Endocrine, *vol. 30, no. 1, 129–138, August 2006* 0969–711X/06/30:129–138/$30.00
ENDO (Online) ISSN 1559-0100

Functional Thyrotropin Receptor Attenuates Malignant Phenotype of Follicular Thyroid Cancer Cells

S. Hoffmann,[1] K. Maschuw,[1] I. Hassan,[1] A. Wunderlich,[1]
S. Lingelbach,[1] A. Ramaswamy,[2] L. C. Hofbauer,[3] and A. Zielke[1]

[1]*Departments of Surgery,* [2]*Pathology, and* [3]*Endocrinology, Philipps-University of Marburg, 35043 Marburg, Germany*

Thyrotropin (TSH) is a thyroid-specific growth factor inducing differentiated function and growth of thyrocytes in vitro. In thyroid cancer, loss of TSH-receptor (TSHR) expression is a sign of de-differentiation and is believed to contribute to the malignant phenotype. The present studies aimed to determine the in vitro and in vivo effects of functioning TSHR in the follicular thyroid cancer cell line HTC, a subclone of FTC133 cells, lacking endogenous expression of TSHR, and HTCtshr+ cells transfected with human TSHR-cDNA. HTCtshr+ cells grew faster in vitro (doubling time 1.15 vs 1.56 d, $p < 0.05$) and TSH caused a dose-dependent growth response. Adhesion to and invasion through reconstituted basement membrane were reduced in HTCtshr+ cells, but when stimulated with TSH increased to levels comparable to naïve HTC cells. In vivo, tumor latency was 11 d for naïve HTC as compared to 21 d for HTCtshr+ xenografts. Smaller tumor volumes were registered for HTCtshr+ cells (250 ± 217 vs 869 ± 427 mm^3, $p < 0.05$). Angiogenesis, as determined by vascular surface density (VSD) of experimental tumors, was enhanced in naïve HTC tumors (VSD 0.87 ± 0.1 μm^{-1} vs 0.55 ± 0.2 μm^{-1} in HTCtshr+, $p < 0.05$). VEGF secretion was more pronounced in naïve HTC cells stimulated with EGF, than in HTCtshr+ cells stimulated with either TSH or EGF. In conclusion, regained expression of functional TSHR in the follicular thyroid cancer cell line HTC alters in vitro features commonly associated with the malignant phenotype. Smaller tumors and reduced angiogenesis of xenotransplanted HTC cells with functioning TSHR suggest a less aggressive in vivo phenotype. The present data highlight the pivotal role of TSHR to affect transformed thyrocytes in vitro and in vivo. They also suggest a role for EGF as a modulator of angiogenesis in thyrocytes devoid of TSHR.

Received June 12, 2006; Revised July 6, 2006; Accepted July 6, 2006.
Author to whom all correspondence and reprint requests should be addressed: Sebastian Hoffmann, MD, Department of Surgery, Endocrine Surgery Research, Philipps-University of Marburg, Baldingerstrasse, 35043 Marburg, Germany. E-mail: hoffmans@mailer.uni-marburg.de

Key Words: Differentiated thyroid cancer; thyroid cancer cell line; thyrotropin receptor; in vitro invasion; in vivo growth; VEGF; angiogenesis; EGF.

Introduction

The therapeutic usefulness of thyroid hormone administration in differentiated thyroid cancer was first outlined in 1937 when thyroid extract was given to children with differentiated thyroid cancer *(1)*. It is now well established that suppression of thyrotropin (TSH) is important for prevention of recurrent disease *(2,3)*. However, despite attempts to define the growth regulating effect of TSH, the results remain controversial to date. Commonly, TSH is regarded as a specific growth factor inducing differentiation and, at concentrations higher than necessary for differentiated function, growth of thyroid cells in vitro *(4)*. In human thyroid cancer cells, TSH stimulates differentiated function and growth by activation of TSH-dependent adenylate cyclase–protein kinase A pathways, independently of insulin or IGF *(5–7)*. However, TSH also stimulates adhesion, migration, and invasion of thyroid cancer cells in vitro mediated by the phospholipase C (PLC-β)–protein kinase C (PKC) pathway *(8–12)*.

Other growth factors that are involved in malignant thyroid disease are tyrosine kinase–mediated growth factors, in particular epidermal growth factor (EGF). In fact, constitutively activated EGF receptor and enhanced EGF receptor expression are frequently observed in undifferentiated thyroid cancer and have been established as independent indicators of tumor recurrence and survival *(13–15)*. While differentiated function decreased upon exposure of thyrocytes to EGF, proliferation, migration, and invasion of thyroid carcinoma cells increased in response to EGF in vitro and in vivo *(8–10,12,16–18)*. Accordingly, in vitro growth was inhibited when anti-EGFR antibodies were applied to thyroid carcinoma cell lines *(19)*.

Such findings supported the concept that less differentiated thyroid carcinoma cells escape from the control of growth factors thought to inhibit the malignant phenotype in vitro. Indeed, loss of responsiveness to TSH is considered a sign of de-differentiation of thyroid cancer and loss of the TSH receptor (TSHR) has been included into the

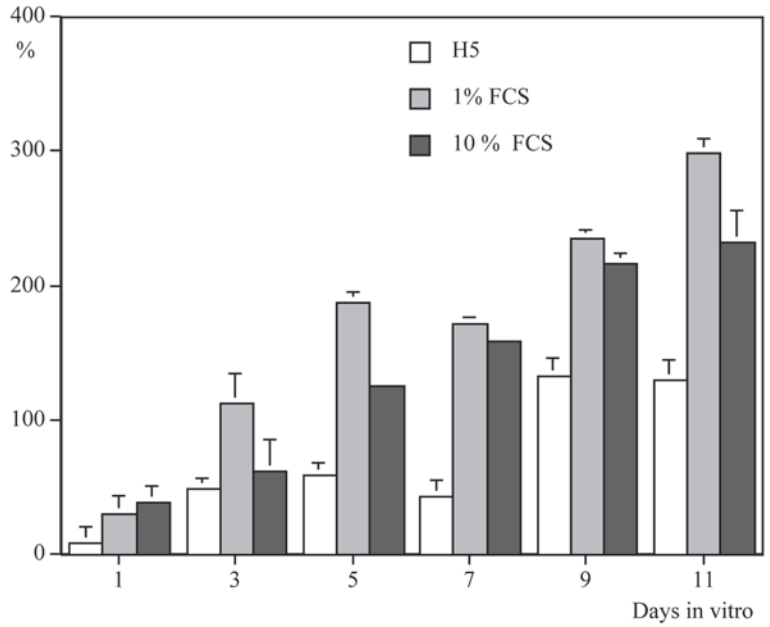

Fig. 1. Percentage difference of cell numbers of HTCtshr+ cells as compared to HTC cells lacking TSH receptor (% diff.). HTCtshr+ cells grew faster at all times and all media conditions. Both cell lines were grown in chemically defined serum-free growth medium (H5) and regular growth medium containing 1% and 10% of fetal calf serum (FCS).

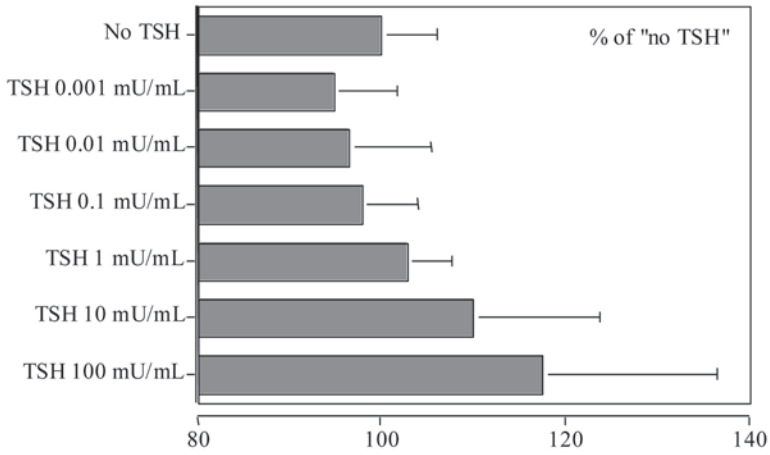

Fig. 2. Dose response of HTC cells transfected with functioning TSH receptor: percentage difference of numbers of HTCtshr+ cells cultured in the presence of bovine TSH at the indicated concentrations for 72 h, as compared to the TSH-free control (No TSH; 100%, MTT-assay).

concept of malignant progression of thyroid cancers *(20)*. This view is supported by clinical reports of reduced survival of patients with thyroid carcinomas not expressing TSHR *(21)*.

Irrespective of this empirical evidence, it has yet to be shown that the presence of functioning TSHR affects the malignant phenotype of thyroid tumor cells in vitro and in vivo. The few in vivo studies published to date fail to convincingly show any effect of endogenous TSH, exogenous administration of TSHR antibodies (Graves' serum), or exogenous TSH administration on the growth of thyroid carcinoma tissue xenografts to nude mice *(22–24)*. We therefore evaluated the effect of TSHR by a comparative evaluation of a follicular thyroid carcinoma cell line expressing functioning recombinant TSHR following stable transfection (HTCtshr+) and the naïve parental cell line, lacking endogenous expression of the TSHR (HTC).

Results

In Vitro Proliferation

HTC cells transfected with TSH receptor cDNA (HTC tshr+) grew faster than naïve HTC cells in vitro (Fig. 1). Population doubling times of HTCtshr+ cells maintained in growth medium supplemented with 1% and 10% FCS averaged 27.6 and 30.96 h as compared to 37.44 and 35.28 h for naïve HTC cells ($p < 0.05$). In the presence of TSH (0.001–100 mU/mL) proliferation of HTCtshr+ cells increased (Fig. 2), whereas that of HTC cells remained unchanged (minimum 90.5 ± 10.4% and maximum 103.5 ±

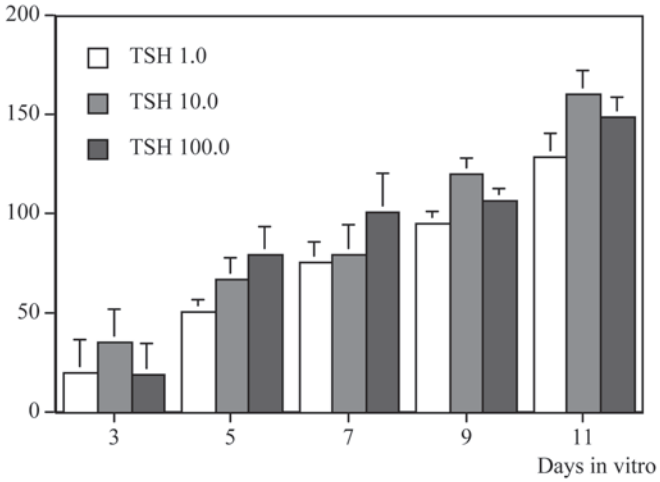

Fig. 3. Percentage difference of cell numbers of HTC cells cultured in the presence of bovine TSH at the indicated concentrations (mU/mL), as compared to HTC cells lacking TSH receptor in chemically defined serum-free growth medium (H5). Experiments were conducted over the entire range of TSH concentrations (0.001–100 mU/mL). For convenience, only the results of unequivocally stimulating concentrations of TSH are depicted.

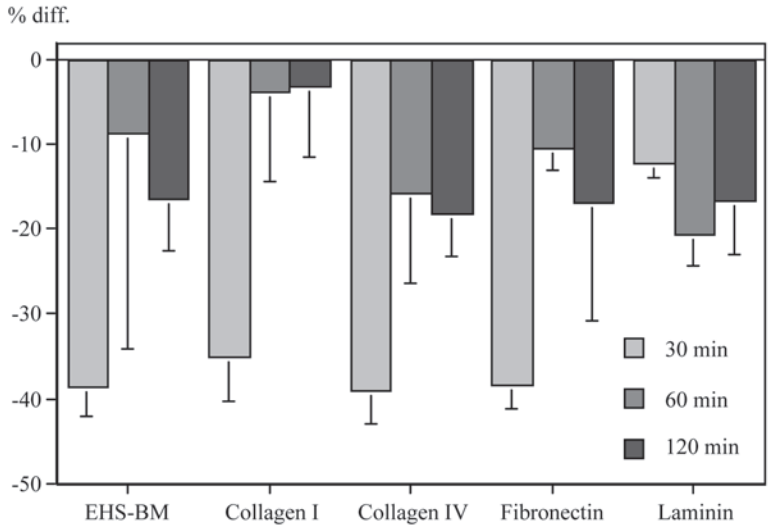

Fig. 4. Differential attachment (% diff.) of HTCtshr+ cells as compared to naïve HTC cells. HTC cells without a functioning TSH receptor adhere faster and better to purified extracellular matrix proteins (EHS-BM, reconstituted basement membrane).

9.6% of controls). Proliferation of HTCtshr+ cells decreased at concentrations of TSH considered physiologic (TSH 0.001–1.0 mU/mL), and increased at supraphysiologic concentrations 10–100 mU/mL). Figure 3 displays the differential proliferation of HTC and HTCtshr+ cells and documents a dose-dependent increase of the number of HTCtshr+ cells. After five generation times, the number of HTCtshr+ cells had increased between 80 and 150% over HTC cells ($p < 0.05$, Fig. 3).

In Vitro Cell–Substratum Adhesion

HTC cells lacking TSH receptor adhered significantly better to uncoated culture dishes and to purified proteins of the extracellular matrix (ECM). This was particularly observed in the early phase of attachment. However, even after 120 min, TSHR-positive HTC cells displayed decreased attachment of some 15% on average (Fig. 4). When HTCtshr+ cells were incubated with rising doses of bTSH, attachment to uncoated dishes increased and, at concentrations above 1 mU/mL of TSH, was no longer different from that of naïve HTC cells. Naïve HTC cells remained unaffected by TSH (Fig. 5). Likewise, TSH induced adhesion of HTCtshr+ cells to purified proteins of the ECM. For example, when incubated with 10 and 100 mU/mL of TSH, the differences of attachment to Matrigel, fibronectin, and collagen IV previously observed between HTCtshr+ and naïve HTC cells were reduced by more than 50% and completely abolished to laminin and collagen I. Induction of adhesion by TSH was already apparent after 30 min (Table 1), i.e. the initial phase of cell–substratum interaction, but

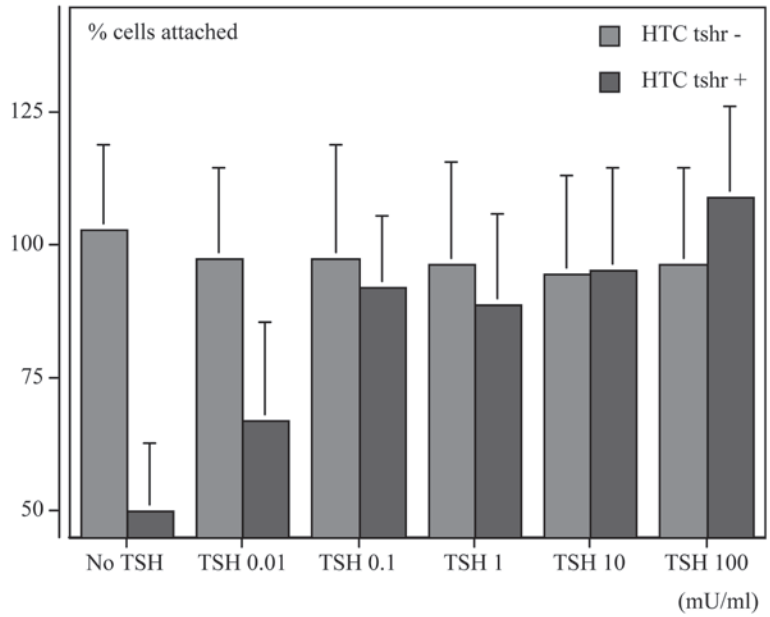

Fig. 5. Attachment of HTCtshr+ cells and naïve HTC cells (HTCtshr–) in the presence of bovine TSH (serum-free H5 medium, 0.01–100 mU/mL) to uncoated dished at 120 min. An increase in a dose-dependent fashion is appreciated for HTCtshr+ cells as compared to insignificant changes of HTC cells devoid of the TSH receptor.

Table 1
Differential Adhesion of HTC Cells to Purified ECM Proteins,
Following Stimulation of Both HTC CellLines with TSH at the Indicated Concentrations at 30 Min[a]

	Laminin	Fibronectin	Collagen I	Collagen IV	Matrigel
100 mU/mL TSH	–6.9	–13.6	–0.5	–10	–16
10 mU/mL TSH	4.4	–21.1	3.3	–21	–29
1 mU/mL TSH	9.7	–37.1	–8.4	–19.8	–22.4
0.1 mU/mL TSH	8.8	–21.3	–5.7	–19.7	–29.4
0.01 mU/mL TSH	–1.7	–23.8	–14.1	–24.7	–42.4
No TSH	–15	–37.1	–33.3	–42	–39.4

[a]Values represent the percentage difference of adhesion of HTCtshr+ cells as compared to HTC cells lacking TSH receptor.

(Standard deviation between individual experiments averaged less than 17% in both cell lines.)

likewise observed at 60 and 120 min (data not shown). These results suggest that TSH bound to its receptor gradually shifted the adhesive properties of HTCtshr+ cells gradually toward that of naïve HTC cells.

In Vitro Invasion

HTCtshr+ cells invaded reconstituted basement membrane to a lesser extent than naïve HTC cells, although these results were significant only for media containing 10% FCS ($p < 0.05$, Fig. 6). Upon incubation with TSH in serum-free H5 medium, invasion of naïve HTC cells remained grossly unaffected, whereas HTCtshr+ cells showed a dose-dependent increase of invasion. Maximum stimulation of invasion occurred at 10 mU/mL TSH, where it exceeded that of naïve HTC cells by more than 30%. In accordance with previous reports, invasion of HTCtshr+ cells decreased at higher concentrations of TSH.

Tumorigenicity and In Vivo Growth of HTC Xenografts

The tumor take-rate was 100% in both groups. Tumor latency was 11 d for naïve HTC cells and 21 d for HTCtshr+ cells. Final tumor volumes were calculated with 869 ± 427 mm^3 (median 880 mm^3) for xenografts grown from naïve HTC cells and averaged 250 ± 217 mm^3 (median 293 mm^3, $p < 0.05$) for tumors grown from HTCtshr+ cells. Tumors from naïve HTC xenografts weighed three times heavier than HTCtshr+ xenografts (HTC: median 1.1, mean 0.99 ± 0.46, range 0.5–1.8 g; HTCtshr+: median 0.3, mean 0.34 ± 0.26, range 0.1–0.7 g, $p < 0.05$).

Angiogenesis and Markers of Proliferation

VEGF and CD34 expression was confirmed in all tumors. With respect to the average intensity and distribution of VEGF immunoreactivity, the differences were not signifi-

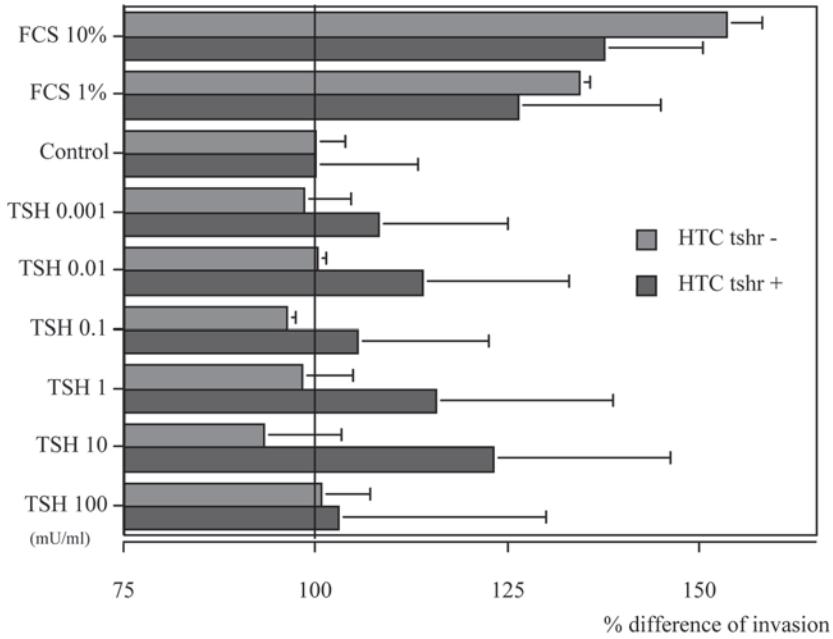

Fig. 6. Percentage difference of cell numbers of HTCtshr+ cells invading reconstituted basement membrane in the presence of bovine TSH at the indicated concentrations (mU/mL) and fetal calf serum (FCS), as compared to serum-free H5 growth medium (Control, 100%). HTCtshr+ cells appeared to invade to a lesser extent when kept in FCS media. A dose-dependent increase of invasion of HTCtshr+ cells as compared to insignificant changes of naïve HTC cells (HTCtshr–) was noted in serum-free H5 medium.

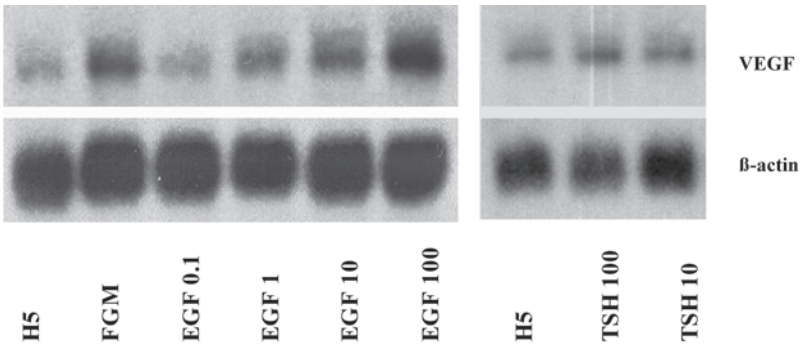

Fig. 7. Northern blot analysis of growth-factor stimulated VEGF gene expression (steady-state levels) of naïve HTC cells lacking TSHR expression. VEGF gene expression increased following a single pulse of EGF at the indicated concentrations (ng/mL, respectively), whereas it was largely unaffected by TSH (mU/mL).

cant between the two cell lines. Tumors had a percentage area of positive VEGF stains of 1.7 ± 0.54 for tumors grown from HTCtshr+ cells vs 1.2 ± 0.88 for naïve HTC tumors. Vascular surface density was determined with an average 0.547 ± 0.16 μm^{-1} for HTCtshr+ tumors and was significantly less than in naïve HTC tumors, (VSD: 0.873 ± 0.08 μm^{-1}, $p < 0.05$. Rate of mitoses and Ki-67 staining were similar between both groups. Both cell lines grew tumors that stained positively for EGFR without discernible differences (data not shown).

In Vitro VEGF Production

VEGF gene expression and accumulation of VEGF protein were documented in HTC cells, confirming their ability to synthesize and secrete VEGF. EGF induced VEGF gene expression in both cell lines, but TSH did not induce VEGF gene expression in naïve HTC cells (Fig. 7). Unstimulated HTCthsr+ cells secreted more VEGF than naïve HTC cells, both into regular growth medium as well as serum-free H5 medium. The cell-number-adjusted average basal VEGF protein accumulation into H5 medium was 388 ± 55 pg/mL for naïve HTC cells and 1143 ± 361 pg/mL for HTCtshr+ cells. VEGF protein accumulation increased by 20% when HTCtshr+ cells were exposed to bTSH, whereas HTC cells without TSHR were largely unaffected (Fig. 8). However, EGF was far more effective in stimulating VEGF gene expression and secretion of VEGF protein than TSH. For instance, the amount of VEGF protein following stimulation with a single pulse of EGF exceeded that of the TSH response by 2.5-fold in HTCtshr+ cells and up to 10-fold in naïve HTC cells. Overall, the highest levels of VEGF protein were measured following stimulation of naïve HTC

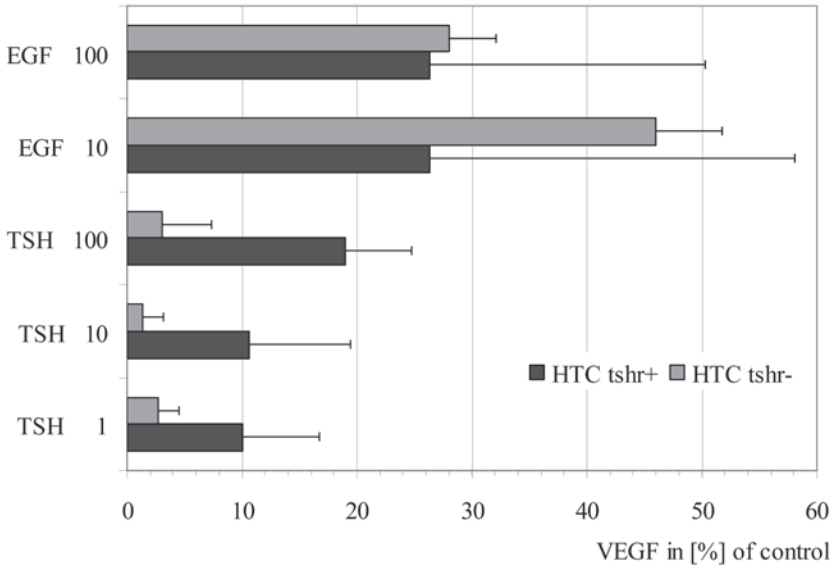

Fig. 8. Percentage difference of the magnitude of stimulation of VEGF into serum-free, defined medium of HTCtshr+ and naïve HTC cells (HTCtshr–). VEGF protein accumulation was enhanced by TSH in TSH-receptor positive HTC cells and by EGF in both cell lines. Note that in the absence of TSHR, EGF enhanced VEGF accumulation more effectively than TSH.

cells with EGF. Thus, in the HTC thyroid carcinoma cell lines, EGF increased VEGF gene expression and secretion of protein more effectively than TSH and particularly in the absence of a functioning TSHR.

Discussion

The HTC cell line, which lacks endogenous TSHR, has been subcultured from the follicular FTC 133 cell line by Derwahl and Bröcker *(4)*. Following transfection with human TSHR-cDNA, the resultant HTCtshr+ cell line has been shown to express some 3000–5000 TSHR per cell, which is within the range of non-neoplastic thyroid cells *(34)*. Moreover, functional TSHR was confirmed by cAMP accumulation in response to TSH as well as studies on TSHR–G protein–coupled signal transduction *(26,27,34)*.

Most of the follicular thyroid cancer cell lines studied to date were stimulated by exogenous TSH with respect to in vitro growth, adhesion, and tumor cell invasion through reconstituted basement membranes *(8–10,12,25,26,34–36)*. Increasingly de-differentiated follicular cell lines have been shown to be less sensitive to TSH *(9,10)*. This evidence led us to hypothesize that undifferentiated thyroid tumor cells may have escaped from the control of their differentiating growth factor, TSH. Such loss of control was observed in human thyroid cells immortalized by SV40 large T antigen or following the loss of tumor suppressor genes such as p53, p16, and p15 *(25)*. In accordance with this hypothesis, a moderate reduction of in vitro growth of HTCtshr+ cells had been noted previously when these cells were exposed to TSH at physiologic concentrations *(34)*. A similar observation was made in the anaplastic thyroid cell line C643, transfected with TSHR-cDNA. In this cell line, decrease of thymidine incorporation of some 10% had been reported after exposure to TSH, although no mention was made with regard to the effects of TSH on the naïve cell line *(36)*.

The results presented here suggest that low and presumably physiologic concentrations of TSH appear to moderately inhibit proliferation, whereas concentrations of TSH considered supraphysiologic apparently stimulate proliferation of HTC follicular thyroid cancer cells. This is in accordance with previous reports and the established dual effect of TSH on human thyrocytes: one that affects cell function (mediated by cAMP and phosphokinase A) and another that affects cell growth. The latter requires considerably higher concentrations of TSH and involves phosphokinase C *(25,37)*. In the current study the presence of TSHR already increased unstimulated in vitro proliferation, which was further enhanced when cells were stimulated with the ligand, TSH.

With respect to adhesion and invasion, the presence of TSHR resulted in less adhesive and invasive potential, suggesting attenuated malignant phenotype in vitro. However, TSH entirely compensated for any loss of adhesive or invasive propensity of TSHR-positive HTC cells. Although most of these effects were observed at concentrations of TSH considered to be supraphysiologic, it is evident that activation of transfected TSHR by its ligand shifted the in vitro phenotype of TSHR-positive HTC cells toward that of their originally more malignant TSHR-deficient naïve parental cells. In accordance with previous observations, this study has documented that functioning TSHR stimulates follicular thyroid tumor cell proliferation, adhesion and invasion in vitro, and all of these properties are attributes

of the malignant phenotype *(9,12,38)*. This study has also documented that thyroid cancer cells without functioning TSHR have enhanced ability to adhere to and invade through basement membrane, which is considered to be a hallmark of malignant cells.

However, whether TSH stimulates growth of differentiated thyroid cancer in vivo has been a matter of controversy *(39)*. In the current paper, we have documented significantly slower growth of xenografted follicular thyroid cancer cells transfected with functioning TSHR. These findings to our knowledge are the first to describe growth inhibition of thyroid tumor cells in vivo by functioning TSHR. A possible explanation for the divergence to historic studies may be the fact that we have made use of a well-characterized established in vitro model of follicular thyroid cancer, rather than poorly defined tumor tissue transplanted to nude mice *(22–24)*. A study involving anaplastic C643 cells is, to our knowledge, the only other study employing a thyroid cancer line transfected with TSHR-cDNA. Although only small groups of three and four grafts had been studied and the naïve cell line was unavailable for comparison, the authors discussed a TSH-dependent inhibition of tumor growth, because there was a trend toward smaller tumors when mice were treated with PTU *(36)*.

Further evidence in support of TSH-regulated in vivo growth of neoplastic thyroid cells comes from another study employing the FRTL5 transformed rat thyroid cell line. Successful establishment of xenografts was, with one exception, only achieved in hypothyroid mice, resulting in cell lines that like the parental line were TSH dependent. Of the one exception of a tumor arising in an euthyroid animal, two cell lines were established and found to be TSH independent for growth. The cell lines displayed rapid in vivo growth, undifferentiated histology and were unable to concentrate radioiodine *(40)*. In another study, growth of thyroid carcinomas from mice bearing a thyroid-targeted RET/PTC1 oncogene was enhanced when animals received a low iodine diet, suggesting that these carcinomas had retained TSH responsiveness *(41)*. Moreover, continuously elevated serum TSH levels cause the TSHR or the second messenger to downregulate in non-neoplastic thyroid cells, resulting in time-limited TSH effects. Lack of such "desensitization" was convincingly shown in thyroid carcinoma cell lines and could account for a continuous stimulation of proliferation in vivo *(42,43)*. Finally, there have been reports of tumor expansion following L-thyroxine withdrawal and enlargement of thyroid cancer remnants in response to the administration of recombinant TSH *(44)*. Together with the results of the present study, this accumulated evidence supports the concept of TSH to be a modulator of in vivo growth of neoplastic thyroid cells. Specifically, in the HTC xenograft model, expression of functioning TSHR decreases in vivo growth. If this observation were confirmed in non-experimental tumors, it would seem to call into question the current clinical concept of TSH-suppressive thyroxin therapy for patients with differentiated thyroid cancer. However, empirical clinical evidence argues against such a hypothesis. Thyroid hormone treatment has repeatedly been shown to result in fewer recurrences and a lower mortality rate *(45)*.

This raises the question as to why HTCtshr+ cells grew smaller tumors in vivo albeit significantly enhanced ability to proliferate in vitro. We found the in vivo proliferation of xenografted HTC cells not to be different, as evidenced by a similar rate of mitoses and Ki-67 staining. Apart from in vivo proliferation rates of tumor cells, their ability to induce angiogenesis may be a decisive feature. Recruitment of blood vessels is essential for expanding tumors and VEGF, a specific mitogen for endothelial cells, has been identified as the major autocrine regulator of angiogenesis in the thyroid *(46,47)*. TSH induces VEGF in cultures of non-neoplastic human thyroid cells as well as thyroid cancer cells in vitro and upregulation of VEGF in human thyroid cancer correlates with malignancy and poor prognosis *(48–53)*. Such evidence prompted us to address tumor angiogenesis and we found HTC cells without expression of functioning TSHR to grow tumors with enhanced angiogenesis. Moreover, EGF appeared to be a strong inductor of VEGF, both at mRNA and at protein level. A first indication that EGF may be involved in thyroid angiogenesis was made when EGF was shown to induce thrombospondin and plasminogen activator inhibitor, both of which are associated with the angiogenic cascade *(54)*. We have recently reported that EGF induced VEGF in thyroid carcinoma cell lines and suggest that EGF is a potent stimulator of VEGF in follicular thyroid carcinoma cells, more effective than TSH, particularly in cell lines not expressing TSHR *(55)*. This may be an explanation for a previous observation that "priming" of thyroid cancer cells with EGF resulted in accelerated in vivo growth of FTC133 follicular thyroid cancer xenografts *(9)*. It is likely that the effects of EGF on thyroid cancer cells are mediated by autocrine or paracrine pathways as suggested by the synchronous expression of EGF, TGF-α, and EGFR *(56)*. In this context it is noteworthy that increased synthesis of EGF or expression of EGFR as well as resistance to the inhibitory action of TGF β have been shown to become dominating pathways in the absence of TSHR and that the autocrine stimulation of tyrosine kinase receptors such as the EGFR found in many human thyroid cancer cells has also been detected in HTCtshr+ cells *(9,57–59)*. It is thus conceivable that EGF may have contributed to the growth of xenotransplanted TSHR-negative HTC cells through enhanced VEGF-mediated angiogenesis in vivo. However, EGF did not affect in vitro proliferation of HTC cells, suggesting that in the HTC in vivo model angiogenesis may be more important than proliferation.

In conclusion, regained expression of functional TSHR in the follicular thyroid cancer cell line HTC had a profound effect on biological in vitro features associated with the malignant phenotype. Restoring TSHR was associated

with reduced in vivo growth, suggesting a less aggressive in vivo phenotype. Taken together, the present data highlight the crucial role of TSHR to modulate the biological properties of transformed thyrocytes and suggest a novel role for EGF as a modulator of angiogenesis in undifferentiated thyroid cancer cells devoid of TSHR.

Materials and Methods

Materials

Culture flasks, dishes, and Boyden-type transwell chambers were obtained from Nunc (Nunc, NY), cell culture medium was from Sigma (Rödermark, Germany). EHS-basement membrane preparations (Matrigel), and all other matrix proteins, were purchased from Collaborative Research (Bedford, MA). Bovine thyroid stimulating hormone (bTSH) was from Sigma. Random primer labeling kit (Decaprime II) was from Ambion (Austin, TX) and [α-^{32}P]-dCTP from DuPont-NEN (Boston, MA). The human β-actin cDNA insert and ExpressHyb solution were purchased from Clontech (Palo Alto, CA). All other reagents including MTT and EGF were from Sigma.

HTC Thyroid Tumor Cell Lines

The HTC cell line is a follicular thyroid cancer cell line, subcultured from the well-described FTC 133 cell line *(25)*. HTC cells have no endogenous expression of the TSHR as documented by functional assays as well as PCR *(26)*. These cells were transfected with a human TSHR cDNA and isolated after G418 selection. Expression of functional TSHR was established by specific binding of radiolabeled TSH and cAMP production. The resultant cell line was designated HTCtshr+ and has been shown to express a number of functioning TSHR comparable to non-neoplastic differentiated thyroid cells *(27)*. These cell lines were generously provided by Dr. Derwahl, Berlin, Germany. HTC cells were maintained in DMEM-h21/Ham's F12 1:1 (v/v), with 25 m*M* HEPES, 0.055 g/L sodium pyruvate, 0.365 g/L glutamine, 10% bovine serum ("full growth medium," FGM), 10,000 U/L penicillin, and 100 mg/L streptomycin at 37°C in a 100% humidified, 5% CO_2 atmosphere. Transfected HTC cells were expanded in the presence of G418 (100 μg/mL). Cells planned for experiments with stimulators were switched to serum-free H5 medium for 48 h *(28)*. H5 contains bovine insulin (10 μg/mL), human transferrin (5 μg/mL), somatostatin (10 ng/mL), glycyl-L-histidyl-L-lysine (2 ng/mL), and hydrocortisone (10^{-8} *M*). For experiments, cells in exponential growth were harvested by ice-cold trypsin-EDTA, resuspended in H5, and vitality assessed by tryphan blue exclusion.

In Vitro Proliferation Assays

HTC cells were repeatedly washed with calcium- and magnesium-free PBS (CMF), resuspended, and plated at a density of 1×10^4 viable cells into triplicate wells of microtitration plates. Cells were allowed to adhere and resume full growth over 24 h. Bovine TSH (0.001–100 mU/mL) was added to H5 and incubations were continued for five generation times. Cell numbers were determined every 24–72 h by MTT assay as described *(9)*. Optical densities were read at 570 nm with a reference filter of 630 nm (Emax, Molecular Devices, Munich, Germany).

In Vitro Cell–Substratum Adhesion

Adhesion assays to purified constituents of the extracellular matrix (ECM) were carried out as published previously *(9)*. Briefly, 100 μL of laminin (20 μg/mL PBS), collagen I (20 μg/mL 0.1% acetic acid), collagen IV (20 μg/mL 0.4 *N* NaHCl), and fibronectin (20 μg/mL PBS) were added to microtitration plates and allowed to air dry at 4°C over night. Wells were blocked with bovine serum albumin (BSA, Cohn fraction V, 20 mg/mL) for 2 h at 37°C and rinsed twice with CMF, before adding 50 μL of H5 supplemented with 200 μg/mL BSA (adhesion medium: AM). Single cell suspensions were repeatedly washed with CMF, resuspended in AM, and plated at a density of 7.5×10^4 cells. Plates were spun at 200*g* to synchronize attachment and allowed to adhere for 30, 60, and 120 min. Plates were then inverted and spun at 40*g* for 2 min, washed to completely remove detached cells, and the number of attached cells was quantified by MTT assay. Assays evaluating cell substratum attachment in the presence of bTSH (0.01–100 mU/mL) represent data from two experiments in triplicates.

In Vitro Invasion

Boyden chamber translocation assays were performed as reported earlier *(9)*. Briefly, HTC cells (1×10^5 viable cells/100 μL) were plated to upper wells of 10 mm Transwell chambers with 8 μm pore membranes in H5 supplemented with bTSH (0.001–100 mU/mL) and allowed to penetrate for 48 h. For invasion assays, membranes were coated with Matrigel (300 μg protein/mL). The number of cells in the upper and lower wells was determined by MTT assay.

Tumorigenicity and In Vivo Growth of HTC Xenografts

Four- to five-wk old female, pathogen-free BALB/c NCR-NU congenitally dysthymic mice (Harlan–Winkelman, Borchen, Germany) were housed in a semisterile environment with a 12 h light/dark cycle, and fed an autoclaved standard breeding chow and water *ad libitum*. Groups of nine mice each had either 1×10^6 HTCtshr+ or naïve HTC cells injected to the interscapular space. After 7 wk, mice were sacrificed and tumor weights and sizes measured using calibrated micrometer calipers. Volumes were calculated according to (length × width × high) × 0.5236 *(29)*. Tumors were harvested and fixed in formalin. All procedures involving animals were approved and monitored by the local ethics committee and animal welfare authorities and conducted in accordance to the guidelines for the welfare of animals in experimental neoplasia.

Morphometric Analysis of In Vivo Angiogenesis

Three representative tumors of each HTC line were submitted to morphometric analysis of VEGF expression and tumor angiogenesis. Immunostains of 3 μm slides were obtained using a polyclonal anti-VEGF-antibody (SC152, Santa Curz, Biotechnology, CA) and a polyclonal anti-CD34-antibody (MEC13.3, Pharmingen, Heidelberg, Germany). Vascular surface densities (VSD) of the tumors were evaluated as described *(30,31)*. To this end, contiguous tumor sections were analyzed for the number of CD34-stained vessels and the area they covered, as well as the relational areas of tumor cells allowing for a calculation of the area covered by microvessels in relation to a given tumor volume (vascular surface density, VSD, 1/mm). Twenty random areas were analyzed for each tumor (Leica Q500 MC/Leica imaging system QWin).

Northern Blot Analysis

Total RNA of HTC cells from experiments described above was isolated using the RNeasy-kit (Qiagen, Hilden, Germany). Ten micrograms of total RNA was separated on 1.5% agarose/formaldehyde (2.2 *M*) gel and transferred to Hybond N+ nylon membranes by capillary blotting. Random prime labeling and hybridization procedures were carried out as reported *(32,33)*. A 320-bp ^{32}P-labeled VEGF-probe hybridizing to three mRNA species of VEGF was kindly provided by Dr. Sander, Regensburg, Germany. Hybridizations with human β-actin cDNA verified equal amounts of RNA were loaded.

VEGF Protein Measurement

HTC cells were plated at a density of 1–5 × 10^5 into triplicate wells of multiwell plates and allowed to adhere. They were then shifted to H5 for 24 h, medium was renewed, and cultures continued for 48 h to obtain conditioned medium (CM). CM was centrifuged (15,000*g*, 20 min, 4°C) and aliquots stored at –80°C until analyzed. In some experiments, cells were incubated in the presence of EGF (10–100 ng/mL) or TSH (1–100 mU/mL). VEGF was quantified by ELISA (VEGF-DuoSet, R&D Systems; Emax, Molecular Devices, Munich, Germany, 450–570 nm).

Statistics

Descriptive statistics were computed for all parameter. Naïve HTC cells were compared to HTCtshr+ cells (test condition) by means of repeated measures analysis of variance. Whenever significant differences occurred, paired Student's *t*-test was performed for further analysis. To evaluate microvessel densities, Fisher's exact test was used. A probability value of $p < 0.05$ was accepted to indicate significance. Values are presented as mean ± standard deviation if not stated otherwise. With the exception of TSH-stimulated adhesion assays, all experiments were carried out three times in triplicates or duplicates (tumor cell invasion assays).

References

1. Dunhill, T. (1937). *Trans. Med. Soc. London* **60,** 234–248.
2. Balme, H. W. (1954). *Lancet* **266,** 812–813.
3. Pujol, P., Daures, J. P., Nsakala, N., Baldet, L., Bringer, J., and Jaffiol, C. (1996). *J. Clin. Endocrinol. Metab.* **81,** 4318–4323.
4. Derwahl, M., Bröcker, M., Meyer, K., Schatz, H., and Studer, H. (1992). *Acta Endocrinol.* **126,** 50–55.
5. Clark, O. H. and Gerend, P. L. (1985). *J. Clin. Endocrinol. Metab.* **61,** 773–778.
6. Duh, Q. Y., Gum, E. T., Gerend, P. L., Raper, S. E., and Clark, O. H. (1990). *World J. Surgery* **14,** 410–418.
7. Wyllie, F. S., Dawson, T., Bond, J. A., et al. (1998). *Mol. Cell. Endocrinol.* **76,** 13–18.
8. Holting, T., Tezelman, S., Siperstein, A. E., Duh, Q. Y., and Clark, O. H. (1993). *Biochem. Biophys. Res. Commun.* **195,** 1230–1234.
9. Holting, T., Zielke, A., Siperstein, A. E., Clark, O. H., and Duh, Q. Y. (1994). *J. Clin. Endocrinol. Metab.* **79,** 806–813.
10. Holting, T., Zielke, A., Siperstein, A. E., Clark, O. H., and Duh, Q. Y. (1994). *Clin. Exp. Metastasis.* **12,** 315–323.
11. Duh, Q. Y. and Grossman, R. F. (1995). *Surg. Clin. North Am.* **75,** 421–437.
12. Zielke, A., Hoffmann, S., Plaul, U., Duh, Q. Y., Clark, O. H., and Rothmund, M. (1999). *Exp. Clin. Endocrinol. Diabetes* **107,** 361–369.
13. Bergstrohm, J. D., Westermark, B., and Heldin, N. E. (2000). *Exp. Cell Res.* **259,** 293–299.
14. Akslen, L. A., Myking, A. O., Salvesen, H., and Varhaug, J. E. (1993). *Br. J. Cancer* **68,** 808–812.
15. Ness, G. O., Haugen, D. R., Varhaug, J. E., Akslen, L. A., and Lillehaug, J. R. (1996). *Int. J. Cancer* **65,** 161–167.
16. Mastuda, H., Sugenoya, A., Kobajashi, S., Kasuga, Y., and Iida, F. (1988). *World J. Surgery* **12,** 616–622.
17. Westermark, K., Westermark, B., Karlson, K. A., and Erickson, L. E. (1986). *Endocrinology* **118,** 1040–1046.
18. Asmis, L. M., Gerber, H., Kaempf, J., and Studer, H. (1995). *J. Endocrinol.* **145,** 513–520.
19. Gabler, B., Aicher, T., Heiss, P., and Senekowitsch-Schmidtke, R. (1997). *Anticancer Res.* **17,** 3157–3159.
20. Schlumberger, M. J. (1998). *N. Engl. J. Med.* **338,** 297–306.
21. Tanaka, K., Sonoo, H., Yamamoto, Y., et al. (2000). *J. Surg. Oncol.* **75,** 108–116.
22. Dralle, H. (1989). In: *Growth regulation of the thyroid gland and thyroid tumors*. Goretzki, P. E. and Röher, H. D. (eds.), Karger: München, pp. 109–122.
23. Smeds, S., Boeryd, B., Jöstro, E., and Lennquist, S. (1989). In*: Growth regulation of the thyroid gland and thyroid tumors*. Goretzki, P. E. and Röher, H. D. (eds.), Karger: München, pp. 98–108.
24. Wenisch, H. J. C., Schumm-Dräger, P. M., and Encke, A. (1992). *Exp. Clin. Endocrinol. Diabetes* **100,** 48–50.
25. Goretzki, P. E., Simon, D., Dotzenrath, C., Schulte, K. M., and Röher, H. D. (2000). *World J. Surg.* **24,** 913–922.
26. Derwahl, M., Kuemmel, M. Goretzki, P. E., Schatz, H., and Broecker, M. (1993). *Biochem. Biophys. Res. Commun.* **191,** 1131–1138.
27. Broecker, M., Mayr, G. W., and Derwahl, M. (1997). *Endocrinology* **138,** 3787–3796.
28. Ambiosi-Impiombato, F. S., Parks, L. A. M., and Coon, H. G. (1980). *Proc. Natl. Acad. Sci. USA* **77,** 3455–3459.
29. Isaacs, T. and Coffey, D. S. (1981). *Cancer Res.* **41,** 5070–5074.
30. Barth, P. J., Weingärtner, K., Köhler, H. H., and Bittinger, A. (1996). *Hum. Pathol.* **27,** 1306–1310.
31. Zielke, A., Middeke, M., Hoffmann, S., et al. (2002). *Surgery* **6,** 1056–1063.
32. Hofbauer, L. C., Kluger, S., Kühne C. A., et al. (2002). *J. Cell. Biochem.* **86,** 642–650.

33. Middeke, M., Hoffmann, S., Hassan, I., Wunderlich, A., Hofbauer, L. C., and Zielke, A. (2002). *Exp. Clin. Endocrinol. Diabetes* **110,** 386–392.
34. Brocker, M., deBuhr, I., Papageorgiou, G., Schatz, H., and Derwahl, M. (1996). *Exp. Clin. Endocrinol. Diabetes* **104,** 20–23.
35. Wright, P. A., Lemoine, N. R., Goretzki, P., et al. (1991). *Oncogene* **6,** 1693–1697.
36. Gustavsson, B., Hermansson, A., Andersson, A. C., et al. (1996). *Mol. Cell. Endocrinol.* **121,** 143–151.
37. Derwahl, M., Hamacher, C., Russo, D., et al. (1996). *J. Clin. Endocrinol. Metab.* **81,** 1898–1904.
38. Demeure, M. J., Damsky, C. H., Elfman, F., Goretzki, P. E., Wong, M. G., and Clark, O. H. (1992). *World J. Surg.* **16,** 770–776.
39. Wenisch, H. J. (1996). *Exp. Clin. Endocrinol. Diabetes* **3,** 61–63.
40. Ossendorp, F. A., Bruining, P. F., Schuuring, E. M., et al. (1990). *Endocrinology* **127,** 419–430.
41. Sagartz, J. E., Jhiang, S. M., Tong, Q., and Capen, C. C. (1997). *Lab. Invest.* **76,** 307–318.
42. Al-Sobhi, S. S., Soh, W. Y., Wong, M. G., Siperstein, A. E., Duh, Q. Y., and Clark, O. H. (1998). *World J. Surg.* **22,** 552–554.
43. Tezelman, S. T., Holting, T., Jossart, G. H., et al. (1998). *World J. Surg.* **22,** 544–548.
44. Braga, M., Ringel, M. D., and Cooper, D. S. (2001). *J. Clin. Endocrinol. Metab.* **86,** 5148–5151.
45. Dulgeroff, A. J. and Hershman, J. M. (1994). *Endocr. Rev.* **15,** 500–515.
46. Klein, M., Picard, E., Vignaud, J. M., et al. (1999). *J. Endocrinol.* **161,** 41–49.
47. Katoh, R., Miyagi, E., Kawaoi, A., et al. (1999). *Hum. Pathol.* **30,** 891–897.
48. Biglietto, G., Maglione, D., Rambaldi, M., et al. (1995). *Oncogene* **11,** 1569–1179.
49. Sato, K., Yamazaki, K., Shizume, K., et al. (1995). *J. Clin. Invest.* **96,** 1295–1302.
50. Soh, E. Y., Sobhi, S. A., Wong, M. G., et al. (1996). *Surgery* **120,** 944–947.
51. Fenton, C., Patel, A., Dinauer, C., Robie, D. K., Tuttle, R. M., and Francis, G. L. (2000). *Thyroid* **10,** 349–357.
52. Klein, M., Vignaud, J. M., Hennequin, V., et al. (2001). *J. Clin. Endocrinol. Metab.* **86,** 656–658.
53. Lennard, C. M., Patel, A., Wilson, J., et al. (2001). *Surgery* **129,** 552–558.
54. Kotlarz, G., Wegrowski, Y., Martiny, L., Declerck, P. J., and Bellon, G. (2002). *Biochem. Biophys. Res. Commun.* **295,** 737–743.
55. Hoffmann, S., Hofbauer, L. C., Scharrenbach, S., et al. (2004). *J. Clin. Endocrinol. Metab.* **89,** 6139–6145.
56. Gorgoulis, V., Aninos, D., Priftis, C., et al. (1992). *In Vivo* **6,** 291–296.
57. Lemoine, N. R., Hughes, C. M., Gullik, W. J., Brown, C. L., and Wynford-Thomans, D. (1991). *Int. J. Cancer* **49,** 558–561.
58. Brocker, M., Hammer, J., and Derwahl, M. (1996). *Thyroid (Suppl 1)* **5,** S4.
59. Derwahl, M., Broecker, M., and Kraiem, Z. (1999). *J. Clin. Endocrinol. Metab.* **84,** 829–834.