Biological Effects of Thyrotropin Receptor Activation on Human Orbital Preadipocytes

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Purpose. Thyrotropin receptor (TSHR) expression is upregulated in the orbits of patients with Graves ophthalmopathy (GO), most of whom have TSHR-stimulating antibodies. The authors investigated the biological effects of TSHR activation in vitro in adipose tissue, the site of orbital TSHR expression.

Methods. Activating mutant TSHR (TSHR*) or wild-type (WT) was introduced into human orbital preadipocytes using retroviral vectors. Their proliferation (Coulter counting), basal cAMP accumulation (radioimmunoassay), and spontaneous and peroxisome proliferator-activated receptor (PPARγ)–induced adipogenesis (quantitative oil red O staining) were assessed and compared with those of nonmodified cells. QRT-PCR was used to measure transcripts of CCAT/enhancer binding protein (C/EBP)β, PPARγ, and lipoprotein lipase (LPL; early, intermediate, and late markers of adipogenesis) and for uncoupling protein (UCP)-1 (brown adipose tissue [BAT]).

Results. Expression of TSHR* significantly inhibited the proliferation of preadipocytes and produced an increase in unstimulated cAMP of 200% to 600%. Basal lipid levels were significantly increased in TSHR* (127%–275%) compared with nonmodified (100%) or WT-expressing (104%–187%) cells. This was accompanied by 2- to 10-fold increases in early-intermediate markers and UCP-1 transcripts (2- to 8-fold); LPL was at the limit of detection. In nonmodified cells, adipogenesis produced significant increases in transcripts of all markers, including LPL (approximately 30-fold). This was not the case in TSHR*-expressing cells, which also displayed 67% to 84% reductions in lipid levels.

Conclusions. TSHR activation stimulates early differentiation (favoring BAT formation?) but renders preadipocytes refractory to PPARγ-induced adipogenesis. In neither case did lipid-containing vacuoles accumulate, suggesting that terminal stages of differentiation were inhibited. (*Invest Ophthalmol Vis Sci.* 2006;47:5197–5203) DOI:10.1167/iovs.06-0596

Graves disease (GD) is caused by thyrotropin (TSH) receptor (TSHR)– stimulating antibodies (TSABs) that mimic the action of TSH and produce hyperthyroidism.[1] A proportion of GD patients have ophthalmopathy (GO), in which expansion of the orbital contents by adipogenesis, overproduction of extracellular matrix, and edema lead to proptosis.[2] The target autoantigen of GD, the TSHR, is expressed in the orbits of GO patients.[3,4] Specifically, it is upregulated during adipogenesis.[5,6] Recent improvements in patient management, especially in avoiding elevated TSH levels, have decreased the incidence of GO.[7] Furthermore, a positive correlation has been reported between TSAB titer and GO activity,[8] as assessed using a clinical activity score (CAS). These facts suggest that TSHR activation (TSHR*), either by excess hormones or TSABs, contribute to the disease process.

The receptor can also be activated by gain-of-function mutations, with somatic changes responsible for toxic adenoma and germline mutations causing familial hyperthyroidism.[1] Of interest, two newborns harboring different activating germline TSHR mutations, L629F and M453T, displayed proptosis.[9,10] We have exploited this activation mechanism to establish an in vitro model to investigate the resultant biological effects, beginning with adipose tissue, the site of TSHR expression in the orbit.

Methods

Sample Collection, Preadipocyte Culture, Generation of TSHR*-Expressing Cells

Orbital adipose tissue was obtained with informed consent and relevant local ethical approval. The research adhered to the tenets of the Declaration of Helsinki.

Six tissue samples were collected, three from GO patients (undergoing decompression surgery) and three from patients free of GO and thyroid autoimmunity (undergoing blepharoplasty with fat pad excision).

Preadipocytes were obtained from explants of the adipose tissues, cultured in DMEM/F12 10% FCS (complete medium). Adipogenesis was induced in confluent cells by replacement with medium having reduced FCS and containing a range of hormones and PPARγ agonists (differentiation medium), for 10 days, as previously described.[11]

Activating mutant TSHR, L629F and M453T, and wild-type (WT) were introduced using retroviral vectors previously produced in our laboratory.[12] Geneticin selection resulted in mixed populations stably expressing the various TSHRs, all as previously described.[12]

Effect on Proliferation and cAMP Levels

WT, TSHR*-expressing, and nonmodified preadipocytes were plated at 5×10^4/well in 12-well plates. They were counted with a Coulter particle counter 3 and 5 days after plating; the difference indicated proliferation in 48 hours. Results were expressed as total increased cell counts or as population doubling time (PDT).

The same plating conditions were used for cAMP estimates, but, once attached, the cells were incubated for 4 hours in medium containing 10^{-4} M isobutylmethylxanthine (IBMX) with no further addition (basal conditions) plus 10^{-5} M forskolin or 10 mU/mL bovine TSH. Cyclic AMP was extracted in 0.1 M HCl and measured using an in-house radioimmunoassay capable of detecting femtogram quantities of the second messenger, as previously described.[13] Coulter counting of

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TABLE 1. Forward and Reverse Primer Sequences, Exon Locations, and Amplicon Sizes for Genes Analyzed by QRT-PCR

		Forward		Reverse	
Gene	bp	Primer	Exon	Primer	Exon
C/EBPβ	304	AACTTTGGCACTGGGG	1	GGCCCGGCTGACAGTT	1
PPARγ	390	CAGTGGGGATGTCTCATA	3	CTTTTGGCATACTCTGTGAT	5
LPL	275	GAGATTTCTCTGTATGGACC	7	CTGCAAATGAGACACTTTCTC	9
UCP-1	540	CGGATGAAACTCTACAGCGG	2	CACTTTTGTACTGTCCTGGTGG	5
APRT	247	GCTGCGTGCTCATCCGAAAG	3	CTTTAAGCGAGGTCAGCTGC	5

adjacent wells provided an accurate cell number for results to be expressed as picomole cAMP per 10^4 cells.

Effect on Spontaneous and PPARγ-Induced Adipogenesis

Various cell populations (in 12-well plates) were examined in complete and differentiation medium. Microscopic examination provided a means of determining whether morphologic changes, e.g., rounding up of cells or acquisition of lipid-filled droplets, had occurred. In addition, the cells were subjected to oil red O staining, followed by extraction of the absorbed dye with 100% isopropanol and measurement of the OD_{490}. Coulter counting of adjacent wells provided an accurate cell number for standardization of OD values.

QRT-PCR of Transcripts for Early, Intermediate, and Late Adipogenesis and BAT

The various cell populations were plated in six-well plates in complete or differentiation medium. Ten days later, RNA was extracted and reverse transcribed, and transcript copy numbers for C/EBPβ, PPARγ, LPL, and UCP-1 were measured using Sybr green and a light cycler (MX3000; Stratagene, La Jolla, CA). Primer details are provided in Table 1.

Comparative analyses were performed with reference to the housekeeping gene *APRT*. Results are expressed as fold differences in transcript copy number calculated from the ratio of crossing threshold (Ct) value gene of interest: Ct value APRT.

Statistical Analysis

Means were compared nonparametrically by Kruskal-Wallis, post hoc by Dunn's multiple comparison test, and parametrically by ANOVA with post hoc Tukey HSD.

RESULTS

Adipose Explants Can Be Maintained in Culture for Long Periods

It was possible to harvest preadipocytes for up to 9 months, during which time the explants shrank. Preadipocytes close to the explant had oil red O–positive lipid-filled vacuoles (Fig. 1A) that persisted. If the explants were detached from the culture dish, lipid-filled vacuoles in the adherent preadipocytes decreased (Fig. 1B), whereas in dishes in which the explant was completely removed, all cells assumed a uniform fibroblast appearance (Fig. 1C). Cells from this last condition were used for subsequent experimentation.

TSHR Activation Increases Intracellular cAMP in Preadipocytes

In thyroid cells, the TSHR is coupled to Gsα, which activates adenylate cyclase and produces increased cAMP on ligand binding or in the absence of ligand for gain-of-function mutants such as M453T and L629F.[1] To investigate whether the TSHR is functional and similarly coupled in preadipocytes, we measured TSH-induced cAMP, whereas unstimulated cAMP levels would indicate whether the gain-of-function mutants were active. Forskolin provided a positive control and in all cases induced a robust increase in cAMP corresponding to at least a 10-fold increase compared with basal.

As shown in Figures 2A and 2B, the nonmodified cells responded minimally (twofold increase) to TSH, in keeping with the very low proportion of these cells expressing the receptor. In contrast, TSH induced a 4- to 16-fold increase in cells expressing the WT or mutant forms of TSHR, indicating that the receptor was coupled to Gsα in these cells. Furthermore, unstimulated (basal) cAMP was significantly ($P < 0.05$, GO; $P < 0.01$ non-GO) increased in the L629F- and M453T-expressing cells compared with the nonmodified or WT, indicating that the gain-of-function mutants were active in preadipocytes from normal orbits and patients with GO.

TSHR Activation Inhibits Preadipocyte Proliferation

The effects of TSHR activation on proliferation were studied. In all cases proliferation was significantly inhibited when comparing cells expressing the mutant TSHR and the nonmodified cells, and in some instances between the WT and nonmodified cells (representative examples are shown in Figs. 3A and 3B). Effects were always most apparent with mutant M453T. In two instances (1 GO, 1 non-GO) these cells proliferated so slowly that insufficient numbers of cells were obtained for experimentation.

GO and non-GO preadipocytes displayed inhibited proliferation that resulted in a significant increase in PDT (hours) from,

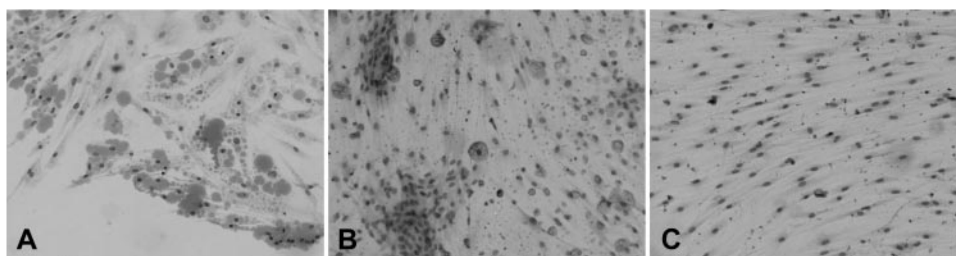

FIGURE 1. Lipid-filled vacuoles in preadipocytes close to adipose tissue explant (**A**), in cultures in which the explant was detached (**B**), and in cultures in which the explant had been removed from the dish (**C**).

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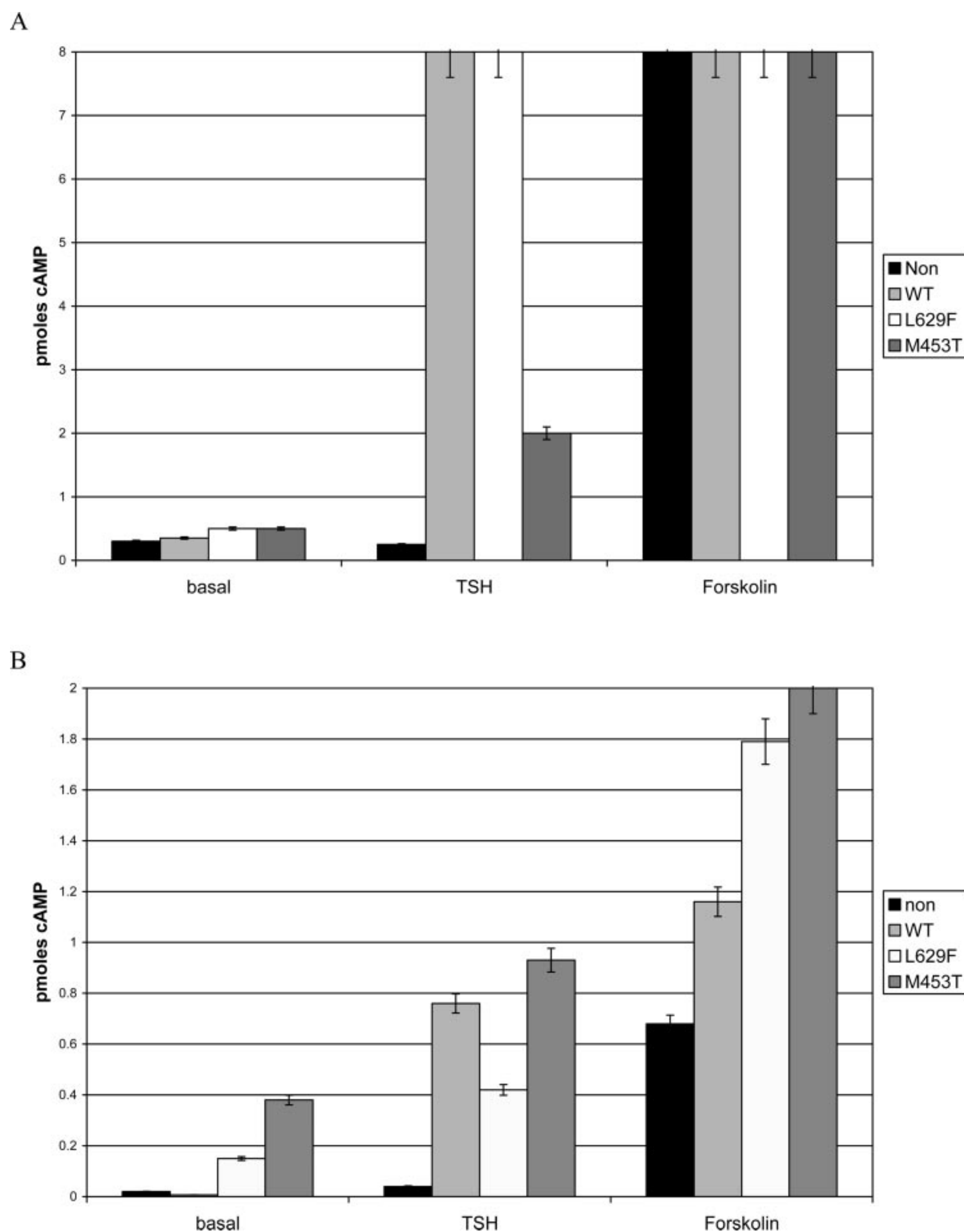

FIGURE 2. cAMP levels in nonmodified or WT/activating mutant TSHR (L629F, M453T) expressing preadipocytes from patients with GO (**A**) and non-GO (**B**) orbits. Results are expressed as picomoles cAMP per 10^4 cells measured in basal conditions or in response to 10 mU/mL TSH or 10^{-5} M forskolin.

for example, 38 ± 2.8 (nonmodified) to 43 ± 4 (WT), 86 ± 7.7 (L629F), and 160 ± 15.2 (M453T) in preadipocytes from a non-GO orbit.

TSHR Activation Induces Morphologic Changes in Preadipocytes

In complete medium, orbital preadipocytes expressing mutant TSHR had a more rounded appearance than their WT-expressing or nonmodified counterparts, but no obvious lipid vacuoles were observed, either microscopically or after oil red O staining. A representative example is shown in Figure 4.

TSHR Activation Increases the Lipid Content of Preadipocytes

Visual examination of the various preadipocyte populations cultured in complete medium revealed no obvious oil red O–staining droplets. However, when quantifying the lipid content, the mutant (and, to a lesser extent, WT) TSHR–expressing cells, from GO and non-GO orbits, displayed increased oil red O staining compared with nonmodified cells ($P < 0.05$, GO; $P < 0.01$, non-GO), as illustrated in representative examples in Figure 5.

To mimic more closely the situation in GD, the various preadipocyte populations were cultured in complete medium supplemented with T3 (1 nM). Because insulin is reported to be essential for adipogenesis, experiments were also performed in complete medium supplemented with insulin (500 nM) alone or combined with T3. Neither set of conditions resulted in the appearance of lipid vacuoles, nor was there any further increase in the lipid content of the cells, assessed using oil red O. A representative example is shown in Figure 6.

TSHR Activation Renders Preadipocytes Refractory to PPARγ-Induced Adipogenesis

The various preadipocyte populations were allowed to reach confluence and then were maintained in differentiation medium for 10 days. In the nonmodified cells, morphologic evidence of differentiation and visible oil red O–stained droplets

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Figure 3. Proliferation (increase in cell number in 48 hours) in nonmodified or WT/activating mutant (L629F, M453T) TSHR–expressing preadipocytes from patients with Graves ophthalmopathy (GO) (**A**) and non-GO orbits (**B**) cultured in complete medium.

were apparent in all microscopic fields examined and was accompanied by a significant increase in lipid content. The behavior of the preadipocytes transduced with the WT receptor was similar to the equivalent nonmodified population. In contrast, in cells expressing the most potent activating mutant, TSHR oil red O–stained vacuoles were absent, and there was little or no increase in the lipid content. A representative example (non-GO preadipocytes) is shown in Figure 6. Representative examples (GO preadipocytes) of the morphologic changes and oil red O–stained vacuoles are shown in Figure 7.

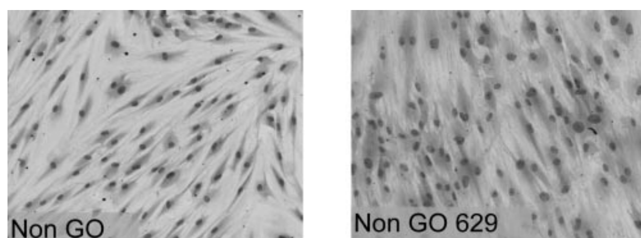

Figure 4. Nonmodified preadipocytes from a non-GO orbit appear as elongated fibroblasts. The same cells expressing mutant TSHR L629F have a more rounded appearance (non-GO 629).

TSHR Activation Induces Early and Intermediate Markers of Adipogenesis

Results obtained with oil red O staining of the mutant-expressing preadipocytes were paradoxical. Microscopic examination revealed no positive vacuoles in complete medium and reduced or no vacuoles in differentiation medium, yet the basal lipid content of the cells was higher but failed to increase in response to a PPARγ agonist. To investigate further, the transcript copy number of genes whose expression is upregulated during adipogenesis was calculated.

In nonmodified preadipocytes, PPARγ-induced adipogenesis (visible oil red O–positive lipid droplets) was accompanied by an increase in all the markers of differentiation tested

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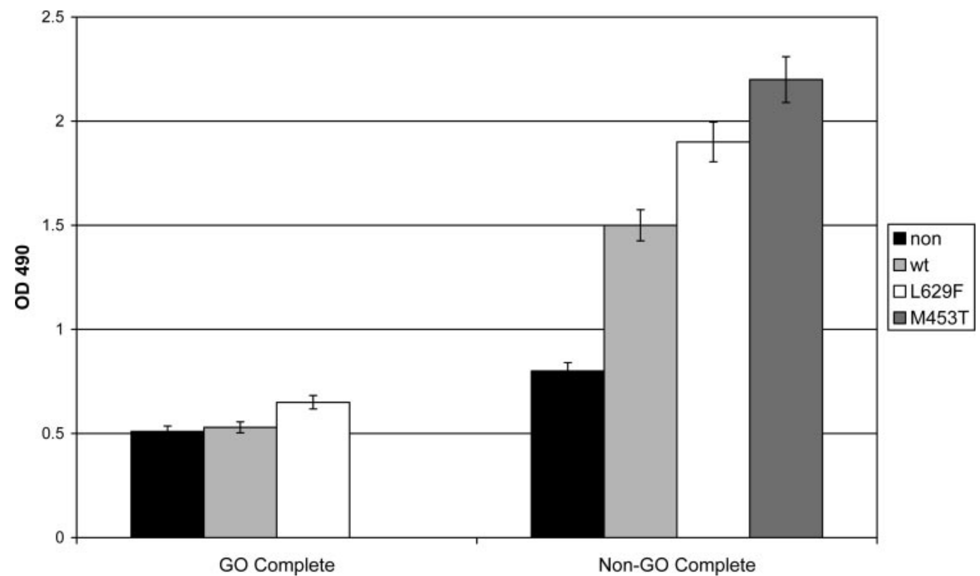

FIGURE 5. Lipid accumulation, expressed as OD_{490} of oil red O staining, in nonmodified or WT/activating mutant (L629F, M453T) TSHR–expressing preadipocytes from patients with GO and non-GO orbits cultured in complete medium.

compared with the equivalent transcript copy number obtained in these cells in complete medium.

In the TSHR*-expressing cells, no PPARγ-induced adipogenesis was apparent, and the transcript copy number of the differentiation markers was not increased. In fact, in some instances it was reduced compared with the same cells in complete medium.

When comparing the nonmodified cells and the TSHR*-expressing cells, all in complete medium, transcript copy numbers for markers of early to intermediate differentiation and brown adipose tissue formation, but not those for the terminal stages of adipogenesis, were upregulated, as summarized in Table 2. A representative example of the individual Ct values obtained is shown in Table 3.

DISCUSSION

Orbital fat is a scarce commodity, compounded by the loss of phenotype of preadipocytes that have undergone several passages. We modified explant cultures, transferred the adipose tissue fragments to new dishes for reattachment, and thus were able to harvest first-passage preadipocytes for several months.

A heterogeneous population of cells migrates from the explants, including fibroblast-like preadipocytes and cells at various stages of fat maturation. Cells containing lipid vacuoles could be fibroblast-like preadipocytes undergoing adipogenesis or cells at various stages of fat maturation in the process of de-differentiating. Gesta et al.[14] have indicated that de-differentiation occurs within the first 48 hours of culture. We observed cells close to the explants with lipid-filled droplets that persisted for several months. Only detachment or removal of the explant reduced the lipid droplets, indicating the production of pro-adipogenic signals, both soluble and through cell–cell contact, by mature fat.

Our subsequent experiments indicated that TSHR activation stimulates the early stages of adipogenesis but inhibits the terminal stages of differentiation. The absence of lipid-filled droplets, combined with increased cellular lipid content, suggests that lipolysis is upregulated or that the cytoskeletal changes necessary for droplet formation have been inhibited.

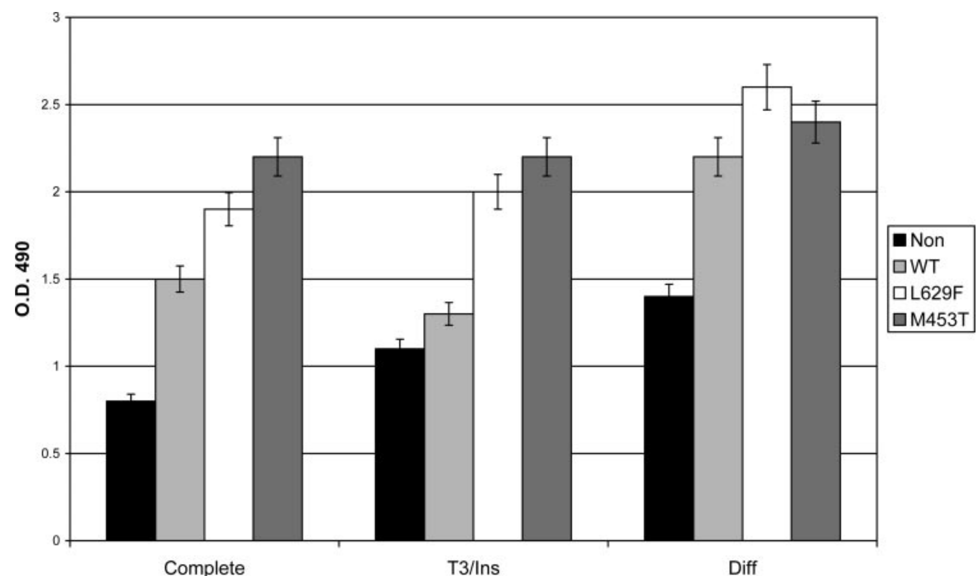

FIGURE 6. Lipid accumulation, expressed as OD_{490} of oil red O staining, in nonmodified or WT/activating mutant TSHR (L629F, M453T) expressing preadipocytes from a non-GO orbit cultured in complete medium alone or supplemented with T3 plus insulin (T3/Ins) or in differentiation medium (Diff).

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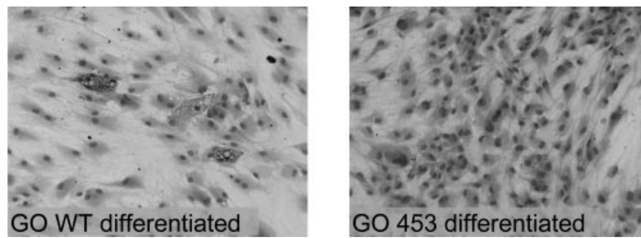

FIGURE 7. Oil red O staining in WT or activating mutant TSHR M453T expressing preadipocytes from a patient with GO in differentiation medium.

The fact that lipoprotein lipase transcripts remain at the limit of detection in cells expressing TSHR* favors the latter mechanism. The results agree with earlier reports of cAMP elevation rapidly reducing a suppressor of adipogenesis, Wnt,[15] thereby promoting differentiation. In contrast, prolonged elevation of cAMP by pharmacologic agents reduced the accumulation of lipids by decreasing key lipogenic enzymes rather than increasing lipolysis.[16]

TSHR activation also seems to favor the development of brown adipose tissue. Although elevated UCP-1 transcripts are a feature of human preadipocytes exposed to PPARγ agonists,[17] we observed an increase in UCP-1 copies in the TSHR*-expressing cells, even in complete medium (basal conditions). It is possible that this feature may be restricted to orbital preadipocytes, a depot having many similarities with brown adipose tissue such as selective expansion (along with intrascapular fat) in mice overexpressing adiponectin.[18]

We opted to use gain-of-function mutants of the TSHR as an activation mechanism rather than adding the pathologic ligands TSAB. At the time this study began, only patient sera containing heterogeneous mixes of TSAB were available because monoclonal TSABs have only recently been developed.[19–22] Furthermore, our previous experiences demonstrated that the vectors transduced a physiological level of TSHR expression because we were unable to distinguish endogenous rat TSHR from transduced human TSHR using flow cytometry in a rat thyroid cell line.[12] The model also has the advantage of generating free α and β/γ subunits of the G protein, unlike an earlier study[23] in which constitutively active Gsα mutants were expressed in the murine 3T3L1 cell line There was no effect on proliferation; basal cAMP or rates of adipogenesis compared with nonmodified cells were not examined.

Recent studies have reported that the activation of CREB (by phosphorylation) is necessary and sufficient to induce adipogenesis in the 3T3L1 preadipocyte cell line.[24] CREB can be activated by the cAMP/PKA route, though there are other activation mechanisms. In the FRTL5 thyroid cell line, we observed increased phosphorylated CREB (p-CREB) in cells expressing TSHR* compared with the WT or nonmodified cells.[25] Thus, we expected that the human preadipocytes expressing the same gain-of-function mutant TSHR would also have increased p-CREB and should thus have exhibited spontaneous adipogenesis. However preliminary experiments indicate that this is not the case, with threefold to fourfold higher levels of pCREB present in WT compared with activating mutant TSHR-expressing cells. This is in agreement with the work of Brunetti et al.,[26] who demonstrated increased adenylate cyclase but reduced pCREB in toxic adenoma (somatic TSHR*) compared with adjacent healthy thyroid tissue. We assume that the discrepancy between cAMP and pCREB is the consequence of upregulation of counterregulatory mechanisms—e.g., the inducible cAMP early repressor[27]—induced by the higher levels of cAMP present in the L629F- and M453T-transduced preadipocytes. The difference illustrates that though they provide useful in vitro models, rodent preadipocyte cell lines do not replicate all aspects of human adipogenesis.

Although we have investigated primary preadipocytes, our model does not faithfully reproduce TSHR expression during adipogenesis. Haraguchi et al.[28] demonstrated that the receptor is upregulated and reaches a maximum at around day 9 of an adipogenesis protocol, including insulin, dexamethasone, and a phosphodiesterase inhibitor. Transcript levels then declined. Our vectors are driven by a viral promoter so that receptor expression is permanently "ON. " We are generating inducible retroviral vectors and siRNA to permit the modulation of activating TSHR expression and to mimic more closely that occurring during adipogenesis.

Apart from this shortcoming, our model has addressed how TSHR activation might affect just one of the mechanisms operating to increase the orbital volume in GO. We are aware that not all GO patients have circulating TSABs and that other processes may be in operation. Furthermore, the TSHR is expressed on only a small proportion of orbital fibroblasts.

Increased cAMP in preadipocytes has been associated with the production of an adipogenic factor, a putative endogenous PPARγ agonist.[29] If this were the case, TSHR activation could have an indirect effect on adipogenesis by elaborating an adipogenic factor having paracrine effects. Experiments are under way using conditioned media from our transduced cells to investigate their proadipogenic activity on primary preadipocytes from various fat depots, including the orbit.

In conclusion, we have demonstrated that TSHR activation stimulates early differentiation and may favor the formation of brown adipose tissue. In contrast, TSHR activation renders preadipocytes refractory to PPARγ-induced adipogenesis. In neither case do lipid-containing vacuoles accumulate, suggesting that terminal stages of differentiation have been inhibited.

TABLE 2. Fold Increases

	Fold Change in TCN PPARγ vs. Basal Medium		Fold Change TCN Basal TSHR* vs. Nonmodified
	Nonmodified Cells	TSHR* Expressing	
C/EBPβ	1.5–5 × increase	2-3 × reduction	2-10 × increase
PPARγ	7–20 × increase	0–4 × increase	2–4 × increase
LPL	20–30 × increase	Limit of detection	Limit of detection
UCP-1	10–150 × increase	0–7 × increase	2–8 × increase

Nonmodified and WT-activating mutant TSHR (L629F, M453T)–expressing preadipocytes from GO and non-GO orbits cultured in complete medium or in differentiation medium. TCN, transcript copy number; C/EBPβ, CAACT enhancer-binding protein beta; PPARγ, peroxisome proliferator activated receptor gamma; LPL, lipoprotein lipase; UCP-1, uncoupling protein 1.

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Table 3. Crossing Threshold Values Obtained in Preadipocytes from a Donor Free of GO and Maintained in Complete Medium or in Differentiation Medium

	Complete Medium		PPARγ-Induced Adipogenesis	
Ct	APRT	PPARγ	APRT	PPARγ
Non-Go	26.5 ± 0.2	30.3 ± 0.6	25 ± 0.3	25.2 ± 0.5
WT	26 ± 0.1	29 ± 0.2	26.6 ± 0.7	29.1 ± 0.8
L629F	25.4 ± 0.3	27 ± 0.6	24.3 ± 0.4	26 ± 0.4
M453T	26 ± 0.5	28 ± 0.5	25 ± 0.2	26 ± 0.6

Values are expressed as mean ± SD. Each experiment was performed in triplicate (these agreed to within 10%), and all were repeated at least twice. Thus, crossing threshold (Ct) values quoted are the mean of at least six measurements.

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