Human melanoma cells express functional receptors for thyroid-stimulating hormone

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Abstract

We have reported a high prevalence of hypothyroidism in the cutaneous melanoma population, suggesting that the pathologic hormonal environment of hypothyroidism promotes melanoma growth. The objective of this study was to test the hypothesis that TSH, which circulates at elevated levels in hypothyroid individuals, stimulates the growth of melanoma cells. Our results show that TSH receptors (TSHR) are expressed by virtually all cutaneous melanocytic lesions, including benign nevi, dysplastic nevi, and melanomas, with higher expression found in malignant and pre-malignant lesions. The finding of TSHR expression by human tumors is confirmed in cultured melanoma cells and melanocytes, in which TSHR expression is demonstrated by immunofluorescent staining, western blotting, and reverse transcriptase-PCR. Melanoma TSHR are functional, as evidenced by the ability of TSH to induce the formation of cAMP and to activate the mitogen-activated protein kinase (MAPK) pathway. Cultured melanoma cells, but not melanocytes, are induced to proliferate at a physiologically relevant concentration of TSH. Taken together, these data support the hypothesis that TSH is a growth factor for human melanoma. Our findings have broad clinical implications for the prevention of melanoma and the management of established disease.

Endocrine-Related Cancer (2006) 13 1269–1277

Introduction

Melanoma, the most aggressive malignancy arising from the skin, is a disease that continues to challenge clinical and laboratory researchers alike. Lethal for the vast majority of patients with advanced metastasis, the behavior of melanoma can otherwise be somewhat unpredictable at intermediate stages. Moreover, although melanoma is considered to be an immunologically sensitive cancer, only a small but reproducible percentage of patients derive lasting clinical benefit from immunotherapeutic approaches. Such findings suggest the existence of distinct biological subsets of human melanoma. The identification of these patient subsets would provide the opportunity for unique, directed therapeutic approaches based on both clinical and molecular tumor characteristics.

We have recently reported a prevalence of hypothyroidism among melanoma patients that exceeds the prevalence in the general population (Ellerhorst et al. 2001, 2003). In this cohort of hypothyroid melanoma patients, the diagnosis of hypothyroidism preceded or was made concurrently with the diagnosis of melanoma in the majority of cases. This chronology suggests that hypothyroidism may in some way promote the development and progression of melanoma, raising the question of mechanisms to account for this growth-promoting effect. One of the diagnostic hallmarks of thyroid failure, even at early stages, is the elevation of circulating levels of thyroid-stimulating hormone (TSH). In the presence of declining concentrations of the thyroid hormone tri-iodothyronine, thyrotropin-releasing hormone (TRH) is secreted by hypothalamic nuclei into the hypothalamic-pituitary portal system. TRH induces the thyrotrope cells of the anterior pituitary gland to secrete TSH into the systemic circulation. TSH binds its receptor on thyroid follicular cells, stimulating the production and secretion of thyroid hormones which provide feedback inhibition...
of TSH secretion. In the case of a failing thyroid gland, unable to respond adequately to TSH, this negative feedback on the hypothalamus and pituitary gland does not occur, and TSH continues to circulate at elevated levels.

TSH is mitogenic for cultured thyrocytes (Dremier et al. 2002). The TSH receptor (TSHR) is a typical G-protein-coupled receptor which, when activated, induces the formation of cAMP. Three distinct signaling pathways lie downstream of cAMP in thyrocytes: the protein kinase A (PKA; Porcellini et al. 1997), mitogen-activated protein kinase (MAPK; Iacovelli et al. 2001), and phosphatidylinositol 3-kinase (PI3K) pathways (De Vita et al. 2000, Suh et al. 2003). Of note, these same pathways play critical roles in the promotion of melanoma growth (Smalley 2003, Poser & Bosserhoff 2004, Robertson 2005). Furthermore, TSHR transcripts have been reported in virtually every cellular component of skin, including melanocytes, and are expressed by some melanoma cell lines (Slominski et al. 2002).

Given the over-representation of hypothyroid individuals in the melanoma population, the elevation of TSH in virtually all cases of primary hypothyroidism, the presence of TSHR transcripts in melanocytic cells, and the importance of TSHR-associated signaling pathways in melanomas, we hypothesized that TSHR are present on the surface of melanoma cells, through which TSH stimulates melanoma growth. We describe here our findings that melanoma cells do, in fact, possess functional TSHR and are induced to proliferate by TSH.

Materials and methods

Patient material

Sections of melanoma tissue were obtained from the MD Anderson Cancer Center (MDACC) Melanoma Tumor Bank. Procurement of all patient materials at MDACC was conducted with IRB approval and in accordance with HIPAA guidelines. Sections of benign and dysplastic nevi were obtained from the Dermatology Clinic, Kiel, Germany. Neonatal foreskin and normal thyroid tissue were obtained as incidental surgical material, with IRB-approved protocols.

Cell lines and primary cultures

The human melanoma MeWo cell line was kindly provided by Dr David Menter (MDACC) and the WM793 line by Dr Robert Kerbel (Sunnybrook Health Science Center, Toronto, Ontario, Canada). The human melanoma cell line A375 was obtained from the American Type Culture Collection (Manassas, VA, USA). TAD2 cells, which are SV40-transformed human fetal thyrocytes (Martin et al. 1990), were kindly provided by Dr Terry F Davies (Mount Sinai School of Medicine) through the laboratory of Dr Jeffrey Myers (MDACC). All melanoma tumor lines and TAD2 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Primary melanocyte culture FMC7C was derived from neonatal foreskin melanocytes. FMC7C was maintained in MCDB-153 media (Sigma), supplemented with 1% FBS, 10 ng/ml phorbal-12-myristate-13-acetate (Calbiochem, San Diego, CA, USA), 1 ng/ml basic fibroblast growth factor (Invitrogen), 5 μg/ml transferrin (Sigma), 10 nM cholera toxin (Calbiochem), 0.1 mM 3-isobutyl-1-methylxanthine (Calbiochem), 30 μg/ml bovine pituitary extract (Sigma), and 5 μg/ml insulin (Sigma). Primary thyrocyte cultures were grown in a media consisting of RPMI 1640 with 1% FBS, 1000 mIU/l TSH (Sigma), 10 ng/ml somatostatin (Sigma), 10 μg/ml insulin, 1 nM hydrocortisone (Sigma), and 5 μg/ml transferrin. RITH media, a serum-free, and TSH-free modification of the thyrocyte media consisted of RPMI 1640 with 10 μg/ml insulin, 5 mg/ml transferrin, and 10 nM hydrocortisone. RITH was used as the basal media for all functional experiments.

Immunohistochemistry

Immunohistochemical (IHC) analysis of melanoma and nevi sections for TSHR expression was performed using a commercially available murine monoclonal IgG2a antibody specific for the extracellular domain of human TSHR (Serotec, Raleigh, NC, USA). Tissue sections were deparaffinized in xylens, and rehydrated in graded concentrations (100–85%) of ethanol. Sections were placed in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) and microwaved intermittently for a total of 10 min, to maintain boiling temperature. After cooling, the slides were placed in 3% H2O2 in cold methanol for 15 min to block endogenous peroxidase activity. This step was followed by permeabilization with 0.05% Triton X-100 (Sigma) in PBS (PBS) for 15 min. An avidin–biotin–peroxidase complex (ABC) kit (Vectastain, Vector Laboratories) was then used for antigen detection. After a 30-min incubation with blocking serum, the primary antibody was applied at a dilution of 1:400 and incubated for 2 h at room temperature. The slides were then washed, incubated for 30 min with secondary biotinylated antibody, followed by a 30-min incubation with the ABC reagent. The immunolabeling was developed with the chromogen 3-amino-9-ethylcarbazole. Hematoxylin was applied as a counter stain.
Immunostaining was scored by a dermatopathologist (AHD), separately for the percentage of immunopositive cells and for the intensity of immunoreactivity. Scoring for percentage of positive tumor cells was defined as follows: '0', <5% positive cells; '1', 5–25% positive cells; '2', 26–75% positive cells; and '3', >75% positive cells. Intensity scoring was defined as follows: '0', no staining; '1', weak staining; '2', moderate staining; and '3', intense staining.

Indirect immunofluorescence

Cells grown on chamber slides were fixed with 2% paraformaldehyde on ice for 30 min, blocked with 5% serum in PBS for 30 min at room temperature, and then incubated with primary antibody diluted in blocking solution for 2 h at 4 °C. Incubation with fluorescein isothiocyanate (FITC)-labeled secondary antibody was performed at room temperature for 1 h. Staining was observed and imaged with a Zeiss Axioplan 2 fluorescent microscope equipped with an Axiocam MRc5 camera.

Western blotting

Antibodies to ERK and phosphorylated ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to TSHR used in western blotting were the same as those used for IHC. Proteins extracted from cell lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked for 1 h in 5% dry milk/PBS. Incubation with primary antibody diluted in 5% dry milk/PBS/0.1% Tween was performed overnight at 4 °C, followed by a 1 h incubation with horseradish peroxidase-labeled secondary antibody, diluted in 5% dry milk/PBS/0.1% Tween. The membrane was developed using enhanced chemiluminescence (Amersham Pharmacia Biotech).

cAMP assay

Levels of intracellular cAMP were quantitated in duplicate samples by means of a commercially available EIA (Amersham), according to the manufacturer’s directions. Both purified bovine TSH (Sigma) and recombinant human TSH (Thyrogen, Genzyme Corporation, Cambridge, MA, USA) were used for the experiments.

Proliferation assay

Cells were plated in RITH media at a density of $3 \times 10^4$ per chamber of a two-chamber tissue culture slide. On the following day, TSH was added at the desired concentration and proliferation was assessed 48 h later by means of a commercially available BrdU immunocytochemistry assay (BrdU Staining Kit, Zymed, Carlsbad, CA, USA). On the evening prior to the assay, BrdU labeling reagent (Zymed) was added at a 1:100 dilution to the cultures. On the following day, the cells were fixed with 70% ethanol and incubated in 3% H$_2$O$_2$ in methanol to block endogenous peroxidase activity. After permeabilization and blocking, the cells were incubated with biotinylated anti-BrdU antibody, followed by streptavidin peroxide, the chromogen diaminobenzidine (DAB), and a hematoxylin counterstain. To calculate the percentage of cells positive for BrdU, the stained sections were examined for brown DAB-stained nuclei. A minimum of 100 cells was counted from two different areas of the slide, and the mean and s.d. calculated from these counts.

PCR and RT-PCR

Oligonucleotide PCR primers were obtained from Sigma Genosys (The Woodlands, TX, USA). PCR was carried out using the GeneAmp Gold PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA) and reverse transcriptase (RT)-PCR with the GeneAmp RNA PCR Kit (Applied Biosystems). PCR primer sequences and conditions for amplification of exons 9 and 10 of the TSHR gene were based on those previously reported (Georgopoulos et al. 2003). Due to the large size of exon 10, two separate amplifications were performed to cover the proximal and distal segments of the exon. Primers designed to amplify a 209 bp fragment of TSHR exon 10 from melanoma cDNA were designed with Oligo Primer Analysis Software (Molecular Biology Insights, Cascade, CO, USA). Control thyroid poly-A RNA for RT-PCR was purchased from BD Biosciences-Clontech. Primers and conditions for PCR are detailed below:

**TSHR** exon 9:
Forward: 5'-TCATCTCCCAATTAACCTCAGG-3'
Reverse: 5'-GCTTCCAATTTCCTCTCCAC-3'
Conditions: 95 °C 15 s, 60 °C 45 s; 72 °C 45 s; magnesium chloride 2.5 mM, 30 cycles

**TSHR** exon 10a:
Forward: 5'-TGGCACTGACTCTTTTCTGT-3'
Reverse: 5'-GTCCATGGGCAGGCAGATAC-3'
Conditions: 95 °C 45 s, 60 °C 45 s; 72 °C 45 s; magnesium chloride 3.0 mM, 30 cycles

**TSHR** exon 10b:
Forward: 5'-ACTGTCTTTGCAAGCCGATT-3'
Reverse: 5'-GTGTCATGGGATTGGAATGC-3'
Conditions: 95 °C 45 s, 60 °C 45 s; 72 °C 45 s; magnesium chloride 3.0 mM, 30 cycles

**TSHR** exon 10, 209 bp:
Forward: 5'-CCGATGAGTTCAACCCGTG-3'
Reverse: 5'-GGCGATGAGAGGAGGTACAT-3'
Conditions: 95 °C 45 s, 57 °C 45 s; 72 °C 45 s; magnesium chloride 2.0 mM, 35 cycles
DNA sequencing
Sequencing of PCR products was performed by the MDACC DNA Core Facility using an ABI Prism 3100 DNA Genetic Analyzer (Applied Biosystems), using Big Dye v.3.1 dye terminator chemistry (Applied Biosystems). Forward and reverse DNA strands were sequenced for all samples.

Statistical analysis
Chi-squared analysis was used to examine differences in IHC scoring between the three sample groups. Differences in cAMP production and in rates of BrdU uptake were tested with unpaired two-sided t-tests. Significance was set at 0.05. Analysis was performed using StatView software (SAS Institute Inc, Cary, NC, USA)

Results
Human melanoma cells express TSHR
To establish the presence of TSHR in melanoma cells and melanocytic lesions, we examined 20 melanomas, 12 dysplastic (pre-malignant) nevi, and 25 benign nevi by immunohistochemistry for TSHR expression (Fig. 1a–d). TSHR was detected in all cases. The percentage of positive cells and the intensity of immunostaining were scored for each lesion as described in Methods. The percentage of positive cells was generally high in most cases and did not differ significantly among the various types of lesions, although there was a trend for a greater number of positive cells in melanomas when compared with benign nevi ($P=0.096$). In contrast, intensity of TSHR immunostaining varied considerably according to lesion type (Table 1). Intensity was significantly increased in melanomas when compared with benign nevi ($P=0.047$); similarly, dysplastic nevi showed a trend of increased intensity relative to benign nevi ($P=0.072$). These findings suggest that virtually all cells of melanocytic origin express TSHR, and that receptor expression is enhanced in malignant and pre-malignant lesions.

To expand the above findings, immunofluorescent staining for TSHR was performed on cultured human melanoma cells, primary human melanocytes, and primary human thyrocytes. To detect surface TSHR, the experiments were performed without a permeabilization step. Surface TSHR were easily detected on melanoma cells and proliferating melanocytes (Fig. 1e–g), and to a lesser degree on cultured primary thyrocytes (Fig. 1h). Immunofluorescent staining of thyrocytes for thyroglobulin confirmed the identity of this cell type (Fig. 1i).

Specificity of the TSHR antibody was confirmed by western blotting of protein extracts from TAD2 thyroid cells and two human melanoma cell lines, WM793 and MeWo. TSHR is composed of two subunits, A and B, joined by disulfide linkage. Fig. 2a shows recognition by the antibody of the holoreceptor (100 kDa) and the A subunit (weak bands around 62 kDa), as well as a doublet of unclear origin at 52 kDa, a pattern that has been previously described in numerous reports (Misrahi et al. 1994, Chazenbalk et al. 1996, Brokken et al. 2005). The appearance of TSHR on western blot was identical in the thyroid and the melanoma cells. As further confirmation, RT-PCR of RNA from thyroid tissue and melanoma cell lines was performed to detect TSHR transcripts. Using primers to amplify a 209 bp fragment of exon 10 cDNA, TSHR expression was demonstrated in the thyroid tissue and in both melanoma cell lines (Fig. 2b). The identity of the PCR product was confirmed by DNA sequencing (data not shown).

Melanoma TSHR are functional
We postulated that melanoma TSHR are functional and, when activated by TSH, initiate molecular events typical of those observed in thyrocytes. To test this hypothesis, TAD2 thyroid cells, melanoma cell lines WM793 and MeWo, and cultured primary melanocytes FMC7C, were treated with purified bovine TSH 10 mIU/L in serum-free, TSH-free RITH media (Methods) for up to 120 min, and examined for cAMP expression. TAD2 cells displayed the expected rapid response to TSH, with elevated cAMP levels observed at 5 min (Fig. 3a). For the melanoma cells and melanocytes, absolute concentration ranges of cAMP varied considerably for a given cell line from one experiment to the next. Regardless, over this time course, WM793 cells produced cAMP with a peak between 30 and 60 min (Fig. 3b). The findings with WM793 were reproducible with recombinant human TSH, ruling out the possibility of cAMP induction by other hormones that might be present as contaminants in the purified bovine preparation (data not shown). MeWo demonstrated a different pattern in the cAMP experiments, showing decreasing levels throughout the time course (Fig. 3c). Even when assayed at a TSH exposure of 5 min, MeWo cAMP concentrations were found to be already declining from baseline (Fig. 3d). Melanocyte cAMP levels were generally lower than those seen in melanoma cells and no particular pattern of response to TSH was observed (data not shown).
The declining cAMP levels in MeWo cells, without a clear initial spike, could be interpreted in two ways: either TSH suppresses cAMP in these cells, or the cAMP peak is too early and transient to be detected in the EIA assay. To address this issue, MeWo cells were examined for activation of the MAPK pathway in response to TSH, an event downstream of cAMP production. Cells were treated with RITH media containing TSH 10 mIU/l for 0, 5, 15, 30, and 60 min. Lysates were then examined for the presence of phosphorylated ERK (pERK) as an indication of MAPK pathway activation. An increase in pERK was

Figure 1 Expression of TSHR by cells of melanocytic origin. Intense immunoreactivity is demonstrated in a melanoma (a) and a dysplastic nevus (b). In contrast, a benign nevus (c) exhibits only faint immunostaining. An IgG control for the melanoma is also shown (d). Surface TSHR are detected on MeWo melanoma cells by immunofluorescence (e); IgG control is shown in (f). Melanocytes also express TSHR on their surface (g). Immunofluorescent staining for TSHR on cultured thyrocytes yields positive results (h), but staining is less intense than for melanoma cells; the fluorescence from the granules in the thyrocyte cytoplasm is non-specific. Thyrocytes stain with antibodies to thyroglobulin, confirming their identity (i). Staining of normal thyroid follicular cells (j and k) and normal skin (l) are included as controls. Magnification is 20× for IHC of melanocytic lesions and skin (a–d and l), 40× for immunofluorescence (e–i), and 100× (oil) for thyroid cells (j and k).
detected at 5 min; levels peaked at 15 min and were relatively stable at the 30 and 60 min time points (Fig. 4). Similar results were seen in identical experiments using WM793 cells. ERK phosphorylation was detected at the earliest time point, with the highest level of pERK appearing at 60 min, in keeping with the cAMP data for this cell line. These findings demonstrate that TSHR are indeed functional in both MeWo and WM793, although the dynamics of activation differ considerably between the two lines.

**TSH induces melanoma proliferation**

We next examined the ability TSH to promote the growth of melanoma cells. Melanoma cell lines WM793 and MeWo, as well as FMC7C melanocytes were plated on chamber slides in RITH media. TSH was added the following day at a concentration of 10 mIU/l. Proliferation was measured at 48 h by BrdU uptake. Both melanoma cell lines showed an increase in proliferation in response to TSH; in contrast, no change was noted for the melanocytes (Fig. 5).

**Melanoma TSHR are not mutated**

Activating mutations of TSHR are well documented in hyperfunctioning thyroid nodules (Porcellini et al. 1997). The majority of these mutations are located in exons 9 and 10, which code for the transmembrane and intracellular domains of the receptor. To determine if activating mutations contribute to TSHR activity in melanoma cells, possibly leading to autonomous receptor function, exons 9 and 10 were sequenced for five melanoma cell lines. No mutations were identified in either exon in any of these lines (data not shown).

**Discussion**

We have investigated the hypothesis that elevated levels of circulating TSH, a hallmark of primary hypothyroidism, promote the growth of human melanoma. We now report that all types of lesions of melanocytic origin, benign or malignant, express TSHR, and that receptor expression is increased in melanomas, potentially rendering them more sensitive to TSH. Furthermore, we demonstrate that cultured melanoma cells produce cAMP and activate the MAPK pathway in response to TSH, indicating that the receptor is functional. Finally, we show that TSH induces proliferation of melanoma cells, but not melanocytes, providing direct evidence for TSH as a melanoma growth factor. It is important to note that the concentration of TSH used in the cAMP, MAPK, and proliferation experiments was 10 mIU/l, a concentration typical of levels found in patients with early hypothyroidism. Therefore, these findings are highly relevant to the clinical population.

A number of interesting similarities exist between cells of melanocytic and thyrocytic origin. Similar pathways are used by both to induce growth, the MAPK pathway showing the greatest overlap. Identical V600E activating mutations of BRAF, a MAPK pathway component, are found in 60% of melanomas, 45% of papillary thyroid carcinomas, and 25% of anaplastic thyroid carcinomas (Goydos et al. 2005, Xing 2005). Ras, the signaling protein directly upstream of B-Raf, is also frequently mutated in both tumor types. Melanomas carry a 20% rate of NRAS mutations (Goydos et al.). Although RAS is mutated in thyroid carcinomas at a somewhat lower frequency, the findings of mutated

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Figure 2 Expression of TSHR by melanoma cell lines. (a) Immunoblotting of protein extracts from cultured WM793 and MeWo melanoma cells (60 μg) reveals the presence of TSHR. Lysates of TAD2 thyroid cells (40 μg) serve as a positive control. (b) TSHR transcripts are found in the same melanoma cell lines by RT-PCR. The (+) sign indicates that both RT and PCR were performed, whereas (−) indicates a control PCR without RT to document any contribution of genomic DNA to the final product. RNA from thyroid tissue serves as a positive control.
HRAS, KRAS, and NRAS are not uncommon in follicular carcinomas (Garcia-Rostan et al. 2003, Goydos et al. 2005). The presence of functional TSHR in both cell types, which we now report, adds to the list of biologically relevant similarities and raises the likelihood that additional similarities exist.

An important area of future research will be further characterization of the TSH-induced signaling pathways in melanoma cells. Our data show that MeWo and WM793 cells activate the MAPK pathway in response to TSH. In many melanoma cell lines, however, this pathway is constitutively active as a consequence of BRAF or NRAS mutations. MeWo is wild type for both genes and thus, increments in ERK phosphorylation are demonstrable. WM793 carries mutated BRAF, indicating some melanoma cells with

Figure 3  Induction of cAMP by TSH in melanoma cells. TSH induces cAMP formation in TAD2 cells by 5 min (a) and in WM793 melanoma cells by 60 min (b). Over the same time period, cAMP is found to decline from baseline (Bl) in MeWo cells (c). Even over a shorter time course, a cAMP peak is not detected for MeWo (d). Figures are representative of duplicate or triplicate experiments. *P=0.075 when compared with baseline; †P=0.038 when compared with baseline.

Figure 4  TSH activates the MAPK pathway in melanoma cells. Western blotting for phosphorylated ERK (pERK) reveals a peak increase at the 15 min time point for MeWo, while levels are still rising at 60 min for WM793. No change in total ERK (tERK) is observed. Data are representative of duplicate experiments.
mutations may also respond to TSH through enhanced MAPK activation. However, it is likely that melanoma cells with high basal mutation-driven MAPK activity alternatively respond through the PI3K and/or PKA pathways. Such findings in cell lines may ultimately be relevant to clinical tumors, i.e. the signaling response to TSH is influenced by the BRAF/NRAS mutational status of the tumor.

The clinical implications of melanoma TSHR are numerous. The development of thyroid failure during immunotherapy of melanoma is well described and is thought to be secondary to cross-reactive antigens (Krouse et al. 1995). In light of our data, melanoma TSHR becomes a reasonable candidate for the antigen of interest. If our finding that TSH promotes melanoma growth is confirmed, TSH screening should be included in the initial evaluation of every new melanoma patient and become part of their regular follow-up. Patients with TSH levels in the upper normal range should begin exogenous thyroid hormone replacement to suppress circulating TSH concentrations, and overt hypothyroidism should be managed aggressively to avoid intermittent TSH elevations. Recommendations should be made for regular skin examinations for hypothyroid individuals, particularly those with melanoma risk factors, and for aggressive management of suspicious cutaneous lesions. In terms of melanoma treatment, TSHR could prove to be an effective therapeutic target, providing the opportunity to complex drugs to TSH or to TSHR antibodies. Furthermore, TSH suppression with thyroid hormone may be an effective, inexpensive, and minimally toxic therapy for patients with TSH-sensitive tumors and advanced disease.

In conclusion, we have identified TSH as a novel growth factor for human melanoma. These findings open the entirely unexplored field of melanoma regulation by metabolic hormones, and provide the opportunity for numerous, unique interventions that may be effective in the management and prevention of this disease.

Acknowledgements

The authors would like to thank George Paraskevopoulos, Dr Jeffrey Myers, and Dr Adel El-Naggar for their efforts in accruing normal thyroid tissue; William Spohn and Dr Eugene T Walch for technical advice; Marcella Johnson for biostatistical consultation; and Dr Elizabeth Grimm for critical review of the manuscript. This work was partially supported by the following: NIH K22 CA097983 (JAE); a career development award from the UT MDACC SPORE in Melanoma, NIH P50 CA093459 (JAE); MDACC Institutional Research Grant (JAE); and NIH CA16672 (DNA Analysis Facility). The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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