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TSH stimulates adipogenesis in mouse embryonic stem cells

Min Lu[1] and **Reigh-Yi Lin**[1,2,3,4]

1*Department of Medicine, Mount Sinai School of Medicine, Box 1055, One Gustave L Levy Place, New York, New York 10029, USA*

2*Department of Molecular, Cell and Developmental Biology, Mount Sinai School of Medicine, New York, New York 10029, USA*

3*Department of Gene and Cell Medicine, Mount Sinai School of Medicine, New York, New York 10029, USA*

4*Mount Sinai School of Medicine, The Leon D Black Family Stem Cell Institute, New York, New York 10029, USA*

Abstract

Although TSH is the main regulator of thyroid growth and function, TSH binding activity in fat has long been reported. Since the TSH receptor (TSHR) has been detected in both preadipocytes and adipocytes, we hypothesized that it may play a role in adipose differentiation. Here, we use an *in vitro* model of adipogenesis from mouse embryonic stem (ES) cells to define TSH function. Directed differentiation of ES cells into the adipose lineage can be achieved over a 3-week period. Although adipocyte differentiation is initiated early in the development of cultured ES cells, TSHR up-regulation is precisely correlated with terminal differentiation of those adipocytes. The adipocytes express TSHR on the cell surface and respond to TSH with increased intracellular cAMP production, suggesting the activation of the protein kinase A signaling pathway. To determine whether TSH impacts adipogenesis, we examined how adipocytes responded to TSH at various points during their differentiation from cultured ES cells. We found that TSH greatly increases adipogenesis when added in the presence of adipogenic factors. More importantly, our data suggest that TSH also stimulates adipogenesis in cultured ES cells even in the absence of adipogenic factors. This finding provides the first evidence of TSH being a pro-adipogenic factor that converts ES cells into adipocytes. It further highlights the potential of ES cells as a model system for use in the study of TSH's role in the regulation of physiologically relevant adipose tissue.

Introduction

It is well known that overt hypothyroidism is associated with obesity (Hoogwerf & Nuttall 1984) and overt hyperthyroidism with weight loss (Baron 1956). Obesity caused by hypothyroidism can be distinguished from that resulting primarily from physical inactivity and a reduced resting metabolic rate by virtue of the accompanying dramatic elevations of thyroid-stimulating hormone (TSH) levels. Sub-clinical hypothyroidism, characterized by elevated TSH levels but normal thyroid hormone levels, is associated with dyslipidemia and an increased risk of ischemic heart disease and mortality (Danese *et al.* 2000, Hak *et al.* 2000, Visscher & Seidell 2001, Imaizumi *et al.* 2004, Moulin de Moraes *et al.* 2005). Previous studies have shown that, when adjusted for body mass index, leptin levels are positively correlated with TSH (Iacobellis *et al.* 2005). Likewise, Iacobellis reported that TSH and body mass index were

Correspondence should be addressed to R-Y Lin; Email: E-mail: reigh-yi.lin@mssm.edu.

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positively correlated in obese populations with normal thyroid function (Iacobellis *et al.* 2005). Together, these observations prompt us to speculate that TSH acts directly on the adipose tissue independent of its effect on thyroid function.

TSH binding activity in fat tissue has long been reported (Gorman *et al.* 1975, Mullin *et al.* 1976, Davies *et al.* 1978, Konishi *et al.* 1982). Since the TSH receptor (TSHR) has been detected in both preadipocytes and adipocytes (Trokoudes *et al.* 1979, Haraguchi *et al.* 1996*b*, 1999, Crisp *et al.* 1997), we hypothesize that it may play a role in adipose tissue differentiation. Most current strategies rely on primary cultures of adipose tissues or preadipocyte cell lines (Haraguchi *et al.* 1996*a*, Bell *et al.* 2002). Because preadipocytes differentiate from multipotent stem cells of mesodermal origin, this approach precludes the study of anything other than terminal differentiation. In contrast, we have developed a murine embryonic stem (ES) cell-based differentiation model to investigate whether TSH directly regulates differentiation of the earliest stages of adipose development. Murine ES cells are pluripotent stem cell lines isolated from the inner cell mass of 3·5-day blastocysts that can be propagated indefinitely in an undifferentiated state (Evans & Kaufman 1981, Martin 1981). When ES cells are induced to differentiation *in vitro*, they form three-dimensional cellular aggregates called embryoid bodies (EBs) that contain derivatives of the three embryonic germ layers and have the potential to differentiate into all cell types of an entire organism(Keller 1995). Using the ES/EB differentiation models, cells with hemangio-blast potential have been identified (Choi *et al.* 1998). ES cell-derived hematopoietic precursors (Keller *et al.* 1993, Keller 1995), neural precursors (Bain *et al.* 1995, Kawasaki *et al.* 2000), insulin-producing β cells (Lumelsky *et al.* 2001), and cardiomyocytes (Kehat *et al.* 2004) have been characterized and transplanted into recipient animals. The first observation of adipocyte-like cells derived from ES cells was reported by Field *et al.* (1992). Importantly, adipogenic gene expression profiles in differentiating ES cells suggest that they encompass the entire spectrum of adipocyte development *in vivo* (Phillips *et al.* 2003) and *in vitro* differentiation ofES cells provides an accessible model system to study adipose progenitor cells that rarely occur in existing primary and immortalized adipocyte cell lines.

Adipocyte differentiation of ES cells can be carried out through a four-stage strategy separated into permissive and terminal differentiation phases (Phillips *et al.* 2003). Commitment of ES cells to the adipogenic lineage during the permissive phase requires all-*trans* retinoic acid, the biologically active form of vitamin A (Phillips *et al.* 2003). Preadipocytes are then induced to differentiate into mature adipocytes during the terminal differentiation phase through treatment with the adipogenic factors insulin, 3,5,3′-triiodo-L-thyronine (T_3) and the peroxisome proliferation-activated receptor-γ (PPARγ) agonist rosiglitazone (Phillips *et al.* 2003). This protocol produces significant EB outgrowths with lipid droplet-containing adipose cells.

To determine whether TSH plays a critical role in adipogenesis, we investigated i) whether the expression and function of TSHR in ES cell-derived adipocytes correlates with cellular differentiation and ii) whether TSH can replace adipogenic factors and stimulate adipocyte differentiation. We found that TSHR is expressed in a time- and adipogenic-dependent manner in our ES cell differentiation model. In addition, our data suggest that TSH stimulates adipogenesis in cultured ES cells even in the absence of adipogenic factors. This finding provides the first evidence that TSH functions as a pro-adipogenic factor to convert ES cells into adipocytes. It further highlights the potential of cultured ES cells to contribute to the understanding of how TSH regulates physiologically relevant adipose tissue.

Materials and Methods

Growth and maintenance of ES cells

W9.5 ES cells were maintained on irradiated mouse embryonic feeder cells as previously described (Lin *et al*. 2003, Arufe *et al*. 2006) in Dulbecco's modified Eagle medium (DMEM, Gibco-BRL) supplemented with 15% fetal calf serum (FCS), penicillin–streptomycin (100 U/ml, Gibco-BRL), 10 ng/ml leukemia inhibitory factor (StemCell Technologies Inc., Vancouver, Canada), and $1{\cdot}5 \times 10^{-4}$ M monothioglycerol (Sigma). Cultures were maintained in a humidified chamber in a 5%CO_2/air mixture at 37 °C. ES cell cultures were monitored daily and the cells were passaged at 1:3 ratios every 2 days.

Differentiation of ES cell-derived EBs into adipocytes

To induce formation of EBs, ES cells were trypsinized into a single-cell suspension and plated at varying densities (10^3–8×10^4 cells/ml) in 60 mm Petri-grade dishes in EB differentiation medium containing Iscove's modified Dulbecco's medium supplemented with penicillin/streptomycin, 15% fetal bovine serum (FBS), 2 mM L-glutamine, 5% protein-free hybridoma medium (Gibco-BRL), 0·5 mM ascorbic acid (Sigma), transferrin (200 μg/ml, Boehringer Mannheim), and $1{\cdot}5\times10^{-4}$ M monothioglycerol for 2 days. Day 2 EBs were then harvested and transferred to new Petri-grade dishes containing DMEM supplemented with 15% FBS and 1 mM retinoic acid (Fisher Scientific, Pittsburg, PA, USA). Day 5 EBs cultured in the indicated conditions were replated on gelatin-coated six-well plates in DMEM with 15% Knock-Out Serum Replacement Media (SR, Gibco/BRL) supplemented with 0·5 mg/ml insulin (Sigma), 2 nM T_3 (Sigma), and 0·5 mM rosiglitazone (GlaxoSmithKline) for 15 days. In some experiments, TSH induction was carried out with 1 mU/ml human recombinant TSH (Fitzgerald Industries, Concord, MA, USA).

Gene expression analysis

Total RNA was isolated with an RNeasy kit (Qiagen) and treated with RNase-free DNase (Qiagen). Two micrograms of total RNA were reverse transcribed into cDNA using Thermoscript First-Strand Synthesis System (Invitrogen). PCR was performed using standard protocols with 2·5 U Platinum *Taq* polymerase (Invitrogen). Amplification conditions were as follows: initial denaturation at 94 °C for 2 min followed by 35–40 cycles of denaturation at 94 °C for 30 s, annealing at 50–61 °C for 45 s, extension at 72 °C for 45 s, and final extension at 72 °C for 7 min. In all cases, the annealing temperatures were set at 2 °C below the calculated denaturation temperature. The amount of cDNA in each sample was normalized using β-actin as a control. RNA controls were included to monitor genomic contamination. The amplified PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The identity of related PCR products was confirmed by direct sequencing. The primers used in this study were as follows:

Adipocyte lipid-binding protein (*ALBP*; forward) 5′-GATGCCTTTGTGGGAACCTGG-3′

ALBP (reverse) 5′-TTCATCGAATTCCACGCCCAG-3′

CCAAT/enhancer-binding protein (*C/EBP*α; forward) 5′-CGCAAGAGCCGAGATAAAGC-3′

*C/EBP*α (reverse) 5′-GCGGTCATTGTCACTGGTCA-3′

*PPAR*γ (forward) 5′-ATCATCTACACGATGCTGGAA-3′

*PPAR*γ (reverse) 5′-CTCCCTGGTCATGAATCCTTG-3′

TSHR(forward) 5′-GAGTGTGCGTCTCCACCCTGTGA-3′

TSHR (reverse) 5′-TTCCAGCCGCTGCAGAGTTGCAT-3′

Oct4 (forward) 5′-GGCGTTCTCTTTGGAAAGGTGTTC-3′

Oct4 (reverse) 5′-CTCGAACCACATCCTTCTCT-3′

β-*actin* (forward) 5′-ATGAAGATCCTGACCGAGCG-3′

β-*actin* (reverse) 5′-TACTTGCGCTCAGGAGGAGC-3′

Oil red O staining and lipid accumulation assay

Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were washed with PBS twice at room temperature. Cells were then stained with 0·5% oil red O (Sigma) for 30 min at room temperature. Next, cells were washed twice with H_2O for 15 min to remove the staining solution. Images were captured using a Nikon Eclipse TE2000-S microscope (Morrell Instrument Company Inc., Melville, NY, USA). To evaluate lipid accumulation during differentiation, stained lipids were extracted by 100% isopropyl alcohol for 5 min, and the optical density of the solution at 540 nm was measured.

Immunofluorescent microscopy

Cells were fixed in 4% paraformaldehyde in PBS. Immunofluorescent staining was carried out using standard protocols as described previously (Lin *et al.* 2003). In brief, fixed cells were pre-blocked with 3% BSA in PBS, followed by 1-h incubation with goat anti-mouse TSHR antibody (M-20; 1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature. For detection of primary antibody, the cells were washed and then incubated with Cy3 affinipure donkey anti-goat IgG (1:10 000; Santa Cruz Biotechnology Inc.) for 30 min at room temperature. The stained cells were washed before mounting with 10 μl Vectashield mounting media (Vector Laboratory, Burlingame, CA, USA). Images were captured using a Nikon Eclipse TE2000-S microscope.

Intracellular cAMP measurement

cAMP responses were measured with the Biotrak cAMP enzyme immunoassay system (Amersham Pharmacia Biotech), as previously described (Lin *et al.* 2003). Briefly, cells were seeded at 4×10^4 cells/well on flat-bottomed 96-well microtiter plates (tissue-culture grade) 1 day before the assay. The plates were incubated overnight in a 5% CO_2/air mixture at 37 °C. Cells were stimulated with TSH for 1 h at 37 °C. Next, 20 μl lysis buffer (2·5% dodecyltrimethylammonium bromide) was added to each well. Following the addition of lysis buffer, cells were incubated at room temperature for 10 min and agitated by vigorous, successive pipetting to facilitate cell lysis. Aliquots (100 μl) of lysed cells were transferred to the donkey anti-rabbit Ig coated plate for cAMP assay. Intracellular cAMP was measured with the Biotrak cAMP enzyme immunoassay system according to manufacturers' protocol (Amersham Pharmacia Biotech). A dose-dependent working standard curve (ranging from 12·5 to 3200 fmol) was generated according to manufacturers' instruction.

Statistical analysis

Numerical data are expressed as mean±S.E.M. An unpaired, two-tailed *t*-test was used for comparison. $P<0·05$ were considered significant.

Results

TSHR gene expression and function correlate with adipocyte differentiation in cultured ES cells

Although ES cells have been shown to differentiate spontaneously into adipocytes in culture, this occurs relatively rarely. It was necessary to optimize the culture conditions to enhance adipocyte differentiation before ES cells could serve as a useful experimental model. We used a four-stage, two-phase differentiation protocol to direct the differentiation of murine ES cells into adipocytes. We began with the expansion of undifferentiated ES cells in the presence of leukemia inhibitory factor (stage 1). When leukemia inhibitory factor is withdrawn, ES cells spontaneously differentiate into three-dimensional cellular aggregates or EBs in suspension (stage 2). Between 2 and 5 days later, the EBs are treated with all-*trans* retinoic acid. This stage (stage 3), known as the permissive phase, is a prerequisite for commitment of ES cells to the adipose lineage. The final stage (stage 4), known as the terminal differentiation phase, begins when the cells are exposed to the classic adipogenic factors: insulin, T_3, and rosiglitazone. This method allows the efficient production of mature adipocyte colonies from the center of EB outgrowths, which are easily identified by oil red O, a specific stain for triglycerides. Figure 1A shows photomicrographs depicting the four stages and two phases of adipocyte differentiation from ES cells.

In order to assess the function of TSH in adipogenesis, we first used semi-quantitative RT-PCR to examine the expression patterns of both TSHR and adipose lineage-associated genes at each stage of the differentiation process. We found that stage 1 ES cells and stage 2 EBs expressed the undifferentiated stem cell marker *Oct4* but not *TSHR* or any of the adipogenic genes (Fig. 1B). After 5 days of differentiation, stage 3 EBs expressed *C/EBPα*, *PPARγ*, and *TSHR*. *C/EBPα*, a member of the C/EBP family, is a pleiotropic transcription factor for adipocyte-specific genes. *PPARγ*, the main regulator of adipogenesis, is induced during differentiation and is responsible for activating a number of genes involved in fatty acid binding and storage. Because *C/EBPα* and *PPARγ* act synergistically to activate the transcription of genes that produce the adipocyte phenotype, these findings suggest that subpopulations of stage 3 EBs consist of adipocyte progenitor cells committed to the adipose lineage. Further analysis of cells from stage 4 EBs revealed that *C/EBPα PPARγ*, and *TSHR* gene expression is up-regulated during terminal differentiation (Fig. 1B). Stage 4 EBs also express *ALBP* (also named adipocyte fatty acid-binding protein, or *a-FABP*; Matarese & Bernlohr 1988,Baxa *et al.* 1989), a marker of terminal differentiation in adipogenesis (Fig. 1B). Our data indicate that adipocyte differentiation and the expression of *TSHR* are initiated as early as stage 3 in the development of cultured ES cells and that up-regulation of the *TSHR* gene correlates with the terminal differentiation of adipocytes.

To investigate the time course of lipid accumulation, we performed oil red O staining followed by extraction of stained lipids with isopropyl alcohol. The optical density (540 nm) of the solution was measured after 7, 10, 12, 16, and 20 days of differentiation (Fig. 1C). Oil red O as indicator of triglyceride accumulation increased significantly over the 20-day period, reaching 0·81 ± 0·01 (arbitrary unit) at day 10 ($P<0·005$), 0·89 ± 0·03 at day 12 ($P<0·005$), 1·04 ± 0·05 at day 16 ($P<0·005$), and 1·10 ± 0·06 at day 20 ($P<0·005$). Consistent with adipose gene expression profile (Fig. 1B), our results demonstrate a steady and time-dependent increase in extracted lipids in adipocytes during terminal differentiation.

TSHR protein is expressed and localized on the cell surface of adipocytes

We next used indirect immunofluorescence to determine whether TSHR protein is detectable in day 20 EB-derived mature adipocytes. We stained these cells with goat anti-mouse TSHR antibody followed by cy3 affinipure-labeled donkey anti-goat IgG and observed them with

fluorescent microscopy. As shown in Fig. 2, TSHR-positive cells were evident on the surface of the adipocytes that contained lipid droplets, but not in surrounding cells. No immunofluorescence was detected in a control experiment (Fig. 2). It should be noted that ES cell-derived mature adipocytes contain one large lipid droplet.

Adipogenic factors stimulate adipocyte differentiation and TSHR function

Treatment with adipogenic factors during stage 4 EB differentiation caused a profound increase in adipocyte differentiation (Fig. 3). To investigate how adipogenic factors regulate the expression of *TSHR* and genes related to adipocyte differentiation, we compared mRNA levels of adipocyte differentiation markers in cells treated with adipogenic factors with those in untreated cells. Semi-quantitative RT-PCR analysis revealed an intense increase in *ALBP* gene expression in cells treated with adipogenic factors (Fig. 4). Levels of *TSHR* gene expression in these cultures were also increased relative to control cultures (Fig. 4) but *C/EBP*α and *PPAR*γ gene expression levels did not differ significantly between treated and untreated cultures (Fig. 4). Next, we assessed the functionality of TSHR in these adipocytes. EB-derived adipocytes from cultures treated with or without adipogenic factors were challenged with TSH and assayed for intracellular cAMP production using the Biotrack cAMP immunoassay system as described in Materials and Methods. Our results show that cells cultured in the terminal differentiation phase without adipogenic factors had a higher intracellular cAMP content when challenged with TSH than did the non-TSH-challenged group (51·1 ± 2.0 vs 24·2 ± 0·7 fmol/cell×10^{-3}, P<0·0005; Fig. 5). Likewise, cells cultured with adipogenic factors during terminal differentiation achieved higher intracellular cAMP levels when challenged with TSH than did the non-TSH-challenged group (84·4 ± 3·4 vs 20·2 ± 2·0 fmol/cell×10^{-3}, P<0·0005). More importantly, the cells cultured with adipogenic factors showed nearly 1·5-fold higher intracellular cAMP content than cells cultured without adipogenic factors (P<0.005). This finding implies that adipogenic factors stimulate TSH-dependent cAMP production, perhaps due to more abundant TSHR expression in the adipocytes in these cultures. Our data also suggest that adenylyl cyclase is involved in the activation of TSH via the protein kinase A signaling pathway in mature adipocytes. Together, these results suggest that adipogenic factors play an important role in the conversion of ES cells to adipocytes. Furthermore, our data suggest that TSH signaling contributes to adipocyte differentiation via TSHR function.

Effect of TSH treatment on adipocyte differentiation in the presence of adipogenic factors

To investigate the role of TSH signaling in adipocyte differentiation, we added human recombinant TSH to ES cell cultures at various time points during stage 4 differentiation. After 20 days of differentiation, we measured lipid accumulation in these cultures and compared it with untreated cultures. Our results show that cultures treated with TSH at days 7, 10, 12, 16, and 18 of differentiation accumulated more lipids than did control cultures, indicating that TSH stimulates adipocyte differentiation at this stage (Fig. 6). We next assessed expression of *ALBP*, *TSHR*, and *PPAR*γ genes to determine whether TSH-induced changes in lipid accumulation were reflected in the mRNA levels of adipogenic and *TSHR* genes. We found that although *ALBP* and *TSHR* genes exhibited the greatest changes at day 12 (Fig. 6), no significant differences were observed for *PPAR*γ. These observations suggest that TSH is most able to stimulate adipocyte differentiation in the presence of adipogenic factors, and that TSH significantly increases the expression of *TSHR* and *ALBP* (P<0·05), but not *PPAR*γ in these cultures after 12 days of differentiation. Therefore, TSH stimulates adipocyte differentiation, resulting in enhanced adipogenesis and increased *ALBP* and *TSHR* gene expression.

Effect of TSH treatment on adipocyte differentiation in the absence of adipogenic factors

To investigate whether TSH can replace adipogenic factors in the conversion of ES cells into adipocytes, we added TSH to ES cell cultures at various time points during stage 4 EB

differentiation in the absence of adipogenic factors (Fig. 7). We found a significant increase in the lipid accumulation assay when TSH was added at day 12 ($P<0{\cdot}05$; Fig. 7). We also found that *ALBP*, *TSHR*, and *PPARγ* gene expression were enhanced when TSH was added at days 7, 10, and 12 of differentiation; however, these differences were not significant ($P>0{\cdot}05$; Fig. 7). Together, this finding suggests that TSH signaling either directly or indirectly impacts major pathways for adipocyte differentiation.

Discussion

In vitro differentiation ofES cells is a valuable model to study the commitment and differentiation of embryonic adipose cells. The technology to rapidly generate adipocytes has been described, but the use of *in vitro* differentiation of ES cells to analyze the physiological role ofTSH and TSHR in this process is just beginning. In the present study, we use RT-PCR analysis, oil red O staining, lipid accumulation assays, immunofluorescent staining, and intracellular cAMP assays to document the expression and function of TSHR in adipocytes derived from ES cells. The current approach relies on a murine ES cell line and a four-stage differentiation strategy that enables the efficient production of adipocytes in a reproducible and developmentally regulated manner. Although TSHR has not been reported in ES cell-derived adipocytes, we have previously reported that murine ES cells can differentiate into thyrocyte-like cells *in vitro* and that the expression of TSHR may be important in this differentiation (Lin *et al.* 2003, Arufe *et al.* 2006, Lin & Davies 2006). We now report that this ES cell-based system – in addition to producing cells in the thyroid lineage – can generate cells in the adipose lineage that express functional TSHR.

The experimental approach described here emphasizes the power of appropriate stimulatory factors in the generation of adipocytes from ES cells. We have shown that retinoic acid treatment followed by stimulation with insulin, T_3, and rosiglitazone generates many EB outgrowths containing adipocytes with fat droplets. RT-PCR analysis of the differentiating EB cells revealed the temporal appearance of mRNA transcripts for a number of adipocyte differentiation genes including *ALAP*, *C/EBPα*, and *PPARγ*. Importantly, we found that EB-derived preadipocyte differentiation is closely correlated with TSHR expression and function. This finding is consistent with previous studies of $3T_3$-L1 preadipocytes and rat preadipocyte primary cultures (Haraguchi *et al.* 1996*a*, Bell *et al.* 2002). Our immunofluorescent observations confirmed TSHR expression on the cell surface of mature, ES cell-derived adipocytes. Intracellular cAMP assays further revealed that this TSHR mediates a signal upon TSH stimulation. Our results indicate that TSH is important for late adipogenic differentiation. TSH treatment of differentiating ES cell-derived EBs increased adipogenesis either in the presence or absence of classic adipogenic factors. Together, these observations suggest a pro-adipogenic role for TSH in the conversion of ES cells into adipocytes.

TSH has been implicated in the induction of lipolysis (Vizek *et al.* 1979). It is known that TSH peaks within the first hours after birth at levels 50–100 times higher than that found in adults (Janson *et al.* 1998). *In vitro* studies have demonstrated that TSH can induce lipolysis in adipocytes from neonates, and that this lipolysis can be completely blocked by TSH antiserum and inhibitory TSHR antibodies (Marcus *et al.* 1988, Janson *et al.* 1998). The TSHR is a G-protein-coupled glycoprotein hormone receptor with a large extracellular domain fused to a seven-membrane-spanning segment (Nagayama *et al.* 1989). Since TSHR was first cloned in 1989, it has been believed to be responsible solely for the control of thyroid follicular cell growth and thyroid hormone synthesis (Nagayama *et al.* 1989, Laugwitz *et al.* 1996). However, it is becoming increasingly clear that expression of TSHR is not confined to the thyroid gland, but is widely expressed in a variety of tissues (Francis *et al.* 1991, Endo *et al.* 1993, Feliciello *et al.* 1993, Heufelder *et al.* 1993). TSHR protein expression has been reported in fibroblasts and adipose tissue from the retro-orbital space of Graves' patients, where it may play a role in

thyroid-associated ophthalmopathy (Bahn *et al.* 1998*a*,*b*, Starkey *et al.* 2003, Bahn 2004). A series of reports demonstrated that the orbital preadipocyte fibroblast is the primary target cell for immune response in the orbits of patients with Graves' ophthalmopathy (GO; Valyasevi *et al.* 1999, 2002, Starkey *et al.* 2003, Bahn 2004, Kumar *et al.* 2004). Although orbital preadipocyte fibroblasts do not express the TSHR, a significant increase in functional TSHR expression is evident in differentiating cells and mature adipocytes derived from GO patients (Valyasevi *et al.* 1999, Starkey *et al.* 2003, Kumar *et al.* 2004). Given that the expanded adipose tissue volume is attributable to *de novo* adipogenesis, these facts imply a possible link between adipogenesis and induction of the TSHR expression in orbital preadipocyte fibroblasts in GO. Recently, Zhang *et al.* (2006) reported that TSHR activation induces morphologic changes and lipid content in orbital preadipocytes derived from GO patients. TSHR activation also increases intracellular cAMP accumulation in preadipocytes. Their model suggests that TSHR activation stimulates the early stages of adipogenesis but inhibits the terminal stages of differentiation on human orbital preadipocytes *in vitro* (Zhang *et al.* 2006).

TSHR expression has also been detected in brown and white adipose tissues in the guinea pig (Roselli-Rehfuss *et al.* 1992). Moreover, Bell *et al.* (2000) have detected the expression of TSHR mRNA and protein in human preadipocytes from abdominal subcutaneous and omental tissues. Despite these observations, the specific role of TSHR in adipose biology remains to be elucidated. Haraguchi *et al.* (1996*a*) previously reported that preadipocyte differentiation in rats is accompanied by TSHR expression. Although cultured rat preadipocytes are a good model system for studying the physiological role of TSHR in adipose tissues, they have limitations. In particular, because rat preadipocytes are prepared directly from epididymal, perirenal, and subcutaneous fat from the inguinal regions of male SD rats (Haraguchi *et al.* 1996*a*), they are often contaminated with other cell types and can be difficult to maintain. In addition, both rat preadipocytes and the murine $3T_3$-L1 preadipocyte cell line, a well-established model of adipogenesis, consist of already committed adipose progenitor cells and permit study of terminal differentiation only (Bell *et al.* 2002). To better understand the mechanisms that control adipocyte differentiation, it is essential to generate an enriched population of proliferating adipose progenitor cells and identify genes and factors specifically involved in regulating adipocyte activity. *In vitro* differentiation of ES cells toward the adipogenic lineage could provide an alternative source of adipocytes for study and offers the possibility to study the regulation of the first steps of adipogenesis. This ES cell differentiation approach has significantly impacted on the field of basic research, in which the contributions of key molecules and signaling pathways to the adipogenic differentiation program can be investigated. Furthermore, the ability to genetically engineer stem cell may allow clinicians to test the effects of current drugs and to develop clinically relevant screening assays that would not otherwise be possible. ES cell differentiation model represents an important model system to study the development of adipocytes. The expression of TSHR during early preadipocyte differentiation in our ES cell-based differentiation could provide an ideal cellular model system to delineate the role of TSHR in adipose development.

Our results indicate that TSH can induce differentiation of murine ES cells to adipocytes in the absence of adipogenic factors at a high level. The *in vitro* concentration of TSH in our study is lower than what was reported in literature in mice (60 mU/ml; Abe *et al.* 2003). However, this concentration is much higher than those in humans (0·4–4·2 mU/dl). Although we suggest that this observation may be physiologically relevant, clearly, more studies are needed to elucidate these probabilities as well as to clarify the precise maturation stage of ES cell-derived adipocytes under the effect of TSH. Furthermore, we found that adipocyte differentiation along with *ALBP* and *TSHR* gene expression were greatly enhanced when cultures were treated with TSH at day 12. This finding indicates that TSH stimulates adipocyte differentiation and *TSHR* expression. As reported in literature, TSH in human fetal thyroid cells positively regulated the TSHR *in vitro* (Huber & Davies 1990). We have previously reported that TSH

was necessary to maintain the expression of the *TSHR* gene during EB differentiation into thyrocytes in culture (Lin *et al.* 2003). It is conceivable that a positive feedback cycle is present between TSH and TSHR in this ES cell differentiation model.

In order to further characterize the role of TSHR in adipocyte differentiation, we studied TSH signaling in ES cell-derived adipocytes. Bell *et al.* (2002) reported that TSH acts as a survival factor in $3T_3$-L1 preadipocytes; although it does not stimulate cAMP accumulation in these cells, TSH activates a PI3K-PKB/Akt-p70 S6 Kinase (S6K) signaling pathway. With respect to the present study, although it is clear that TSH stimulates cAMP accumulation in differentiated adipocytes, it is not clear whether TSH modulates preadipocyte survival and whether TSH signaling leads to p70 S6K activation. Further characterization of these ES cell-derived preadipocytes and adipocytes might help us understand the TSH signaling pathway and the mechanisms by which TSH regulates adipogenesis in these cells.

In summary, the present work demonstrates that TSHR is expressed in a time- and adipogenic-dependent manner in differentiating ES cells. Generation of adipocytes from ES cells can be achieved in 20 days using culture conditions previously established for the growth and differentiation of murine ES cells. Based on our studies, this murine ES cell-based system may be an ideal way to optimize adipocyte production and analyze TSH's function in adipose development. In addition, this murine ES cell-based system may allow the dissection of the fine controls of lipolysis, a complex metabolic process occurring during times of stress or nutrient deprivation.

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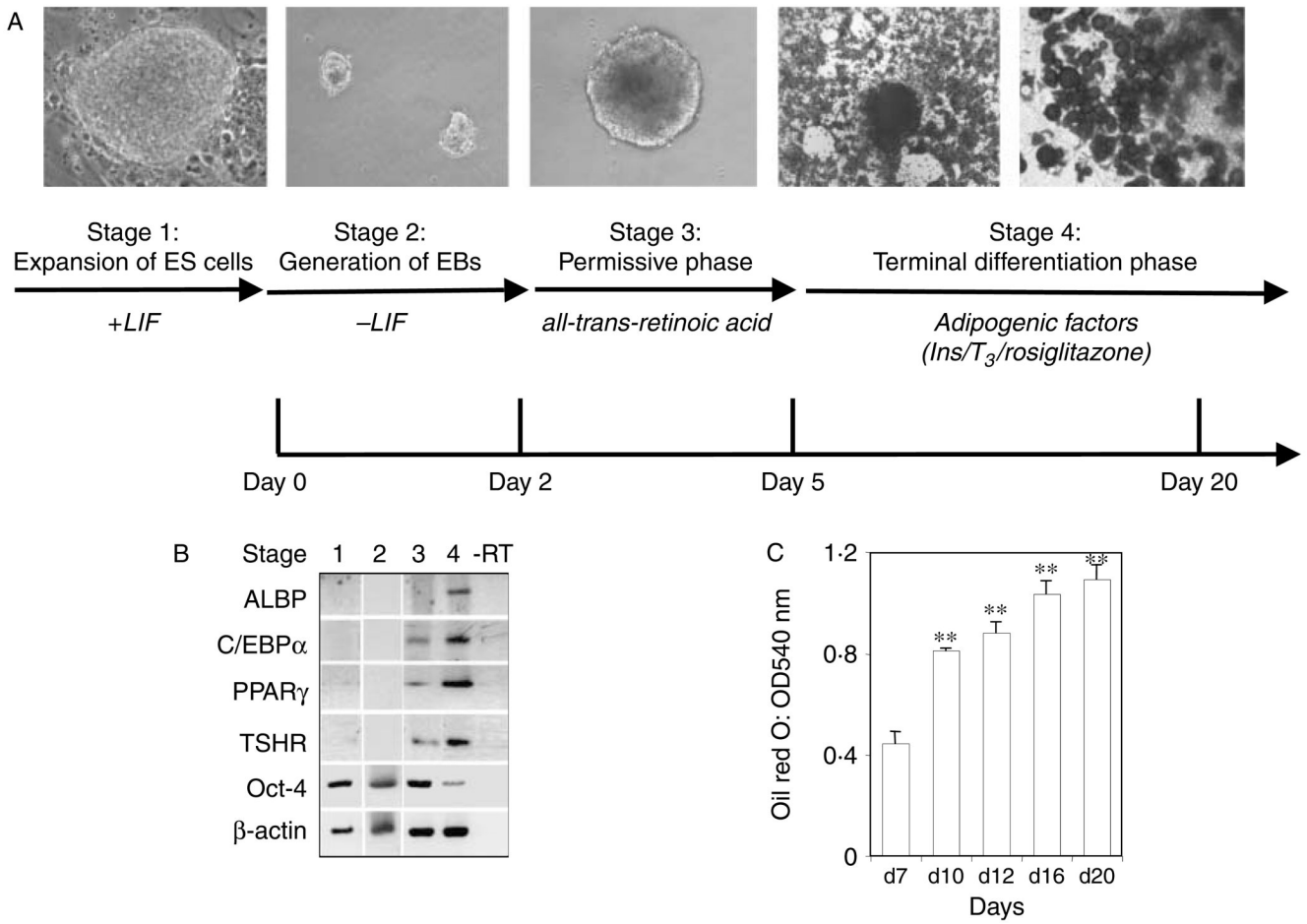

Figure 1.

In vitro differentiation of murine ES cells into the adipocyte lineage. (A) Schematic representation of the strategy for differentiation of murine ES cells into adipocytes. Photomicrographs depicting the four stages of ES cell differentiation: undifferentiated ES cells (stage 1), EBs in suspension (stage 2), EBs in the permissive phase (stage 3), and terminally differentiated adipocytes (stage 4). Note stage 4 adipocytes containing lipid droplets developed on an EB outgrowth, visualized by oil red O staining, and shown at two different magnifications (left, 40×, right, 200×). (B) Gene expression analysis by RT-PCR shows the differentiation of adipocytes from ES cells. RNA was isolated from undifferentiated ES cells (stage 1), and from the cells grown for 2 days (stage 2), 5 days (stage 3), and 20 days (stage 4), and analyzed for expression of *TSHR* and the adipocyte marker genes *ALBP*, *C/EBP*α, and *PPAR*γ. *Oct4* is an undifferentiated ES cell marker. β-*Actin* serves as an internal control. Control experiments contained no reverse transcriptase (−RT). (C) Time course of lipid accumulation. Oil red O-stained lipids were extracted with 100% isopropyl alcohol from individual cultures, and the absorbance at 540 nm was measured. Data are expressed as mean±S.E.M. Values are from three separate experiments each done in duplicate. ***P*<0.005 compared with d7.

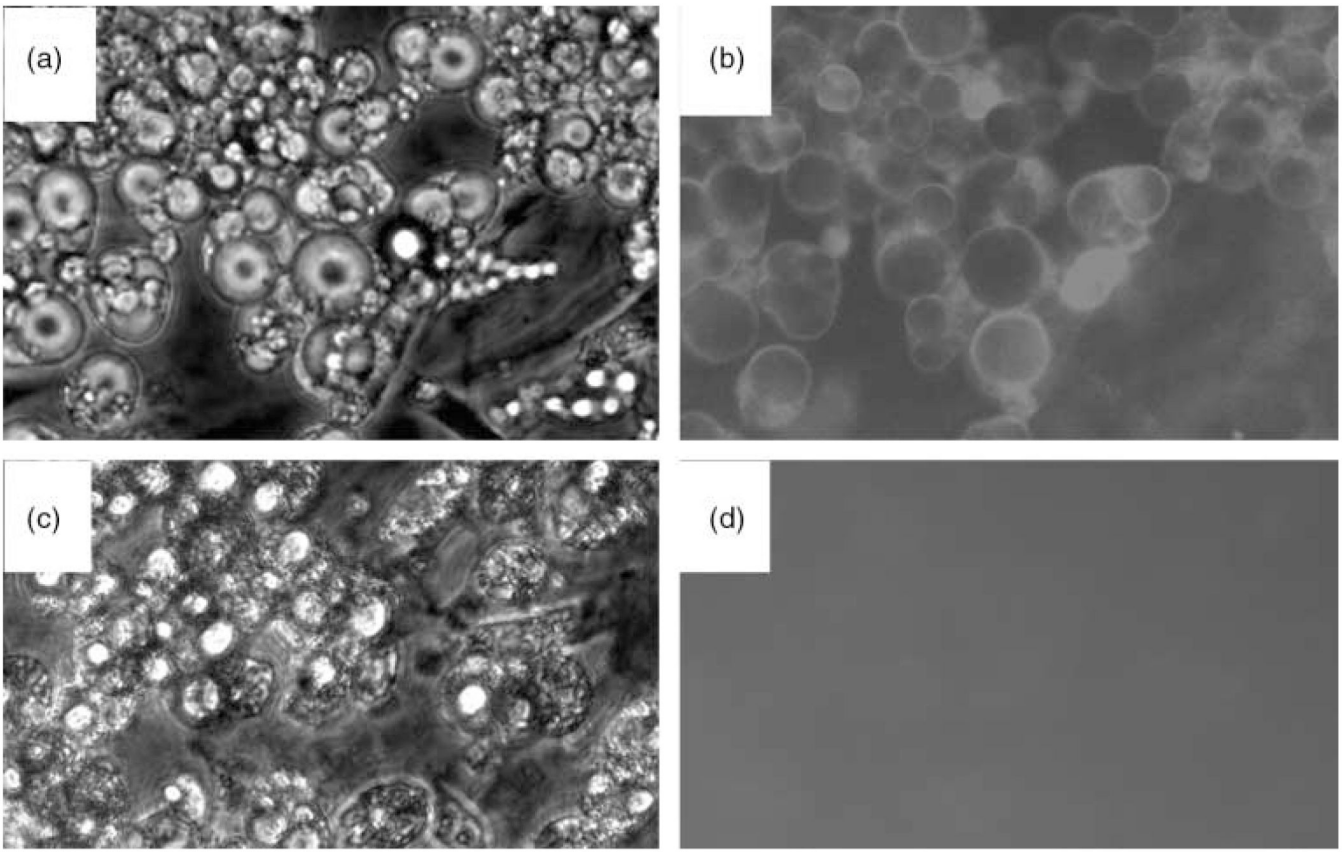

Figure 2.
TSHR is expressed on the cell surface of differentiated adipocytes. The presence of TSHR after 20 days of differentiation was detected by immunofluorescent staining with an antibody to TSHR (red). Note that TSHR is in adipocytes that contained lipid droplets, but not in surrounding cells (b). Immunofluorescence was not detected when the experiment was done with an isotype control (d). Note that *a* and *c* are phase contrast images. Experiments were carried out thrice independently, and each time the same results were obtained. Representative photomicrographs are shown. Magnification, ×200.

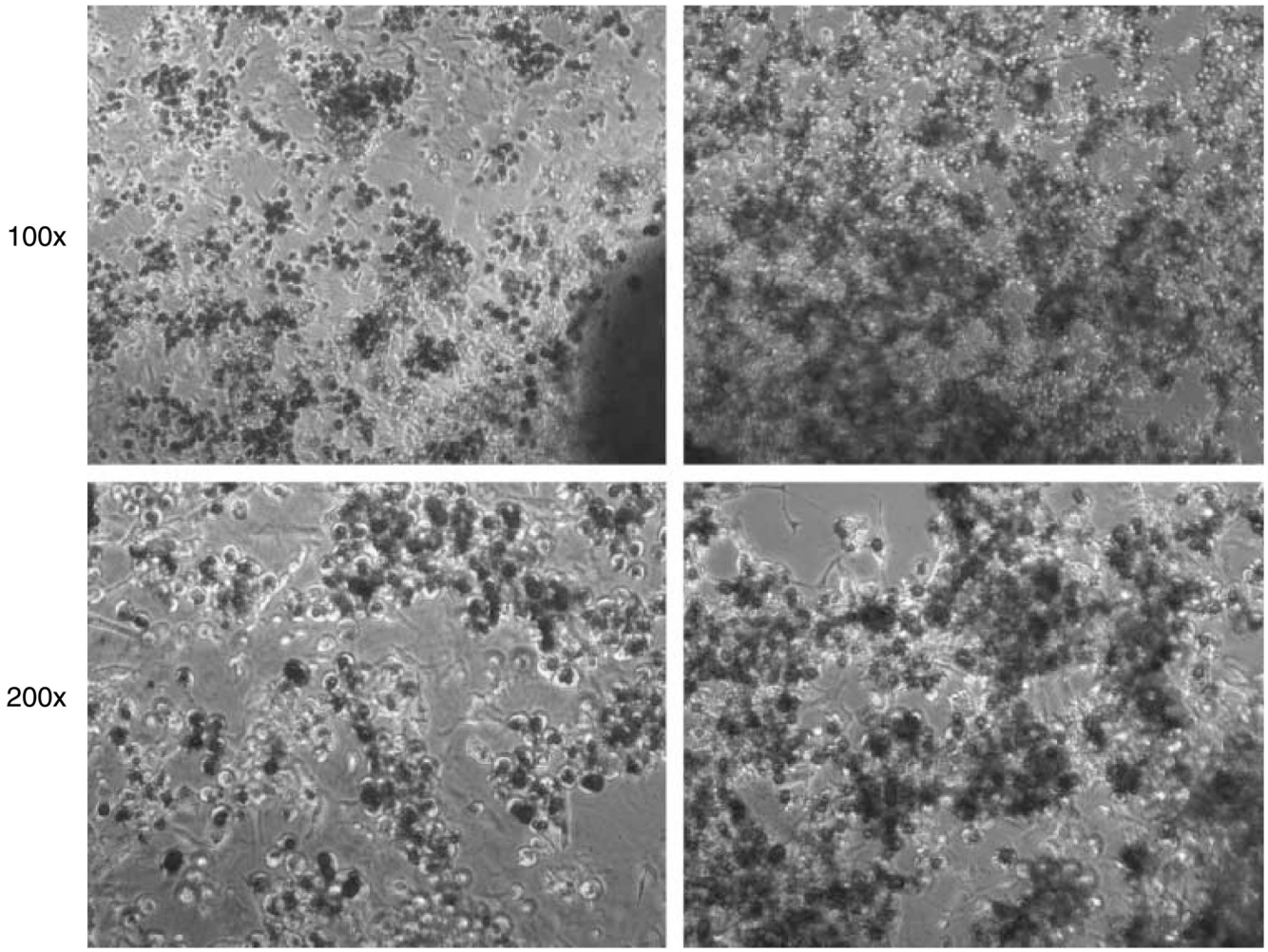

Figure 3.
Effects of adipogenic factors on adipocyte differentiation. Examination of oil red O-stained adipocytes cultured for 15 days in adipocyte differentiation medium containing insulin, T_3, and rosiglitazone (+) by phase contrast microscopy. The cultures not treated with adipogenic factors (−) are used as controls. Experiments were separately carried out at least thrice for each culture condition, and each time the same results were obtained.

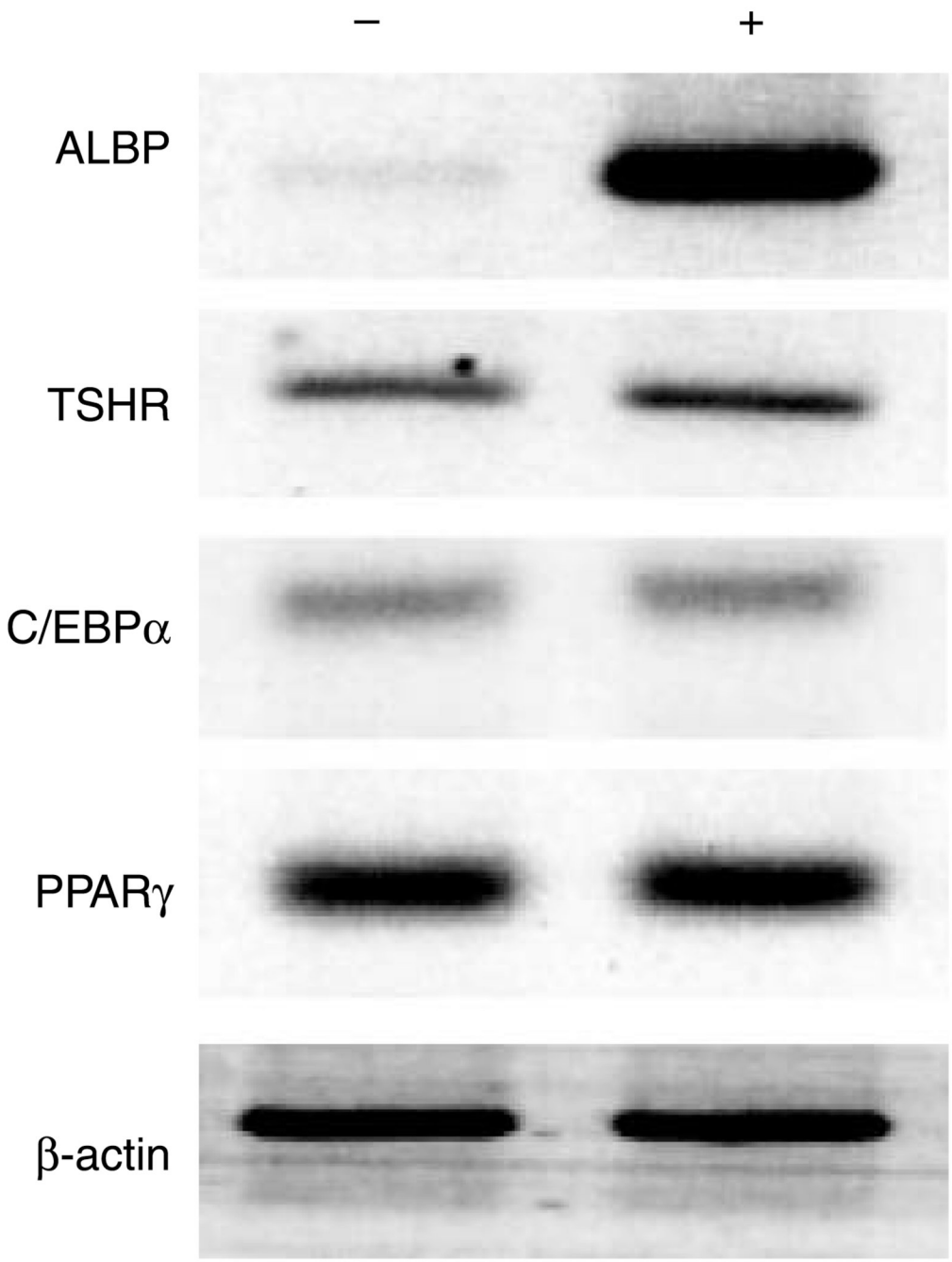

Figure 4.

Adipogenic factors stimulate *ALBP* and *TSHR* gene expression. After 20 days of differentiation, the expression of adipocyte markers (*ALBP*, *C/EBPα*, and *PPARγ*) and *TSHR* were analyzed by RT-PCR in cultures treated with adipogenic factors (+). Untreated cultures (−) serve as controls. Experiments were separately carried out at least thrice for each culture condition, and each time the same results were obtained.

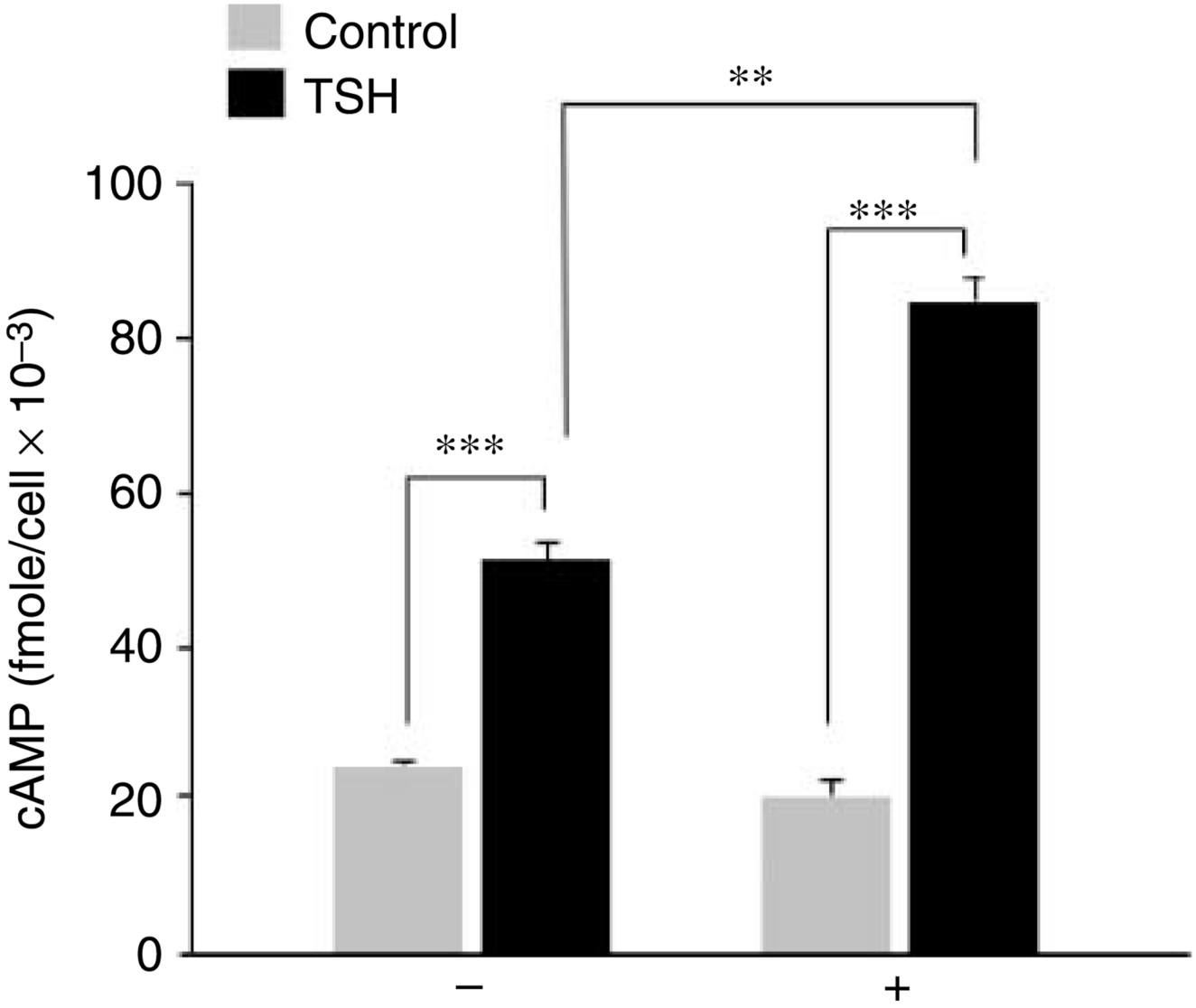

Figure 5.

cAMP response in TSH-stimulated differentiated adipocytes. After 20 days of differentiation, intracellular cAMP levels were measured in cell lysates from cultures treated with adipogenic factors (+) using the Biotrak cAMP immunoassay system as described in Materials and Methods. Untreated cultures (−) were used as controls. Data represent the mean±S.E.M. of three independent experiments, each performed in duplicate. ***P*<0·005, ****P*<0·0005.

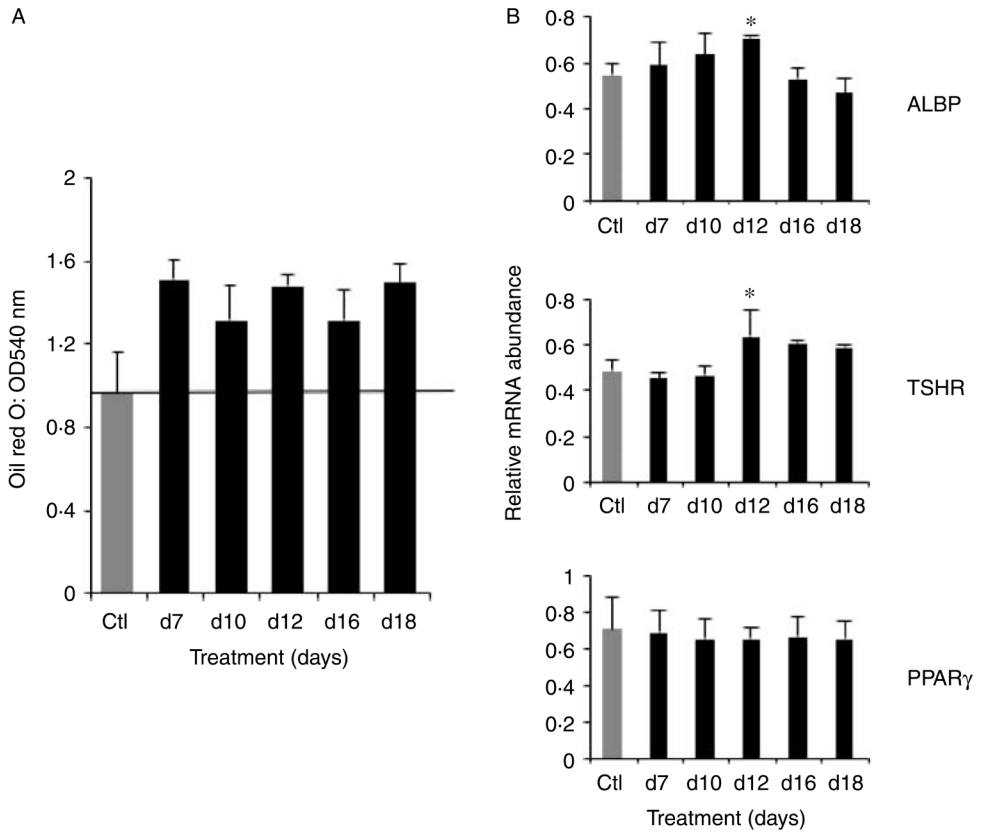

Figure 6.

Effect of TSH treatment on adipocyte differentiation in the presence of adipogenic factors. Cells in duplicate wells were exposed to 1 mU/ml human recombinant TSH in the presence of adipogenic factors at indicated time points and harvested after 20 total days of differentiation. (A) Stained lipids were extracted with isopropyl alcohol and the absorbance at 540 nm was measured. Data are expressed as mean±S.E.M. The values shown are the average from three separate experiments each done in duplicate. **P*<0·05. (B) RT-PCR was performed to detect the expression of *ALBP*, *TSHR*, and *PPARγ* genes. Numbers on the bottom of each figure indicate the day on which TSH was added. Relative gene expression was determined by normalization to that of β-actin control (*Y*-axis). Each experiment was performed with its own control, so to normalize between experiments, the fold increases were divided by the fold increase for each specific control. Error bars represent the standard deviation of triplicate experiments. **P*<0·05.

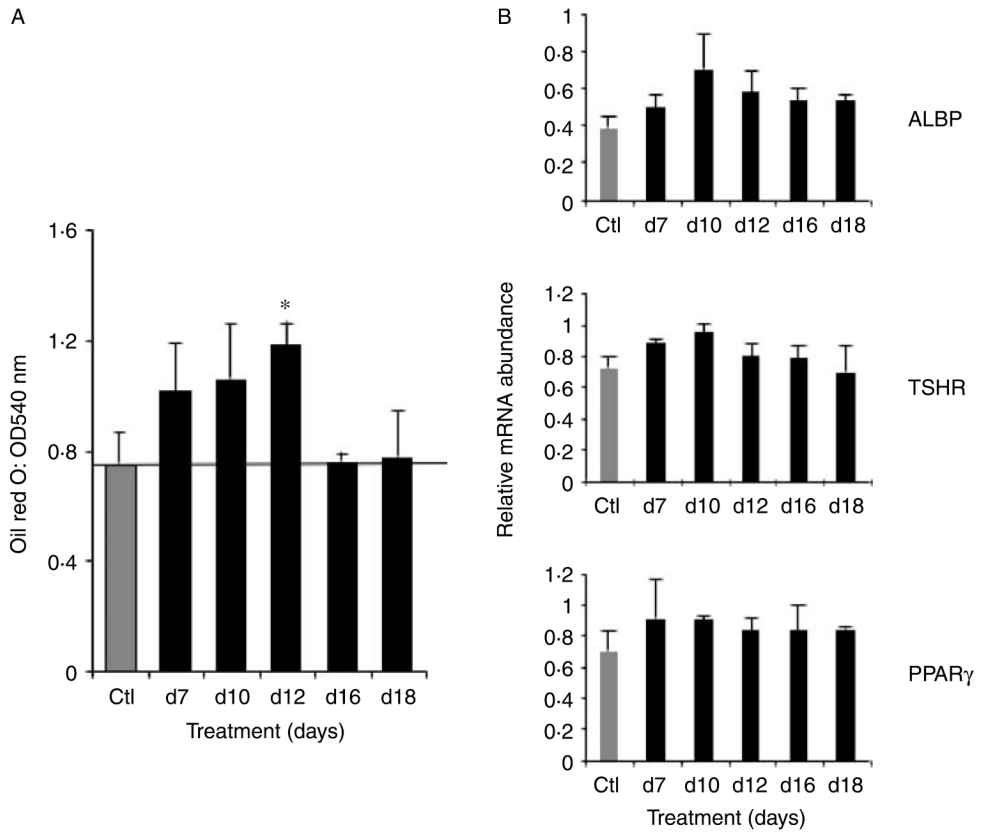

Figure 7.

Effect of TSH treatment on adipocyte differentiation in the absence of adipogenic factors. Cells in duplicate wells were exposed to 1 mU/ml human recombinant TSH in the absence of adipogenic factors at indicated time points and harvested after 20 total days of differentiation. (A) Lipid accumulation by extraction of stained lipid with isopropyl alcohol and the absorbance at 540 nm was measured. Data are expressed as mean±S.E.M. The values shown are the average from three separate experiments each done in duplicate. **P*<0·05. (B) RT-PCR was performed to detect the expression of *ALBP*, *TSHR*, and *PPARγ* genes. Numbers on the bottom of each figure indicate the day on which TSH was added. *Y*-axis indicates relative gene expression normalized with β-actin control. Each experiment was performed with its own control, so to normalize between experiments, the fold increases were divided by the fold increase for each specific control. Error bars represent the standard deviation of triplicate experiments. **P*<0·05.