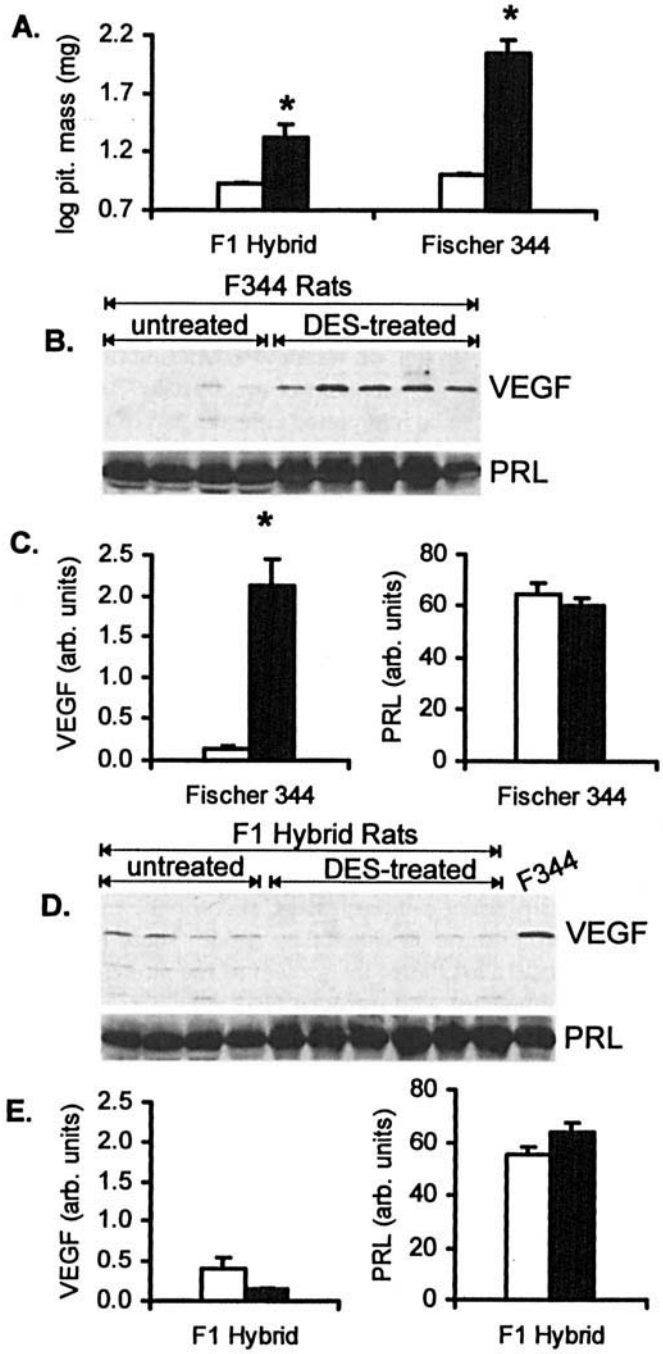

Figure 2. There is a variation in VEGF levels between different individual F344 rats, but this variation is less than the variation between treated and untreated F344 or between treated F344 and treated F_1. In graphs, black bars are estrogen-treated animals and white bars are untreated animals; error bars are 1 SEM and an asterisk indicates a statistically significant difference between treated and untreated of a given genotype ($P < 0.005$). (A) Mean pituitary mass of treated and untreated F_1 hybrid and F344 rats used for the western blots. (B) Western blot of pituitary homogenates of treated and untreated F344 rats probed with anti-VEGF antibody; the bottom panel is from a duplicate blot probed with anti-PRL antibody. (C) Quantitation of VEGF and PRL level in F344 (arbitrary units). (D) Western blot of pituitary homogenates of treated and untreated F_1 hybrid rats probed with anti-VEGF antibody, with DES-treated F344 in the last lane as a positive control; the bottom panel is from a duplicate blot probed with anti-PRL antibody. (E) Quantitation of VEGF and PRL level in F_1 hybrid (arbitrary units).

crease with increased mass of estrogen-induced pituitary tumor, this is due to the presence of a larger pituitary gland [20].) Western blots were probed with anti-PRL antibody (National Hormone and Pituitary Program, NIDDK, NIH; lot number AFP425-10-91) at 1/32,000 dilution. The secondary antibody was horseradish peroxidase-conjugated donkey-anti-rabbit IgG (Amersham Pharmacia Biotech) and was detected using the ECL Plus chemiluminescence detection reagent kit and a STORM fluorimager (Molecular Dynamics, Sunnyvale, CA). (This instrument was not available at the time that the VEGF detection was performed).

The intensity of the VEGF band on films was measured using a Howtek Scanmaster 3+ scanner (Howtek Inc., Hannover, MA) and BioImage software (Millipore Corp., Bedford, MA). The intensity of PRL was measured using Molecular Dynamics ImageQuant software. The absolute intensity of a band will vary from blot to blot, so for comparison of band intensities for treated versus untreated animals of the same genotype (as in Fig. 2), only samples from the same blot were compared, and the significance of differences between group mean values was tested by one-sided Student's t test. Not all BC_1 samples could be analyzed with a single Western blot. Therefore, all Western blots of BC_1 samples carried an internal control in the form of one lane of homogenate from the same DES-treated F344 rat. Band intensities on each blot were indexed relative to this DES-treated F344 so that data could be compared from blot to blot. Because of the method of detection and quantitation used for the BC_1 samples, statistical analysis was performed using nonparametric methods.

Genetic Mapping. Microsatellite DNA was amplified by polymerase chain reaction (PCR) using oligonucleotide primers produced by custom synthesis (Invitrogen Carlsbad, CA). Primer sequences and PCR conditions for microsatellite markers were obtained from public databases. Microsatellite markers with the "*Wox*" designation were developed at the Wellcome Trust Centre for Human Genetics (http://www.well.ox.ac.uk/rat_mapping_resources [21]). Microsatellite markers with the *Got* designation were developed at the Otsuka GEN Research Institute (Otsuka Pharmaceutical Company, (http://ratmap.ims.u-tokyo.ac.jp/ [22,23]). Microsatellite markers with the "*Mgh*," "*Mit*," or "*Rat*" were developed by the Rat Genetic Mapping Project of the Whitehead Institute (http://waldo.wi.mit.edu/rat/public/ [24,25]). Those microsatellites that also serve as genetic markers for protein-encoding genes are indicated in Figure 3.

Linkage maps of microsatellite markers were constructed from BC_1 generation genotype data using MAPMAKER/EXP version 3.0 (26) and marker genotype data from the entire BC_1 generation of 129 rats. The use of markers on Chr 2, 3, and 9 and the Chr 5 markers *D5Mgh3, D5Mgh5, D5Mgh6, D5Mgh11, D4Mit4, D5Mit5,* and *D5Mit7* in these animals has been reported previously (17, 18). Genotype data on all other Chromosome 5 microsatellite markers in this BC_1 generation is new to this study.

QTL mapping of the trait of pituitary VEGF level was performed using microsatellite markers on rat chromosomes where *Edpm* QTL have been previously reported, namely Chr 2 (*Edpm2-1* and *Edpm2-2*), Chr 3 (*Edpm3*), Chr 5 (*Edpm5*), and Chr9 (*Edpm9* and *Edpm9-2*) (17). VEGF level was determined in 48 individual BC_1 generation rats. The rats chosen for analysis were those at the high (30 rats) and low (18 rats) extremes of the phenotypic distribution of pituitary mass in the BC_1 generation (27). The VEGF level determined by densitometry (above) of the selected BC_1 generation rats was used as a nonparametric trait (28). Nonparametric QTL mapping was performed using MAPMAKER/QTL version 1.9 (28). The hypothesis being tested by QTL mapping was that inheritance of a BN allele of a given known QTL would reduce the level of VEGF. The null hypothesis in these experiments was that there is no difference in VEGF level between rats heterozygous for BN and F344 alleles and rats that were homozygous for F344 alleles of a given known QTL. Because each chromosome was viewed independently and the hypothesis being tested was based on there already being a known QTL on the chromosomes tested, single-point significance criteria were used rather then genome wide significance criteria (29).

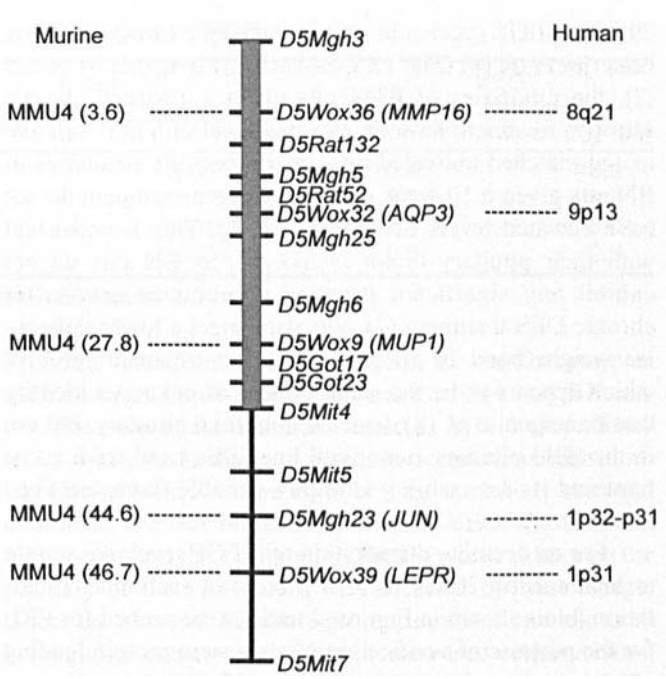

Figure 3. Map of RNO5 with our microsatellite data and with human and mouse homologies indicated. Microsatellites associated with protein-encoding genes were used to identify homologous chromosomal positions in human and mouse as reported by Watanabe *et al.* (23). The purpose of this illustration is to link our mapping of *Edpm5* with possible human and murine homologs. Mouse chromosomal positions were obtained from the Mouse Genome Database (40), and human chromosomal positions were obtained from the Online Mendelian Inheritance in Man database (44).

Results

Estrogen-Induced VEGF Expression Is Strain Dependent. Using Western blots of total rat pituitary protein to assay the level of VEGF protein, we detect the same

29-kDa VEGF species in rat pituitary that was detected by Banerjee *et al.* (8) (Fig. 1). Consistent with reports by others (7), the pituitaries of F344 rats given a 10-week chronic estrogen treatment have an elevated level of VEGF relative to age-matched untreated rats. In contrast, the pituitaries of BN rats given a 10-week chronic estrogen treatment do not have elevated levels of VEGF (Fig. 1). This is consistent with their pituitary tumor phenotype, as BN rats do not exhibit any significant increase in pituitary mass after chronic DES treatment (4). We also detect a lower molecular weight band in all genotypes and treatment groups, which appears to be the same protein of unknown identity that Banergee *et al.* (8) detected in normal pituitary, but not in the GH3 pituitary tumor cell line. This band tends to be faint and its detectability is highly variable (compare Figs. 1 and 2).

For an accurate quantitation of VEGF level, we sought to load exactly 20 μg of total protein in each lane. In addition, blots shown in Figures 1 and 2 were probed for PRL for the purpose of verification of consistent protein loading when comparing treated with treated or comparing untreated with untreated, because prolactin synthesis rate does not vary between tumor-susceptible and tumor-resistant strains (19). However, we also note that there is no statistically significant difference between PRL band intensity between estrogen-treated and untreated rats. Estrogen treatment is known to increase the transcription of the *Prl* gene (30) and increase the rate of prolactin synthesis in cells obtained from estrogen-treated rats (31). However, a Western blot detects the steady-state level of PRL, which is a balance of synthesis and release. A lack of significant difference in PRL as a fraction of total pituitary protein between estrogen-treated and untreated has been reported by others as well (32).

Estrogen-Dependent Pituitary Growth Is Possible Without Elevated VEGF. The F_1 hybrid of F344 and BN develops a pituitary tumor in response chronic estrogen treatment, which is intermediate in mass and DNA content between F344 and BN (4). However, the pituitaries of F_1 hybrid rats given a 10-week DES treatment do not exhibit elevated VEGF levels (Fig. 1). This indicates that estrogen-dependent VEGF expression in pituitary is a recessive trait. More importantly, the results demonstrate that significant (2.5-fold) estrogen-dependent pituitary growth is possible without an elevated VEGF level. We do detect variation between individual pituitary samples of estrogen-treated F344 rat in the expression of VEGF protein. This can be due to either variation in the degree of expression or sample loading, or both. However, the level is consistently and significantly higher in the estrogen-treated F344 than in the estrogen-treated F_1 hybrid animals (Fig. 2).

Control of Estrogen-Induced VEGF Protein Level Is Linked to the Edpm5 QTL. To investigate the genetic basis of the strain difference in estrogen-dependent pituitary VEGF level, we measured VEGF level in DES-treated rats generated by backcrossing the F_1 hybrid to F344 (BC_1 generation). Pituitary homogenates from the animals whose pituitary mass was at either the lower or upper extreme of the mass distribution were assayed for VEGF level. Overall, pituitary VEGF level correlates with pituitary mass in these rats (Table I).

We used QTL mapping to test the hypothesis that one or more of the previously identified *Edpm* QTL for DES-dependent pituitary mass regulated the level of VEGF in response to chronic estrogen treatment. We performed QTL mapping using VEGF level as a trait and genetic markers on rat Chromosomes 2 (*Edpm2-1* and *Edpm2-2*), 3 (*Edpm3*), 5 (*Edpm5*), and 9 (*Edpm9* and *Edpm9-2*). The hypothesis tested was that inheritance of a BN allele of a given QTL corresponded with lowered VEGF level in the BC_1 generation. Because the high level of estrogen-dependent VEGF expression of the F344 strain is a recessive trait (Fig. 1), a gene that affects estrogen-dependent VEGF expression should be detectable in a backcross to F344. Of all of the markers tested, only those on Chromosome 5 exhibit a statistically significant association between marker genotype and the trait of VEGF level (Table II). The peak z score for the association between variation in VEGF level and marker genotype on Chromosome 5 is within the same interval of markers as the peak z score for mass, and both are within the interval previously identified as the *Edpm5* QTL (Fig. 4).

We do not detect any significant association of either *Edpm2* or *Edpm9-2* with the trait of pituitary VEGF level. This is despite the fact that both are associated with a significant effect on mass (Table II). We also do not detect a significant association between *Edpm3* and VEGF. However, when performing QTL mapping for the trait of mass to Chromosome 3 with only the animals for which we have VEGF data, we detect only a moderately significant association of *Edpm3* with mass. Therefore, the negative result is not conclusive.

Discussion

Although our results support an association between estrogen-dependent VEGF expression and estrogen-dependent pituitary tumor growth, we have also identified the existence of a route, or routes, estrogen-dependent pituitary tumor growth that does not involve high-level VEGF

Table I. Average Values of BC_1 Pituitaries

	Median values	
	Low end[a]	High end[a]
Sample size	18	30
Mass (log mg)	1.34	1.89
VEGF value	0.10	0.40
DNA (total μg)	87	394
Hb/DNA[b]	1.07	2.94
MMP-9^M value[c]	0.00	0.24
MMP-9^D value[c]	0.05	0.29

[a] Of distribution of pituitary mass in BC_1 generation.
[b] As described previously (17).
[c] As described previously (18).

Table II. Test of *Edpm* QTL for Trait of VEGF

QTL	Marker interval[a]	Mass		VEGF	
		z score	*P* value	*z* score	*P* value
Edpm2-1	*D2Wox14-D2Mit6* (9 cM)	2.4	0.008	0.4	0.3
Edpm3	*D3Rat81-D3Rat27* (8 cM)	1.7	0.04	1.0	0.2
Edpm5	*D5Mgh5-D5Wox32* (6 cM)	3.1	0.001	2.2	0.01
Edpm9-2	*D9Mit4-D9Rat31* (29 cM)	3.3	0.0005	0.6	0.3

[a] Marker interval within QTL that contains peak *z* scores for both mass and VEGF traits.

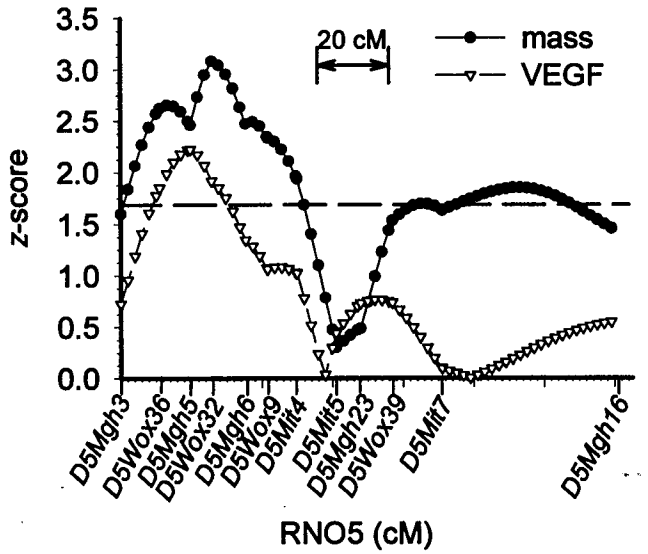

Figure 4. Variation in VEGF level is associated with the *Edpm5* QTL. The quantitative traits of pituitary mass and VEGF level were mapped nonparametrically on RNO5. The horizontal dotted line indicates a *z* score of 1.64 that corresponds with a single-point *P* value of 0.05. The peak *z* scores for both traits are within the interval previously identified as *Edpm5* (16, 17). The peak *z* scores for mass are lower than previously reported for this same backcross (17, 18) because the QTL mapping presented here uses mass data only from animals for which we have VEGF data, rather than the entire backcross generation.

expression. Other authors have reported that estrogen treatment increases the level of VEGF in the pituitary of F344 rats (7, 8). This suggests a role in the growth of that tumor such as promoting angiogenesis to support growth. Consistent with that, we show that chronic estrogen treatment leads to an increased level of VEGF in the pituitary of F344 rats, not in the pituitary of tumor-resistant BN rats. At least some of this genetic difference is linked to the *Edpm5* QTL, with inheritance of the BN allele of markers in the *Edpm5* interval corresponding with a reduced level of VEGF, depsite estrogen treatment. However, two lines of evidence point out that significant growth is possible without an increased level of VEGF expression. The first is the fact that the estrogen-induced pituitary tumor of the F_1 hybrid of F344 and BN does not exhibit an elevated VEGF level. The second is the fact that two QTL, which have a significant effect on estrogen-dependent pituitary mass, do not have any effect on estrogen-dependent VEGF level.

Crosses between F344 and BN indicate that the growth of the pituitary tumor is an additive trait. This means that the F_1 hybrid does form a pituitary tumor, but one that is intermediate mass between estrogen-treated F344 and BN rats (4). The mass of the estrogen-treated F_1 hybrid rat pituitary is 2.5-fold greater than untreated. In contrast, we detect no increase in VEGF in the F_1 hybrid upon chronic estrogen treatment. The lack of increased level of VEGF is consistent with the other traits exhibited by the F_1 hybrid. Although the pituitary of the F344 rat is characterized by hemorrhagic lakes, an outward hemorrhagic appearance, and elevated hemoglobin content indicative of increased relative blood volume, the F_1 hybrid has the morphology and hemoglobin content of a normal pituitary (4).

The QTL mapping work presented here indicates that *Edpm2-1* and *Edpm9-2* function in a growth regulatory pathway(s) that does not involve high level VEGF expression. *Edpm2-1* and *Edpm9-2* have previously been shown to have a highly significant effect on estrogen-dependent pituitary mass in two-generation crosses of F344 and BN (16, 17). This association is strong enough that it is still significant with the smaller subset of the BC_1 rats used in this study. However, we do not detect any association between the trait of VEGF level and the markers linked to *Edpm2-1* and *Edpm9-2* (Table II). Such negative results by themselves would not be meaningful if it were not for the fact that we do find a significant association between the trait of VEGF level and markers linked to *Edpm5*. Thus, although these three QTL all affect mass with similar significance, only *Edpm5* has a significant association with variation in VEGF level. This relationship among the QTL parallels what we have seen previously for the traits of DNA content and hemoglobin content. In previous analysis of the BC_1 generation, *Edpm5* was associated with a highly significant effect on hemoglobin, but *Edpm2-1* and *Edpm9-2* were not, although they did have a significant effect on DNA content (17).

Estrogen is known to directly enhance transcription of the gene encoding VEGF by way of the nuclear estrogen receptors binding to estrogen receptor elements (33). The data presented in Figure 1 show that in the pituitary of estrogen-treated rats, the presence of a high level of VEGF protein is dependent upon additional controls. Likewise, in the BC_1 generation rats, all animals were given the same chronic estrogen treatment but varied significantly in their VEGF protein level. This variation was not random, but was linked to the *Edpm5* locus (Fig. 4).

The pattern of VEGF expression parallels what we have

previously reported for MMP-9. Estrogen treatment increases the level of MMP-9 in the pituitary of F344 rats, but not in BN or F_1 hybrid rat pituitary. Likewise, the difference in control is affected by *Edpm5* genotype with inheritance of the BN allele of *Edpm5* corresponding with lower MMP-9 level, and inheritance of the F344 allele of *Edpm5* corresponding with higher MMP-9 level (18). There are actually two possible mechanisms to explain the correlation of genetic control of MMP-9 and VEGF. The first possible scenario is for the BN allele of *Edpm5* to suppress estrogen-dependent VEGF expression and this then affects the activation of the *Mmp-9* gene. The *Mmp-9* is not directly regulated by estrogen, but rather is regulated by other factors that are in turn regulated by estrogen (34). Among these, VEGF has been shown to upregulate the level of MMP-9 (35) and may be doing so in this tissue. The second scenario has the opposite hierarchy. In mouse pancreatic islets, VEGF mRNA is expressed constitutively, and MMP-9 controls the bioavailability of VEGF by releasing it from the extracellular matrix (36). Of course, because we have measured VEGF in total pituitary homogenates, we would be detecting all 29-kDa VEGF. For our results to connect with VEGF bioavailability, the release of VEGF would have to influence the total pool of VEGF protein in tissue.

The fact that rats that are all treated with estrogen, but differ in genotype, vary dramatically in the expression of VEGF tells us that the control of VEGF level is due to more factors than estrogen alone. Estrogen is known to induce transcription of the gene encoding VEGF (37, 38). As in the case with uterus, short-term estrogen treatment increases expression of VEGF mRNA in the pituitary of F344 rats (15). However, the only other study to compare expression of VEGF in rats of different strains is that of Long *et al.* (14) who investigated the effect of the xenoestrogen Bisphenol A on VEGF transcript level in the pituitary of F344 and tumor-resistant Sprague-Dawley rats. They detected VEGF message in pituitary of both strains at comparable levels. Thus, the difference in VEGF level that we report here must reveal either post-transcriptional regulation, or a level of transcriptional regulation that occurs only after long-term treatment.

Our results clearly show an association of the expression of VEGF with estrogen-induced pituitary tumor growth in the rat. However, we cannot yet say whether VEGF expression plays a primary or secondary role. Estrogen could be inducing growth by way of inducing VEGF. However, it is also possible that the estrogen-induced growth of the pituitary of the F344 rat is generating signals to recruit or remodel vasculature and is these signals that lead to elevated levels of VEGF. Jacubowski *et al.* (12) reported that the relative blood flow in the estrogen-induced pituitary tumor of the F344 rat is actually lower than in normal pituitary. Thus, it is possible that in F344 rats and in BC_1 rats that are homozygous for F344 alleles of *Edpm5* there are conditions such as hypoxia (39) that induce VEGF production.

One objective for future work is to identify the actual *Edpm5* gene, although this will require additional crosses to provide a higher precision of mapping to test candidate genes. A search for candidate genes can involve not only the proximal end of RNO5, but also the genomes of other organisms by use of comparative maps (21). The segment of RNO5 that contains *Edpm5* is part of a large block of conserved linkage with mouse chromosome 4 (MMU4), making it highly likely that the murine homolog of *Edpm5* would be found between positions 0 and 30 cM of MMU4 (Fig. 3). A search of the Mouse Genome Database (40) for genes mapped to the first 30 cM of MMU4 did not reveal any genes with definite ties to VEGF expression. However, there is a QTL for an estrogen-regulated response in that part of MMU4. A QTL for estrogen-induced eosinophil infiltration of the uterus, called *Est1*, was identified by Griffith *et al.* (41) on mouse Chromosome 4 (MMU4). The peak value of their test statistic was around the 19 cM position on the genetic map of MMU4. Though linked by estrogen stimulation, mouse *Est1* and rat *Edpm5* differ in their tissue of action and phenotype. In the rodent uterus, estrogen induces the production of a chemotactic factor, probably an interleukin, that stimulates this influx (42, 43), but the exact mechanism and the role of this response are still not known. One possible commonality, though, is the aspect of inflammation and tissue invasion. *Edpm5* affects the level of VEGF, which can affect vascular permeability, and we have shown previously that *Edpm5* affects the level of MMP-9, which can be involved in tissue reorganization.

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