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Vascular Endothelial Growth Factor, Its Receptor KDR/Flk-1, and Pituitary Tumor Transforming Gene in Pituitary Tumors

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Pituitary tumorigenesis is a poorly understood process involving dysregulation of the cell cycle, proliferation, and angiogenesis. The novel securin pituitary tumor transforming gene (PTTG) disrupts cell division and stimulates fibroblast growth factor (FGF)-2-mediated angiogenesis. We investigated expression of the angiogenic vascular endothelial growth factor (VEGF) and its receptor KDR/Flk-1 in 103 human pituitary tumors, and we assessed functional relationships between these genes *in vitro*. Nonfunctioning tumors (n = 81) demonstrated markedly raised VEGF mRNA (3.2-fold, $P < 0.05$) and protein concentrations, compared with normal pituitaries (n = 10). KDR was also highly induced in nonfunctioning tumors (14-fold, $P < 0.001$, n = 78) as well as in the whole cohort of pituitary tumors, compared with normal pituitary samples (14-fold, $P < 0.0001$, n = 100). *In vitro*, PTTG induced VEGF, but not KDR, expression in fetal neuronal NT2 cells (2.7-fold, $P < 0.001$, n = 8), MCF-7 breast carcinoma cells (1.9-fold, $P = 0.03$, n = 10), and choriocarcinoma JEG-3 cells ($P = 0.0002$, n = 8). A mutated PTTG construct that cannot be phosphorylated showed identical VEGF up-regulation (2.9-fold, $P < 0.001$, n = 8) in NT2 cells, compared with wild-type PTTG, but a further mutated construct with abrogation of the key protein:protein interaction domain of PTTG resulted in a significant reduction in VEGF stimulation, compared with wild-type (0.37-fold reduction, $P < 0.001$, n = 8). FGF-2 findings mirrored those of VEGF, although antibody depletion of secreted FGF-2 in the cell medium failed to influence VEGF up-regulation by PTTG. Overall, our findings implicate altered VEGF and KDR signaling in pituitary tumorigenesis, and we propose that PTTG stimulation of FGF-2 and VEGF expression in the presence of up-regulated growth factor receptors may account for angiogenic growth and progression of human pituitary tumors. (*J Clin Endocrinol Metab* 87: 4238–4244, 2002)

PITUITARY ADENOMA GROWTH, as with all tumors, depends on adequate vascularization once tumor size has exceeded a few millimeters (1). Cytokines and growth factors are important modulators of angiogenesis; and enhanced vascular endothelial growth factor (VEGF) expression, in particular, has been associated with vascular tumors (2). Increased concentrations of VEGF and the VEGF receptor KDR (Flk-1) have previously been reported in rat pituitary tumors (3, 4), and elevated plasma VEGF and fibroblast growth factor (FGF)-2 concentrations have been demonstrated in patients harboring pituitary tumors (5). Approximately 90% of human pituitary tumors cultured *in vitro* show measurable VEGF secretion (6). Taken together, these observations indicate that VEGF and KDR expression may be intimately related to pituitary tumor growth and vascularization. Indeed, the VEGF/KDR receptor system has previously been implicated in other cancers, including carcinoma of the pancreas (7) and liver (8).

Recently, the new oncogene pituitary tumor transforming gene (PTTG) has been linked to pituitary angiogenesis (9). PTTG stimulates FGF-2 expression *in vitro* and *in vivo*. Rat pituitary tumors with higher vascularity show high PTTG expression, and anti-FGF-2 antibodies abrogate PTTG's stimulation of new blood vessel formation *in vitro* (9). FGF-2 is known to regulate endothelial expression of VEGF (10, 11), and both growth factor signaling pathways synergistically modulate angiogenesis, a critical process in tumorigenesis and invasion. The role of angiogenesis in pituitary tumorigenesis is contentious, however. Although some authors have observed increased vascularity in pituitary tumors (12), others have reported fewer intratumoral vessels (13, 14). In the present study, therefore, we have undertaken a detailed assessment of VEGF and KDR mRNA and protein expression in a large cohort of pituitary tumors. Given the suggested link among PTTG, angiogenesis, and pituitary tumorigenesis (9), we have examined PTTG-mediated effects on VEGF and KDR *in vitro*. Further, we have employed mutated PTTG constructs to define the region of functional interaction among PTTG, VEGF, and KDR, as a means of understanding the contribution of VEGF signaling to pituitary tumorigenesis. We show that VEGF and KDR are up-regulated in the majority of pituitary tumors and that mRNA expression of both genes correlates significantly with that of PTTG. Further, we demonstrate that PTTG is able to stimulate VEGF expression *in vitro*. The phosphorylation status of PTTG does not influence VEGF up-regulation, but integrity of the pro-

Abbreviations: AMV, Avian myeloblastosis virus; Ct, cycle number at which logarithmic PCR plots cross a calculated threshold line; FGF, fibroblast growth factor; NFT, nonfunctioning tumor; PRL, prolactin; PTTG, pituitary tumor transforming gene; R^2, Pearson correlation; SH3, src homology 3; VEGF, vascular endothelial growth factor; WT, wild-type.

tein's src homology 3 (SH3)-interacting domain is necessary for this process. We suggest that PTTG stimulates VEGF independently of FGF-2 up-regulation, and that *in vivo*, up-regulated KDR mediates the angiogenic growth and progression of human pituitary tumors.

Materials and Methods

Patients and pituitary tissues

Samples of pituitary tumor tissue from 121 patients with pituitary adenomas were obtained at the time of surgery and immediately snap frozen. Clinical details of patients are summarized in Table 1. All tumors were fully characterized by immunohistochemical staining for anterior pituitary hormones. Ninety-two (77%) were clinically nonfunctioning tumors (NFTs), and all of these were macroadenomas (≥1 cm in diameter). NFTs stained variably for the glycoprotein hormone common α-subunit and other glycoprotein hormone-specific β-subunits. Functioning pituitary tumors comprised 16 GH-secreting (12 macroadenomas), 5 prolactin (PRL)-secreting (3 macroadenomas), 5 ACTH-secreting (1 macroadenoma), and 3 TSH-omas (all macroadenomas). Ten normal human pituitary glands were obtained from postmortem proceedings performed within 24 h of the time of death. All studies were carried out according to local ethical guidelines and approval.

RNA extraction and reverse transcription

Total RNA was extracted from approximately 100 mg tissue after homogenization, using the Trizol kit (a single-step acid guanidinium phenol-chloroform extraction procedure; Sigma, St. Louis, MO), following manufacturer's guidelines. RNA was reverse-transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corp., Madison, WI) in a total reaction vol of 20 μl, with 1 μg pituitary total RNA, 30 pmol random hexamer primers, 4 μl 5× AMV reverse transcriptase buffer, 2 μl deoxynucleotide triphosphate mix (200 μM each), 20 U ribonuclease inhibitor (RNasin, Promega Corp.), and 15 U AMV reverse transcriptase (Promega Corp.).

Contamination with normal pituitary tissue

To determine whether there was significant contamination of NFTs and ACTH-secreting adenoma samples with normal pituitary tissue, we assessed mRNA expression for Pit-1, as previously described (15, 16). Seven NFTs and 1 ACTH-secreting tumor showed evidence of significant Pit-1 expression and were thus excluded from further analyses. To examine normal tissue contamination of other types of functioning tumor, we assessed proopiomelanocortin mRNA expression, which is not present in tumors of the somatotrope/lactotrope lineages. These experiments led us to eliminate 1 GH- and 1 PRL-secreting tumor from our cohort. In total, therefore, we excluded 10 of our 121 tumors, giving a cohort size of 111 samples. Of these, nondegraded RNA of suitable quality was obtained from 103 specimens.

Quantitative PCR

Expression of specific messenger RNAs was determined using the ABI PRISM 7700 Sequence Detection System, as we have described previously (17). Quantitative RT-PCR was carried out in 25-μl vols on 96-well plates, in a reaction buffer containing 1× TaqMan Universal PCR Master Mix, 3 mM $Mn(OAc)_2$, 200 μM deoxynucleotide triphosphates, 1.25 U AmpliTaq Gold polymerase, 1.25 U AmpErase UNG, 100–200 nM TaqMan probe, and 900 nM primers. All reactions were multiplexed with a probe and primers for 18S ribosomal RNA, provided as a preoptimized, primer-limited control system (Applied Biosystems, Warrington, UK), enabling data to be expressed in relation to an internal reference, to allow for differences in reverse transcription efficiency. Primer and probe sequences, which yielded amplicons of less than 150 bp, are given in Table 2. All target genes were tested for amplification efficiency and potential housekeeping gene interference and were found to be suitable for multiplex analysis. As per the manufacturer's guidelines, data were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values [ΔCt = Ct of the target gene (*e.g.* VEGF) minus Ct of the housekeeping gene]. To exclude potential bias attributable to averaging data that had been transformed through the equation $2^{-\Delta\Delta Ct}$ to give fold changes in gene expression, all statistics were performed with ΔCt values. Measurements were carried out a minimum of three times each. Samples that failed to show consistent, repeatable gene expression were excluded from subsequent analyses. All target gene probes were labeled with FAM-6-carboxyfluorescein, and the housekeeping gene was labeled with VIC. Reactions were as follows: 50 C for 2 min, 95 C for 10 min; then 44 cycles of 95 C for 15 sec, and 60 C for 1 min.

TABLE 1. Details of the complete cohort of patients (n = 121) showing tumor subtype, mean age (±SE) at diagnosis, number (%) of females, and number (%) of macroadenomas (≥1 cm diameter)

	Age ± SE	Females (%)	Macroadenomas
All tumors (n = 121)	58.9 ± 1.3	62 (51)	114 (94)
Nonfunctioning (n = 92)	61.4 ± 1.4	43 (47)	92 (100)
GH-secreting (n = 16)	55.0 ± 3.0	11 (69)	12 (75)
PRL-secreting (n = 5)	37.7 ± 0.3	3 (60)	3 (60)
ACTH-secreting (n = 5)	45.5 ± 6.1	3 (60)	1 (20)
TSH-secreting (n = 3)	56.0 ± 6.1	1 (33)	3 (100)

After determination of potential tumor contamination with normal pituitary tissue (see *Materials and Methods*), we excluded 1 GH-, 1 PRL-, and 1 ACTH-secreting tumor, and 7 NFTs.

Western blots

Proteins were prepared from 24 randomly selected pituitary specimens in lysis buffer (100 mM sodium chloride, 0.1% Triton X-100, and 50 mM Tris, pH 8.3) containing enzyme inhibitors (1 mM phenylmethylsulfonylfluoride, 0.3 μM aprotinin, and 0.4 mM leupeptin) and were denatured (2 min, 100 C) in loading buffer. Protein concentration was measured by the Bradford assay, with BSA as standard. Western blot analyses were performed, as we have described previously (15, 18). Briefly, soluble proteins (30 μg) were separated by electrophoresis in 12.5% sodium dodecyl sulfate polyacrylamide gels, transferred to polyvinylidene fluoride membranes, incubated in 5% nonfat milk in PBS with 0.1% Tween, followed by incubation with antibodies to VEGF (1:100) or KDR (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 16 h at 4 C. After washing in PBS plus 0.1% Tween, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. After further washes, antigen-antibody complexes were visualized by the ECL chemiluminescence detection system. Actin expression was used to confirm equivalent total protein loading and was similar in normal and tumorous pituitaries (see *Results*). Coomassie Blue staining demonstrated a similar finding (data not shown).

Cell lines, plasmids, and transfections

Human choriocarcinoma JEG-3 cells (ECACC 92120308) were maintained as previously described (19). Fetal neuronal NT2 cells (Stratagene, Amsterdam, The Netherlands) were grown in Dulbecco's medium NUT F12 (Hams), supplemented with 10% fetal bovine serum, 1× L-glutamine and 1% pen/strep (Life Technologies, Inc., Grand Island, NY). Breast carcinoma MCF-7 cells (ECACC 86012803) were maintained in RPMI 1640 medium, 10% fetal bovine serum, and 1% pen/strep (Life Technologies, Inc.). Before transfection experiments, cells were washed in PBS. A total of 2–3× 10^5 cells were transfected using TransFast reagent (Promega Corp.), as per the manufacturer's instructions but with an optimized ratio of 6 μl per μg plasmid DNA. pCI-neo-PTTG, which housed the full-length human PTTG cDNA, was kindly provided by Prof. Shlomo Melmed (University of California School of Medicine, Los Angeles, CA) (20). Cells were harvested in 1 ml Trizol Reagent, 48 h after transfection. Control transfections used equal amounts of blank plasmid or normal pituitary DNA. After RNA extraction, gene expression was determined as described above. Transfection efficiency was assessed by using quantitative RT-PCR to determine wild-type (WT) and mutant PTTG mRNA expression after transfection. Routinely, we found that WT and mutant plasmids resulted in an approximately 200- to 300-fold induction of PTTG mRNA expression in cells over background, with no obvious differences between WT and mutant sequences, both of which are detected by our primer and probe sequences and which do not

TABLE 2. Oligonucleotide sequences of PCR primers and TaqMan probes used

	Forward primer	Reverse primer
VEGF	TACCTCCACCATGCCAAGTG *Probe*: TCCCAGGCTGCACCCATGGC	TGATTCTGCCCTCCTCCTTCT
KDR	CATGTACGGTCTATGCCATTCCT *Probe*: CATCACATCCACTGGTATTGGCAGTTGG	CGTTGGCGCACTCTTCCT
PTTG	GAGAGAGCTTGAAAAGCTGTTTCAG *Probe*: TGGGAATCCAATCTGTTGCAGTCTCCTTC	TCCAGGGTCGACAGAATGCT
FGF-2	CGACCCTCACATCAAGCTACAA *Probe*: CGACCCTCACATCAAGCTACAA	CCAGGTAACGGTTAGCACACACT
Pit1	AGCAGCGGTTCTCCTTATTTTT	CTTTTCCGCCTGAGTTCCTG
POMC	GGGCAAGCGCTCCTACTCCAT	TGCCCTCACTCGCCCTTCTTGT

All TaqMan primers run at 59 C and yield amplicons of 70–150 bp. PitI annealed at 60 C, and POMC at 62 C. The probe and primers for 18S ribosomal RNA were provided as a preoptimized, primer-limited control system (Applied Biosystems and targeted the 18S sequence of accession number X03205).

overlap the sites of mutation. In this way, data were subsequently corrected directly for PTTG mRNA up-regulation over background in each treatment. FGF-2 depletion studies were carried out essentially as described by Ishikawa *et al.* [2001 (9)]. Briefly, either anti-FGF-2 antibody (100–200 ng/ml; Santa Cruz Biotechnology, Inc.) or normal goat IgG were added to cells grown in 24-well plates, for 48 h. Previously, such treatment has been shown to significantly reduce serum FGF-2 effects (9).

Site-directed mutagenesis of PTTG

The previously reported PTTG 'S165A' mutation (21, 22), which prevents PTTG being phosphorylated and inhibits nuclear entry, was recreated in the pCI-neo-PTTG vector using the GeneEditor System (Promega Corp.), as per the manufacturer's instructions. The mutagenic primer, which resulted in a single amino acid substitution of serine to alanine at position 165, was of sequence: 5′-G CTG GGC CCC CCT GCA CCT GTG AAG ATG CCC. The PTTG 'SH3-' mutation, which disrupts the C-terminal SH3 interacting domain of PTTG responsible for protein: protein interaction, but retains the key phosphorylation site, was mutated with the primer: 5′-AAG CTG TTT CAG CTG GGC GCC GCT TCA GCT GTG AAG ATG GCC TCT GCA GCA TGG GAA TCC AAT CTG TTG. This resulted in the following amino acid changes: P163A, P164A, P166A, P170A, P172A, and P173A.

Statistical analyses

Data were analyzed using Minitab version 13 software. Student's *t* test and the Mann Whitney *U* test were used for comparison between two groups of parametric and nonparametric data, respectively. The Kruskal-Wallis test was used for between-group comparison of more than two groups of nonparametric data. Correlations between levels of mRNA expression were performed using the Pearson rank sum test. Significance was taken as $P < 0.05$.

Results

VEGF and KDR expression in pituitary tumors

VEGF mRNA expression was significantly elevated in NFTs, which represented the largest subset (81 of 103) of informative pituitary tumors, compared with normal pituitaries (3.2-fold induction, $P < 0.05$) (Fig. 1, A and B). TSH-omas were unique in demonstrating significantly reduced expression of VEGF mRNA, compared with normal pituitary (92% reduction, $P = 0.002$) (Fig. 1). Western blot analysis revealed that protein data were in agreement with the mRNA results, with most, but not all, NFTs showing increased VEGF expression (Fig. 1C). Because of the small size of our TSH-omas, we were unable to determine VEGF protein expression in this tumor subtype, however.

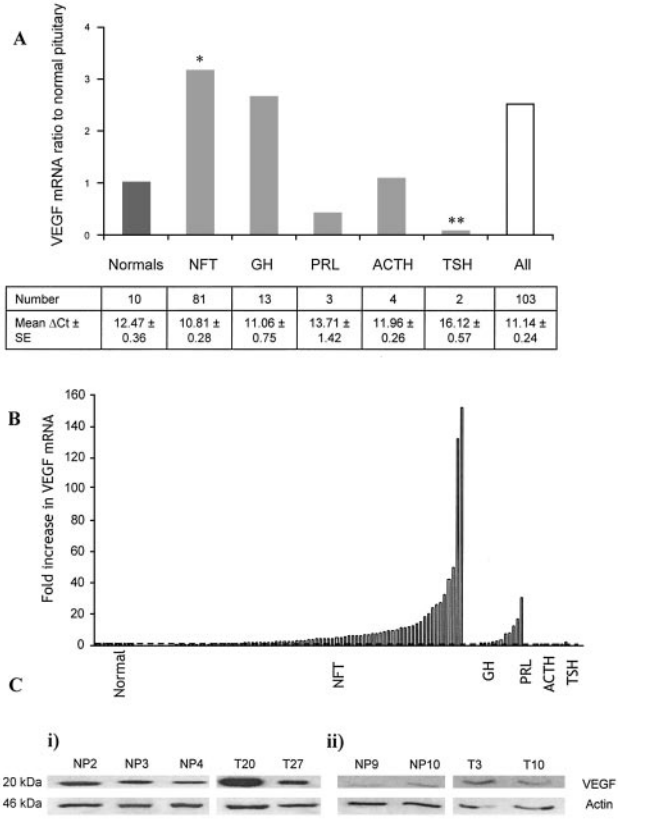
VEGF receptor KDR (Flk-1) mRNA expression was increased 13.9-fold ($P < 0.0001$, n = 100) in pituitary tumors of all types, compared with normal pituitaries (n = 10) (Fig. 2, A and B). In particular, prolactinomas and TSH-omas showed high KDR mRNA levels (27-fold, $P = 0.0012$, n = 4; and 233-fold, $P = 0.0003$, n = 2; respectively). Western blot analysis confirmed elevated KDR in pituitary tumors (Fig. 2C).

VEGF, KDR, and PTTG expression in vivo

In view of our observed increase in VEGF and KDR expression in the majority of pituitary tumors studied, and the previously reported up-regulation of PTTG in pituitary tumors (17, 23), we examined potential relationships between these genes. In pituitary tumors that were informative for both PTTG and VEGF, we noted a significant positive association between mRNA expression of the two (n = 65, $R^2 = 0.22$, $P < 0.001$) (Fig. 3A). When we assessed changes in KDR mRNA expression compared with PTTG in the same tumors, we found that KDR also significantly correlated with PTTG mRNA expression levels (n = 98, $R^2 = 0.14$, $P < 0.001$, Pearson correlation) (Fig. 3B).

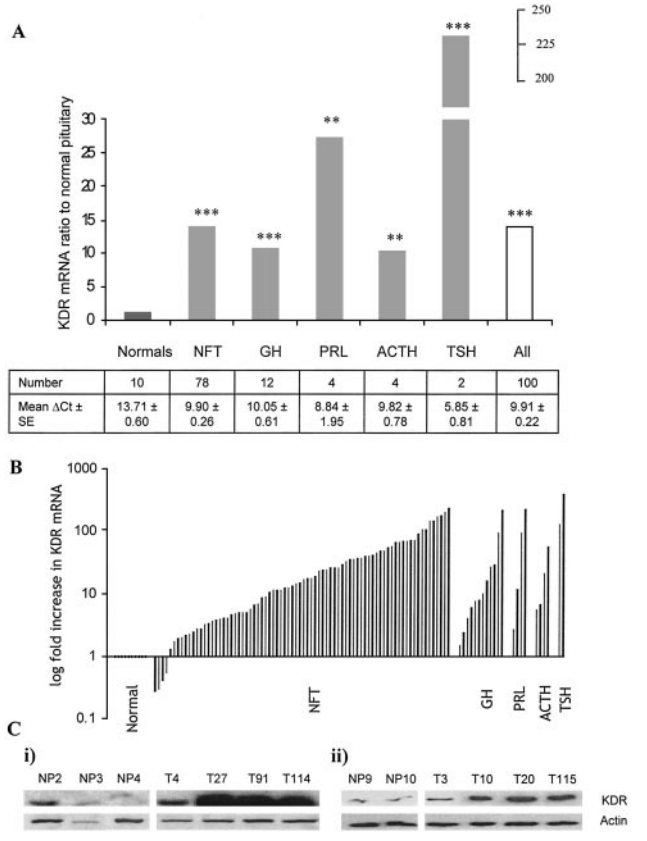
PTTG stimulates VEGF in vitro

Given the apparently close relationship among the expression of VEGF, its receptor KDR, and PTTG, we investigated the functional relationships among these genes *in vitro*. Because well-characterized human pituitary cell lines are not currently available, we performed our experiments in human fetal neuronal NT-2 cells, a cell line that we found to exhibit relatively low background PTTG expression (PTTG Ct ~ 26 for NT-2 cells, ~22 for JEG-3 cells). To confirm whether our findings were cell-type-specific, experiments were also repeated in MCF-7 and JEG-3 cells, as two common transformed cell lines. Transient transfection of WT PTTG cDNA into NT-2 cells led to a significant 2.7-fold induction in VEGF mRNA expression, compared with control ($P < 0.001$, n = 8) (Fig. 4A). When a mutant PTTG incapable of phosphorylation (S165A) was transiently transfected into NT-2 cells, a similar increase in VEGF mRNA expression was observed (2.9-fold, $P < 0.001$, n = 8). Although transient transfection of a PTTG vector harboring a mutation that disrupts the SH3-interacting domain of PTTG led to a 1.7-fold induction



	Normals	NFT	GH	PRL	ACTH	TSH	All
Number	10	81	13	3	4	2	103
Mean ΔCt ± SE	12.47 ± 0.36	10.81 ± 0.28	11.06 ± 0.75	13.71 ± 1.42	11.96 ± 0.26	16.12 ± 0.57	11.14 ± 0.24

FIG. 1. A, VEGF mRNA expression in 10 normal and 103 pituitary tumors (All). GH, Somatotropinomas; PRL, prolactinomas; ACTH, adrenocorticotropinomas; TSH, thyrotropinomas. *, $P < 0.05$; **, $P < 0.01$, compared with normals. In this and following histograms, tumor gene expression is expressed relative to a value of 1.0 for normal pituitaries (NPs). Number of samples and ΔCt values (±SEM) are given *beneath histogram bars* (note: low ΔCt values represent high gene expression). B, The range of VEGF mRNA expression across all samples, given as fold changes, compared with NPs (taken as equal to 1.0; *horizontal dotted line*). C, Two representative Western blot analyses of VEGF protein expression in 5 NPs and 4 NFTs (T3–T27). Actin expression is given below, demonstrating consistent protein loading for normal and tumorous samples.



	Normals	NFT	GH	PRL	ACTH	TSH	All
Number	10	78	12	4	4	2	100
Mean ΔCt ± SE	13.71 ± 0.60	9.90 ± 0.26	10.05 ± 0.61	8.84 ± 1.95	9.82 ± 0.78	5.85 ± 0.81	9.91 ± 0.22

FIG. 2. A, KDR mRNA expression in 10 normal and 100 tumorous pituitaries. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with normals. Number of samples and ΔCt values (± SEM) are given *beneath histogram bars*. All tumor subtypes demonstrated significantly up-regulated KDR mRNA expression, with TSH-omas showing a mean 233-fold increase, compared with normal. B, The range of KDR mRNA expression across all samples, given as $\log_{10}$-fold changes, compared with NPs (fold change = 1.0). C, Two representative Western blot analyses of KDR protein expression in 5 NPs and 8 NFTs (T), with actin expression beneath.

of VEGF, compared with controls ($P = 0.01$, n = 8), this represented a significant reduction of VEGF mRNA stimulation compared with WT (37% reduction, $P < 0.001$, n = 8). Similar findings were apparent in JEG-3 and MCF-7 cells. Transfection of either WT or mutant PTTG, however, failed to stimulate KDR expression (data not shown).

To assess the response of cells to increasing PTTG expression, we transfected JEG-3 cells with increasing amounts of WT-PTTG (0.5–3.5 μg/well) and quantified VEGF and PTTG mRNA expression after 48 h. VEGF demonstrated concordant and significantly correlated mRNA expression in response to PTTG ($P = 0.0002$, $R^2 = 0.91$) (Fig. 4B). We also investigated the potential influence of PTTG phosphorylation on the regulation of VEGF expression. Transient overexpression of S165A-PTTG led to identical VEGF up-regulation, compared with WT, across a range of doses ($P = 0.0003$, $R^2 = 0.85$). Because nuclear entry of PTTG has been reported to be regulated by its phosphorylation at residue S165 (21), these data suggest that PTTG-mediated stimulation of VEGF is not dependent on nuclear entry of PTTG. Unlike its ligand VEGF, KDR failed to demonstrate any response to transient PTTG overexpression in the same transfection experiments. KDR mRNA expression in control treatments was indistinguishable from that of WT PTTG transfectants and was unaffected by either mutation (data not shown).

FGF-2 and VEGF expression in vitro and in vivo

To investigate what role FGF-2 might play (if any) in PTTG's stimulation of VEGF, we first examined expression of each gene in 10 normal pituitary samples. FGF-2 and VEGF mRNA showed a significant positive correlation ($R^2 = 0.68$, $P = 0.003$) (Fig. 5A). Because FGF-2 is able to stimulate VEGF, we next investigated whether PTTG's up-regulation of VEGF was a direct event or occurred indirectly via FGF-2. NT-2 cells were transfected with 3.5 μg/well WT, S165A and SH3- cDNAs, and determined FGF-2 mRNA expression, compared with mock-transfected cells. WT-PTTG induced a significant 1.7-fold increase in FGF-2 mRNA expression ($P <$ 0.01, n = 8), and S165A induced a 1.6-fold up-regulation ($P =$

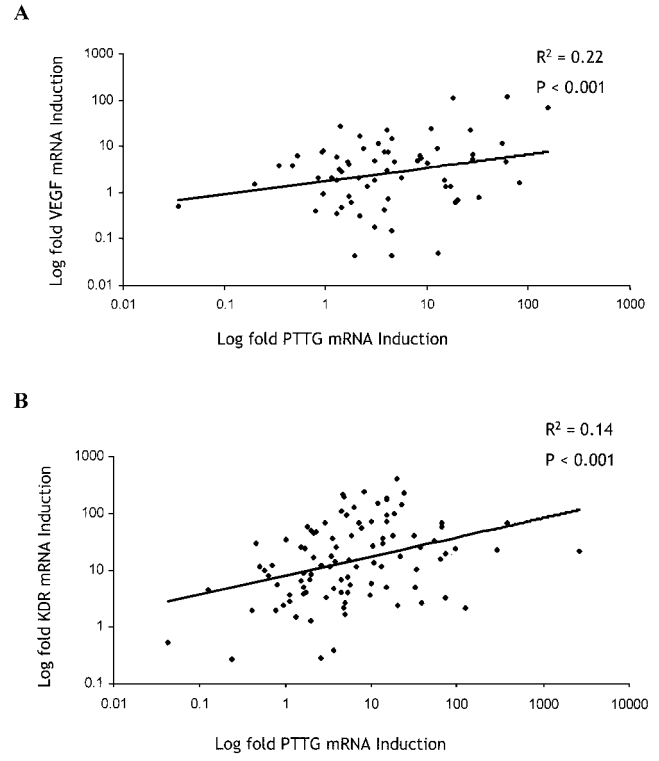
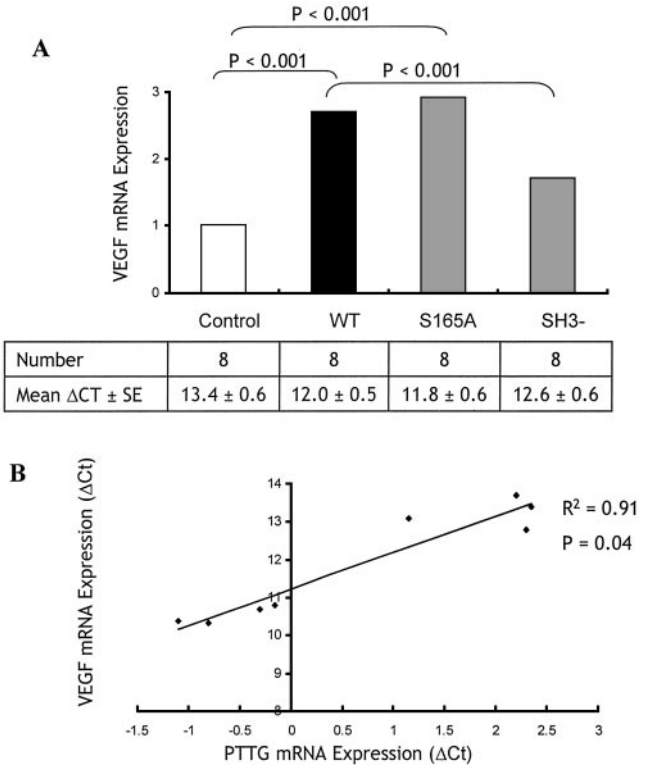


FIG. 3. The correlation among PTTG, VEGF, and KDR mRNA expression *in vivo*. A, VEGF and PTTG mRNA expression was determined in individual pituitary tumors by real time TaqMan RT-PCR, and fold changes in gene expression were calculated after comparison with NP expression. VEGF and PTTG demonstrated significantly correlated levels of expression. B. KDR and PTTG mRNA expression was compared in 98 informative pituitary tumors, again showing a significant positive association.



Number	8	8	8	8
Mean ΔCT ± SE	13.4 ± 0.6	12.0 ± 0.5	11.8 ± 0.6	12.6 ± 0.6

FIG. 4. VEGF is stimulated by PTTG *in vitro*. A, NT-2 cells were transfected with control, WT PTTG, S165A mutant PTTG, or SH3- mutant PTTG DNA; and VEGF expression was determined after 48 h, using quantitative TaqMan RT-PCR. Values were adjusted for 18S housekeeping gene expression and normalized to 1.0 for control treatments. Experiments were repeated twice, with four replicates each. WT PTTG induced a 2.7-fold increase in VEGF mRNA expression, which was unaltered by the phosphorylation status of PTTG. The SH3- mutant, however, which abrogates the SH3-interacting domain of the gene, was less effective in stimulating VEGF. Similar findings were apparent in MCF-7 and JEG-3 cells. B, JEG-3 cells were transfected with a range of WT PTTG doses (0.5–3.5 μg/well), and VEGF and PTTG mRNA expression was assessed in relation to 18S levels. VEGF demonstrated mRNA expression that was concordant and highly significantly correlated with that of WT PTTG ($P < 0.001$, $R^2 = 0.91$), indicating that cells are able to up-regulate VEGF in response to increases in PTTG levels.

0.02, n = 8) (Fig. 5B), compared with control. The SH3- mutation, which prevented PTTG stimulation of VEGF (see Fig. 4A), also abrogated PTTG up-regulation of FGF-2 ($P < 0.001$, n = 8, SH3- compared with WT). Similar results were also found in JEG-3 and MCF-7 cells (data not shown).

Finally, because PTTG causes FGF-2 to be secreted (20), we used an anti-FGF-2 antibody treatment to deplete serum FGF-2, as described by Ishikawa *et al.* [2001 (9)]. Of the cells lines we used, we found that MCF-7 cells consistently expressed detectable FGF-2 mRNA; and hence, these cells were used as our model. WT PTTG induced a 1.9-fold increase in VEGF expression in MCF-7 cells, compared with controls ($P = 0.03$, n = 10) (Fig. 5C). Addition of an anti-FGF-2 antibody (100 ng/ml), however, failed to influence this induction (2.1-fold up-regulation, compared with control, $P = 0.03$, n = 9). Further, a 2-fold-higher dose of antibody (200 ng/ml) similarly did not alter PTTG stimulation of VEGF (data not shown).

Discussion

Pituitary tumorigenesis is a complex and poorly understood process, involving oncogene activation, loss of tumor suppressor function, aberrant pituitary cell growth, and angiogenesis. PTTG is abundantly expressed in pituitary tumors (17, 23), causes cell transformation (20), and stimulates FGF-2-mediated angiogenesis (9). Because FGF-2 regulates endothelial expression of VEGF (10, 11) we have investigated expression of this angiogenic factor and its receptor KDR in a large cohort of pituitary tumors and have sought to determine functional relationships among PTTG, VEGF, KDR, and FGF-2 *in vitro*. In nonfunctioning pituitary tumors, which comprised approximately 80% of our cohort, we demonstrated markedly raised VEGF mRNA and protein expression. KDR mRNA and protein expression was also abundant in all pituitary tumor subtypes, compared with normal pituitaries. PTTG mRNA expression correlated well with both VEGF and KDR levels. *In vitro*, transient overexpression of PTTG led to VEGF induction but did not influence KDR expression. PTTG-mediated VEGF induction was independent of PTTG phosphorylation but was significantly impaired by disruption of the PTTG SH3-interacting domain, suggesting the possibility that PTTG interacts with an as-yet-unknown protein, to directly or indirectly mediate VEGF stimulation.

Although VEGF and FGF-2 signaling pathways act to-

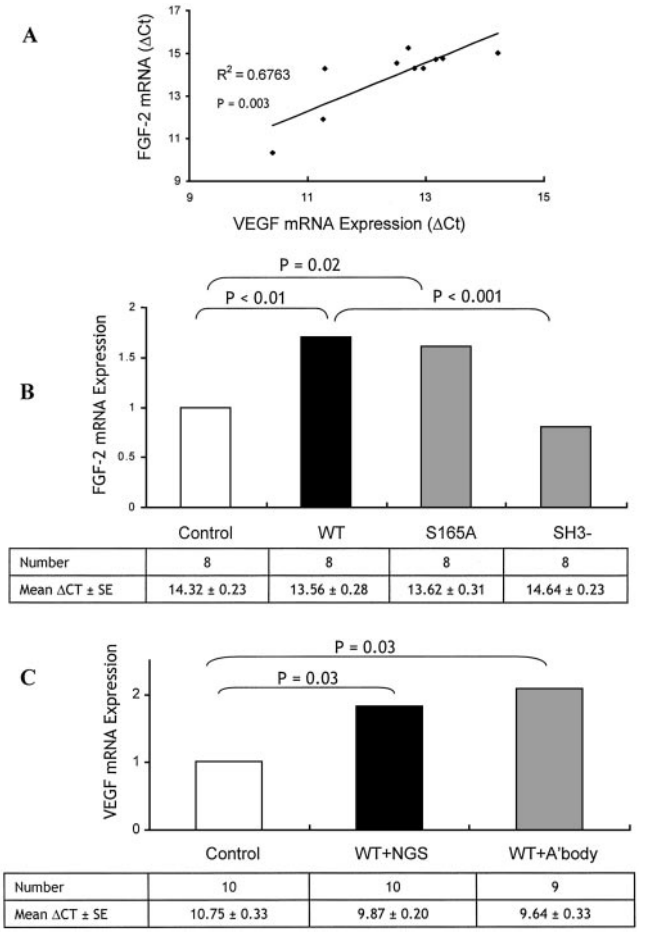


FIG. 5. Investigating the relationship among VEGF, PTTG, and FGF-2. A. VEGF and FGF-2 mRNA expression *in vivo*. In normal human pituitary samples, there was a significant positive association between VEGF and FGF-2 mRNA expression ($R^2 = 0.68$, $P = 0.003$, n = 10). B, NT-2 cells were transfected with control, WT and mutant PTTG constructs, and FGF-2 mRNA expression determined after 48 h, using quantitative TaqMan RT-PCR. Values were adjusted for 18S housekeeping gene expression and normalized to 1.0 for control treatments. Experiments were repeated twice, with four replicates each. C, MCF-7 cells were transfected with control (vector only) or WT PTTG and treated for 48 h with either nonimmune goat serum (NGS) or anti-FGF-2 antibody (A'body), before assessing VEGF mRNA expression.

gether to regulate angiogenesis, which is fundamental to tumorigenesis and invasion, our data suggest that PTTG influences both factors independently. Gene expression data for both genes showed a high level of similarity, with WT and mutated PTTG inducing parallel changes in the two genes. Despite this, a treatment designed to deplete serum FGF-2 failed to influence PTTG-mediated up-regulation of VEGF; and hence, it is unlikely that VEGF stimulation is an indirect consequence of FGF-2 transactivation by PTTG. Alternatively, because we did not measure secreted FGF-2 in antibody-treated experiments, we may have failed to significantly deplete it. However, we used antibody concentrations identical to those used previously (9); and even when we employed significantly increased antibody doses (200 ng/ml), we still saw no influence on VEGF expression. Even if we had only depleted, for example, 25% of secreted FGF-2 at the higher antibody dose, we would still have expected to see some detectable change in VEGF, using our sensitive real time PCR approach. This was clearly not the case, however.

Certainly, the importance of VEGF to pituitary tumorigenesis is more straightforward. In patients with sporadic pituitary tumors, circulating concentrations of VEGF are higher preoperatively than in controls, and levels fall significantly after effective pituitary tumor treatment (5). Increased VEGF and its receptor KDR have been demonstrated in estrogen-induced rat PRL-secreting pituitary tumors (3). Further, around 90% of human pituitary tumors, cultured *in vitro*, secrete measurable VEGF (6). KDR has been shown to be expressed widely in rat pituitary cell types and in transformed GH3 cells (24). Taken together, our results confirm the importance of VEGF signaling in pituitary tumorigenesis, particularly in nonfunctioning adenomas, and provide a link between this angiogenic growth factor and PTTG-mediated actions.

The direct role of VEGF and angiogenesis in pituitary tumorigenesis remains controversial, however, with some authors reporting increased vascularity in pituitary tumors (12), and others observing fewer intratumoral vessels (14). We demonstrate significantly raised VEGF mRNA expression in NFTs and show that, in all tumors overall, PTTG expression and VEGF expression are closely correlated. Highly vascular pituitary tumors have recently been associated with raised PTTG expression, with the gene being shown to induce angiogenic phenotypes in *in vitro* and *in vivo* models via FGF-2 (9). In addition to this, our data now suggest that PTTG-mediated angiogenesis could result from PTTG up-regulation of both FGF-2 and VEGF, to effect new blood vessel growth.

The role of PTTG in the initiation and promotion of tumorigenesis is supported by an increasing number of studies, both in the pituitary and in other tissues (see Ref. 25). Intriguingly, PTTG's stimulation of VEGF was unaffected by a mutation that prevents PTTG phosphorylation (21, 22), a process suggested to facilitate translocation of PTTG to the nucleus (21). This is in contrast to an earlier study that implicated PTTG nuclear localization in FGF-2 regulation (26). Our observations suggest, therefore, that PTTG-mediated induction of VEGF does not seem to be a nuclear transcriptional event. Given that the SH3 mutant significantly reduced PTTG up-regulation of VEGF, however, some form of protein:protein interaction seems to be more crucial to this process. Interestingly, in the first description of human PTTG function, the authors failed to demonstrate VEGF induction in NIH3T3 cells through Northern analysis. However, our own data in human cells, using highly sensitive TaqMan RT-PCR, has consistently shown a 2- to 3-fold induction of the gene. Further, PTTG and VEGF have recently been shown to cycle in abundance together throughout rat estrus (27), and estrogen-mediated increased PTTG and VEGF expression have been demonstrated to be coincident with rat pituitary angiogenesis (28). Our proposed link between VEGF and PTTG expression is therefore perhaps not unexpected.

PTTG was unable to up-regulate KDR expression, despite high VEGF levels and despite the fact that KDR and PTTG mRNA expression were highly correlated in our pituitary tumors, suggesting that KDR does not respond to increased availability of its ligand *in vitro*, or to direct transcriptional stimu-

lation by PTTG. KDR was highly up-regulated in all tumor subtypes, compared with normal pituitaries, showing a 14-fold induction overall, further emphasizing the potential importance of VEGF signaling under such circumstances. Although KDR expression was extremely high in pituitary tumors, it is also important to consider its biological activity. KDR is tyrosine-phosphorylated when stimulated with its ligand VEGF, leading to an intracellular cascade and activation of the MAPK pathway and, hence, stimulation of cell division (29). The present studies were unable to address any potential differences in KDR activity in normal and tumorous pituitaries, however. Interestingly, TSH-omas demonstrated particularly high KDR levels (233-fold, compared with normals), despite low expression of its ligand. We previously demonstrated lowest FGF-2 and FGF-R-1 expression in TSH-omas (17). Why this tumor subtype should exhibit a distinct pattern of growth factor/growth factor receptor expression is unclear, although it is noteworthy that TSH-omas have a comparatively aggressive clinical course (30). Our data suggest very different growth factor signaling in such tumors, and we propose that this may contribute to the observed pattern of clinical behavior.

Taken together, our findings implicate altered VEGF and KDR signaling in pituitary tumorigenesis. In human colon cancer, we have previously correlated PTTG expression with tumor invasiveness and vascularity (18). In the same tissue, expression of VEGF and KDR have also been reported to correlate with vascularity, metastasis, and cell proliferation (31). A model by which PTTG stimulates VEGF and FGF-2, in the presence of up-regulated KDR, may account for angiogenic growth and progression of human pituitary tumors.

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