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Differentiation of murine embryonic stem cells to thyrocytes requires insulin and insulin-like growth factor-1

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Abstract
The mechanisms controlling thyrocyte development during embryonic stem (ES) cell differentiation have only been partially elucidated, although previous studies have suggested the participation of thyroid stimulating hormone (TSH) in these processes. To further define the role of TSH in this context, we have studied a murine ES cell line in which green fluorescent protein (GFP) cDNA is targeted to the TSH receptor (TSHR) gene, linking the expression of GFP to the transcription of the endogenous *TSHR* gene. We demonstrate that, in the initial stages of embryoid body formation, activin A and TSH induce the differentiation of definitive endoderm and thyrocyte progenitors expressing *Sox17*, *Foxa2*, and *TSHR*. These thyrocyte progenitors are then converted into cellular aggregates that, in the presence of insulin and IGF-1, further differentiate into mature thyroglobulin-expressing thyrocytes. Our data suggest that, despite the fact that TSH is important for the induction and specification of thyrocytes from ES cells, insulin and IGF-1 are crucial for thyrocyte maturation. Our method provides a powerful *in vitro* differentiation model for studying the mechanisms of early thyrocyte lineage development.

Keywords
Embryonic stem cell; Thyrocyte; Thyroid stimulating hormone; Insulin; Insulin-like growth factor-1

Introduction

Embryonic stem (ES) cells, isolated from the inner cell mass of developing blastocysts, have the ability to proliferate indefinitely in culture and to generate all cell types in the body [1]. These properties make ES cells not only a novel and unlimited source of cells for the treatment of a broad spectrum of diseases, but also an ideal model of early embryonic development. Among the cell types that can be generated from ES cells, thyrocytes are of particular interest since they are the only cells in the body that can absorb iodine and convert it into the thyroid hormones required for metabolic homeostasis, growth and development. Thyroid organogenesis is a complex and highly regulated process controlled by various key transcription factors, growth factors and hormones. Elucidating the molecular mechanisms of thyroid development during ES cell differentiation could aid in the understanding of how biological processes malfunction and lead to human disease.

More than 70% of the thyroid gland is composed of thyrocytes arranged in follicles. The thyrocyte lineage is one of the least recognizable cell types in a culture of differentiating ES cells. Unlike cardiovascular lineage cells that exhibit characteristic spontaneous, rhythmic contractions, identification of the thyrocyte lineage by conventional differentiation screens is challenging. Our laboratory was the first to demonstrate that mouse CCE ES cells can differentiate into thyrocyte-like cells when cultured with thyroid stimulating hormone (TSH), the major regulator of the thyroid gland [2] and [3]. The effect of TSH on the thyroid gland is mediated by its interaction with a G protein-coupled glycoprotein receptor, TSHR, and activation of the cAMP-adenylate cyclase pathway. In previous studies, we analyzed thyrocyte progenitors in embryoid bodies (EBs) differentiated from ES cells in which the expression of green fluorescent protein (GFP) had been linked to the transcription of the endogenous *TSHR* gene. We demonstrated that these early EBs contained a subpopulation of TSHR-expressing cells that can differentiate into thyrocyte-like cells upon additional culture with TSH [4]. However, we were unable to detect the expression of thyroglobulin (Tg; a key terminal marker and the precursor of thyroid hormones) in these cells. These observations suggest that TSH alone is not sufficient to maintain the thyrocyte phenotype over time and imply that other factors must control the maturation of thyrocytes in ES cell differentiation cultures.

Thyrocytes are derived from the definitive endodermal germ layer, and recent work by Kubo et al. and others demonstrated that a brief challenge of activin A, a member of the TGFβ family, during early differentiation can enhance the formation of definitive endoderm in mouse and human ES cells [5], [6], [7] and [8]. In this report, we describe a reliable and simple way to use activin A, TSH, and other maturation factors in serum-free medium to induce mouse ES cells to differentiate into mature Tg-expressing thyrocytes. We provide evidence that, despite the fact that TSH is important for the induction and specification of thyrocytes from ES cells, insulin and insulin-like growth factor-1 (IGF-1) are crucial for thyrocyte maturation.

Materials and methods

Growth and maintenance of ES cell culture. The development of the *TSHR*$^{+/-}$ ES cell line has been reported [4] and [9]. The undifferentiated *TSHR*$^{+/-}$ ES cell line was maintained on an irradiated mouse embryonic fibroblast feeder layer in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 15% fetal calf serum (FCS), penicillin–streptomycin (100 U/ml, Invitrogen), 10 ng/ml leukemia inhibitory factor (LIF, StemCell Technologies, Vancouver, Canada), and 1.5×10^{-4} M monothioglycerol (MTG, Sigma–Aldrich, St. Louis). Cultures were maintained in a humidified chamber in a 5% CO_2/air mixture at 37 °C.

In vitro differentiation of thyrocytes from ES cells. Differentiation was carried out as shown in Fig. 1. To induce the formation of EBs, ES cells were trypsinized into a single-cell suspension and plated at varying densities (10^3–10^5 cells/ml) in 60-mm Petri-grade dishes in EB differentiation medium (EBDM) containing Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with penicillin/streptomycin, 15% FCS, 2 mM l-glutamine, 5% protein-free hybridoma medium (Invitrogen), 0.5 mM ascorbic acid and 1.5×10^{-4} M MTG. For endoderm differentiation, the EBs were harvested at day 2 of differentiation and cultured in IMDM supplemented with 15% KnockOut serum replacement medium (KSR, Invitrogen), penicillin/streptomycin, 0.5 mM ascorbic acid, 1.5×10^{-4} M MTG and 10 ng human recombinant activin A (R&D Systems, Inc., Minneapolis) [4]. The commercially available KSR medium is a serum-free formulation optimized for mouse ES cells. In some experiments, activin A-induced EBs were grown in the presence of different concentrations of human recombinant TSH (*h*TSH, 1–100 μU/ml; Fitzgerald Industries International, Inc., Concord, MA). For thyrocyte maturation, day 7 EBs were harvested and dissociated to single cells and then re-plated in IMDM with 15% KSR and 100 μU/ml *h*TSH in five different culture conditions for another 9 days: insulin (condition I); IGF-1 (condition II); insulin and IGF-1 (condition III); 6H (six hormones: hydrocortisone, transferrin, glycyl-l-histi-l-lysine acetate, somatostatin, TSH and insulin; condition IV); no treatment (condition V). Cells from re-plated cultures were harvested on days 11 and 16 for RT-PCR analysis and immunostaining.

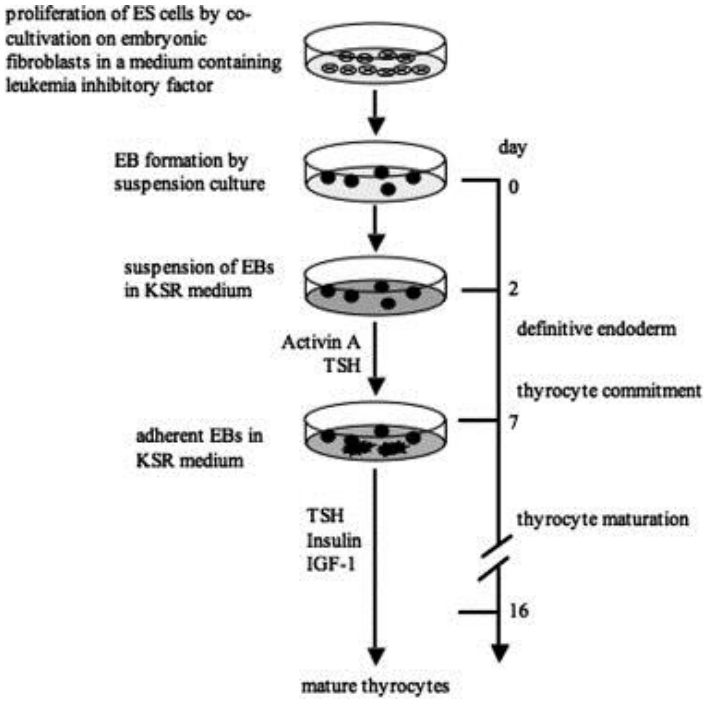

Fig. 1. The protocol for the generation of thyrocytes from ES cells. Undifferentiated mouse ES cells were cultured on irradiated mouse embryonic fibroblasts in IMDM containing LIF. To induce EB formation, ES cells were trypsinized into a single-cell suspension and were differentiated in EBDM for 2 days to form cellular aggregates. The differentiated cells were then further induced with 10 ng/ml activin A and TSH for 4 days in KSR medium. The EB-derived cells were further cultured for 5–10 days in the maturation medium containing TSH, insulin, and IGF-1. *Abbreviation:* LIF, leukemia inhibitory factor; TSH, thyroid stimulating hormone; IGF-1, insulin-growth factor-1; EBDM, embryoid body differentiation medium; KSR, knockout serum replacement medium.

Gene expression analysis. For gene expression analysis, total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) and was treated with ribonuclease-free deoxyribonuclease (Qiagen). Two micrograms of total RNA were reverse transcribed into cDNA using the Thermoscript First Strand Synthesis (Invitrogen). PCR was performed using standard protocols with 2.5 U platinum *Taq* polymerase (Invitrogen). The primers used in this study have been reported previously [2] and [4].

Flow cytometry. The cells were trypsinized and fixed with 2% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBS. The resulting cells were incubated with rabbit anti-rat NIS (1:100) antibody (a gift from Dr. Nancy Carrasco, Albert Einstein College of Medicine, Bronx, NY), rabbit anti-human Tg (1:4000; DakoCytomation, Carpinteria, CA), or isotype-matched control antibody at room temperature for 30 min; washed with PBS; and stained with Alexa Fluro 594 chicken anti-rabbit IgG (1:2000; Molecular Probes, Eugene, OR) at room temperature for 30 min. The cells were then washed and re-suspended in PBS. Flow cytometric analysis was performed on a FACSCalibur flow cytometer (BD Biosciences). FACS data were generated using FlowJo software (FlowJo LLC, Ashland, OR).

Immunostaining. Cells were fixed in 4% paraformaldehyde in PBS. After fixation, the cells were washed and permeabilized in PBS containing 0.1% Triton X-100 for 10 min, then pre-blocked with 3% BSA or 5% normal rabbit serum for 1 h. The following primary antibodies were used: rabbit anti-rat NIS (1:100) and rabbit anti-human Tg (1:4000). For detection of primary antibodies, the cells were washed, and then incubated with Alexa Fluro 594 chicken anti-rabbit IgG (1:2000) for 30 min at room temperature. The stained cells were washed before mounting with 10 μl Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Images were captured using an Axioskop fluorescent microscope (Carl Zeiss, Inc., Thornwood, NJ) at the Microscopy Shared Research Facility at Mount Sinai School of Medicine, New York, using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA).

Statistical analysis. Numerical data are expressed as mean ± SEM. An unpaired, two-tailed *t*-test was used for comparison. $P < 0.05$ was considered significant.

Results

Derivation of thyrocyte progenitors from mouse ES cells by treatment with activin A and TSH

To evaluate the thyrocyte differentiation potential of activin A-treated cell populations, undifferentiated *TSHR*$^{+/-}$ ES cells were cultured in suspension for 2 days with serum to induce EB differentiation. EBs were then transferred to serum-free cultures containing activin A for an additional 4 days. RT-PCR analyses revealed that 10 ng/ml of activin A increased the expression of endoderm-specific genes such as *Sox17* and *Foxa2*. *Foxa2* expression was initiated 4 days after the onset of differentiation, and its expression in concert with the initiation of *Sox17* on day 4 is consistent with endoderm induction and specification (Fig. 2A). As a comparison, we also examined differentiation during EB development by determining the expression of a mesoderm-specific gene, *brachyury*, a T-box domain-containing transcription factor. *Brachyury* expression was initiated 5 days after the onset of differentiation (Fig. 2A), consistent with a previous report [5]. Molecular analysis of the stem cell marker *Rex1*, a zinc finger transcription factor found in ES cells but not in their differentiated progeny [10], showed that *Rex1* was expressed between days 2 and 3 of differentiation and that its expression declined by day 6, also consistent with previous findings [10].

To determine whether thyrocyte progenitors were present in the differentiating cultures, we examined the temporal pattern of *TSHR* gene expression by RT-PCR. *TSHR* expression was initiated 4 days after the onset of differentiation, and was subsequently down-regulated to almost undetectable levels by day 6 (Fig. 2A). Next, we used fluorescence-activated cell sorting (FACS) analysis to analyze the temporal patterns of GFP expression after *in vitro* differentiation of *TSHR* ES cells to explore whether GFP levels could be used to monitor *TSHR* expression. GFP expression was initiated 4 days after the onset of differentiation, and the population of cells expressing GFP was almost undetectable on day 6, consistent with the expression of *TSHR* gene. This suggests that GFP expression faithfully mimics endogenous *TSHR* expression in differentiating EBs, and confirmed that *TSHR* expression can be measured in this ES cell line by monitoring GFP expression with FACS analysis. These findings also indicate that, at the dose used, activin A can stimulate *TSHR* expression in EBs in the absence of serum. However, the reduction in the number of GFP-expressing cells in day 6 EBs suggests that potential of these cells to become thyrocytes is transient.

We have previously reported that TSH is necessary to maintain *TSHR* gene expression during EB differentiation [2]. Given that the *TSHR* gene is transiently expressed in early EBs, we investigated the effects of TSH on the temporal expression of *Foxa2*, *Sox17*, and *brachyury* in activin A-induced endoderm and thyrocyte lineages in this ES cell model. As shown in Fig. 2C, the expression of these genes was not significantly affected by treatment with 1–100 μU/ml of TSH. The expression of *TSHR* and GFP genes was, however, extended from day 5 into day 6 EBs—a finding that was not seen with activin A treatment alone (Fig. 2A and B). These data support the notion that TSH, directly or indirectly, stimulates a program of thyrocyte differentiation that is not induced at lower concentration of activin A. To further explore this observation, day 7 EBs were re-plated in a serum-free culture with TSH for an additional 10 days. Cells from these cultures were then stained with specific antibodies to two terminal thyrocyte differentiation markers, NIS and Tg, and analyzed *via* FACS. As shown in Fig. 2D, the number of NIS$^+$ cells increased in EBs stimulated with TSH (26.5% ± 0.2% vs 16.6% ± 1.4%, TSH vs control, respectively) after 10 days of differentiation. In contrast, the numbers of Tg$^+$ (4.8% ± 0.9% vs 3.9% ± 0.2%; TSH vs control) or GFP$^+$ (1.3% ± 0.1% vs 1.4% ± 0.3%, TSH vs control) cells were not significantly different. Moreover, NIS$^+$, Tg$^+$ or GFP$^+$ cells were either undetectable or extremely rare in the cultures after 12 total days of differentiation. Together these observations suggest that although activin A and TSH can stimulate thyrocyte induction and specification, they are not sufficient to maintain the thyrocyte phenotype in the long run. We therefore set out to identify maturation factors that facilitate the terminal differentiation of thyrocytes in ES cell cultures.

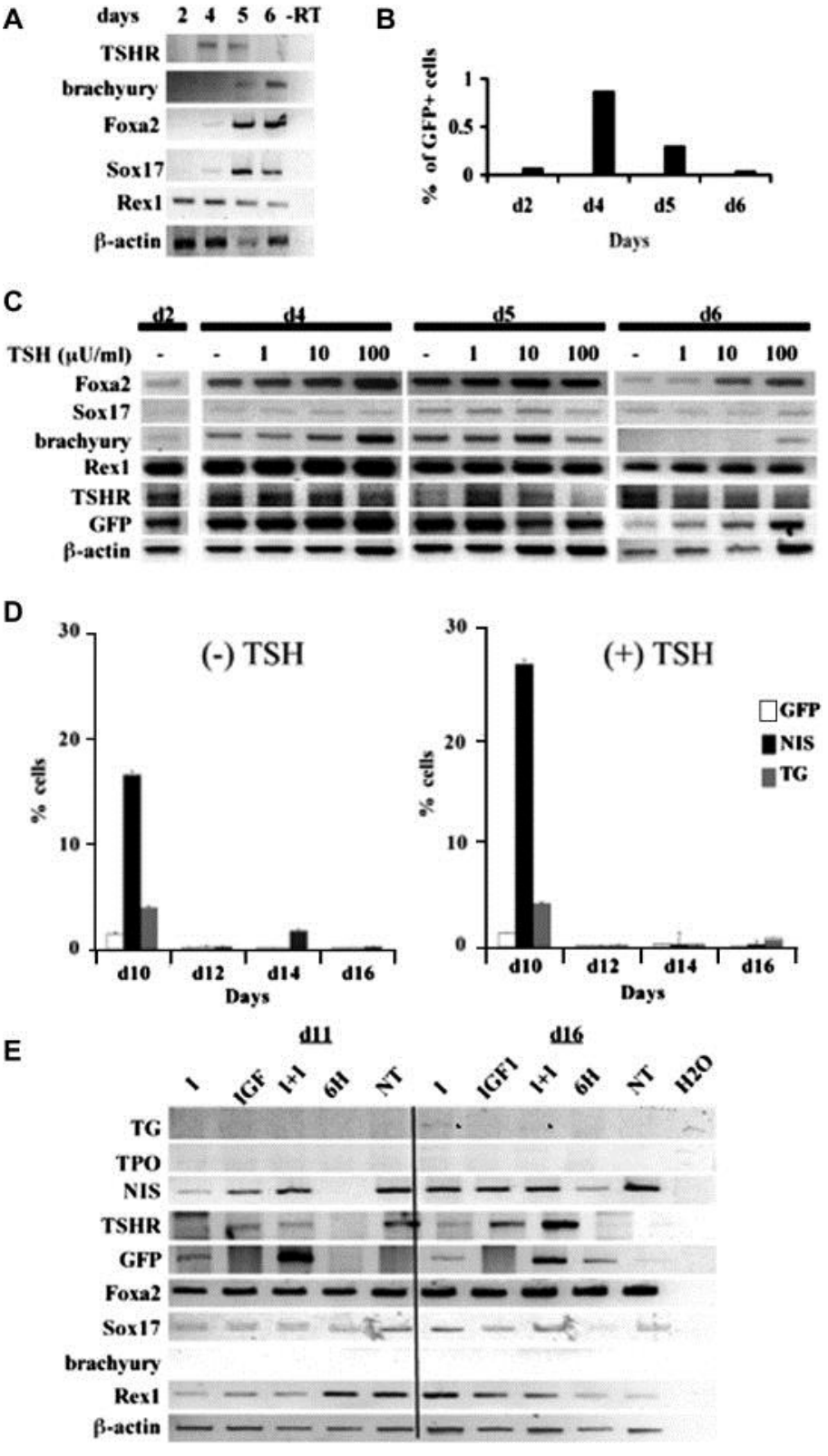

Fig. 2. Differentiation capacity of mouse *TSHR*$^{+/-}$ ES cells treated with activin A and TSH. (A) RT-PCR expression analysis of various markers characteristic of endoderm and mesoderm germ layers, including *Foxa2*, *Sox17*, and *brachyury*, in *TSHR*$^{+/-}$ EBs differentiated in serum-containing medium followed by serum-free medium supplemented with activin A. *TSHR* is a thyrocyte marker. *Rex 1* is a stem cell marker. Numbers on top of the figure indicate days of differentiation. β-Actin serves as an internal control. Control experiments contained no reverse transcriptase (-RT). (B) Kinetics of GFP expression in early EBs. (C) RT-PCR analysis of gene expression profiles in activin A-treated EBs exposed to varying concentrations of TSH. (D) FACS analysis of GFP expression in EBs differentiated in serum-free medium supplemented with TSH (right panel). Control groups were not treated with TSH (left panel). (E) RT-PCR analysis of gene expression profiles in day 11 and day 16 EBs treated with different conditions. *Abbreviations:* I + I, insulin and IGF-1; 6H, 6 hormone medium; NT, no treatment (∗ indicates the band for Tg transcript).

Insulin and IGF-1 are critical for thyrocyte maturation

The growth factors insulin and IGF-1, in concert with TSH, are known to promote the proliferation of FRTL-5 cells, a line of rat thyrocytes [11] and [12]. Therefore, we transferred day 7 EBs co-stimulated with activin A and TSH to a serum-free medium containing TSH. We then added insulin and IGF-1 alone or in combination to the cultures in an attempt to induce the maturation of thyrocytes. Some cells were cultured in the presence of 6H medium, which promotes growth in FRTL-5 cells [13]. As shown in Fig. 2E, the expression of *NIS*, *TSHR*, *GFP*, *Foxa2*, and *Sox17* can be detected after a total of 11 days of differentiation. The expression levels were higher in insulin and IGF-1 treated groups. The expression of *NIS*, *TSHR*, *GFP*, *Foxa2*, and *Sox17* was also found more intense in day 16 cultures. Furthermore, Tg expression was detected in day 16 cell cultures treated with insulin alone or with an insulin and IGF-1 combination. However, we could not detect thyroperoxidase (*TPO*, the enzyme responsible for Tg iodination) expression in all samples. The addition of a 6H medium did not stimulate the expression of all thyroid genes examined in day 11 cultures, and with only a slight increase of the expression of NIS in the cultures found on day 16 cells (Fig. 2E).

Next, we analyzed the cell aggregates for the presence and location of TSHR (*via* GFP) and NIS. We stained these cells with anti-NIS antibody, followed by staining with Alexa Fluro 594 chicken anti-rabbit IgG and observation with fluorescent microscopy. We found that NIS was expressed at day 11 of differentiation (Fig. 3). These data demonstrate that, at the dose used, insulin and IGF-1, either alone or in concert, can stimulate NIS and TSHR expression in EBs in a no-serum culture medium. The addition of 6H medium, however, did not enhance thyrocyte generation (Fig. 3). Furthermore, as shown in Fig. 4, in many cell clusters, GFP (green) and NIS (red) were localized to either the cytoplasm (GFP) or the surface (NIS) of these cells, respectively, they also co-localized in some clusters (yellow). Because co-expression of NIS and TSHR is a hallmark of thyrocytes, this observation suggests that these culture conditions can support the maturation of thyrocytes. Upon further differentiation (day 16) in the presence of insulin and IGF-1, the cells exhibit robust GFP expression and the cultures were strongly positive for NIS (Fig. 4A). A significant up-regulation of Tg was also observed in many differentiated cells (Fig. 4B). Taken together, these observations suggest that addition of insulin and IGF-1 to late cultures enables the long-term propagation and differentiation of mature thyrocytes.

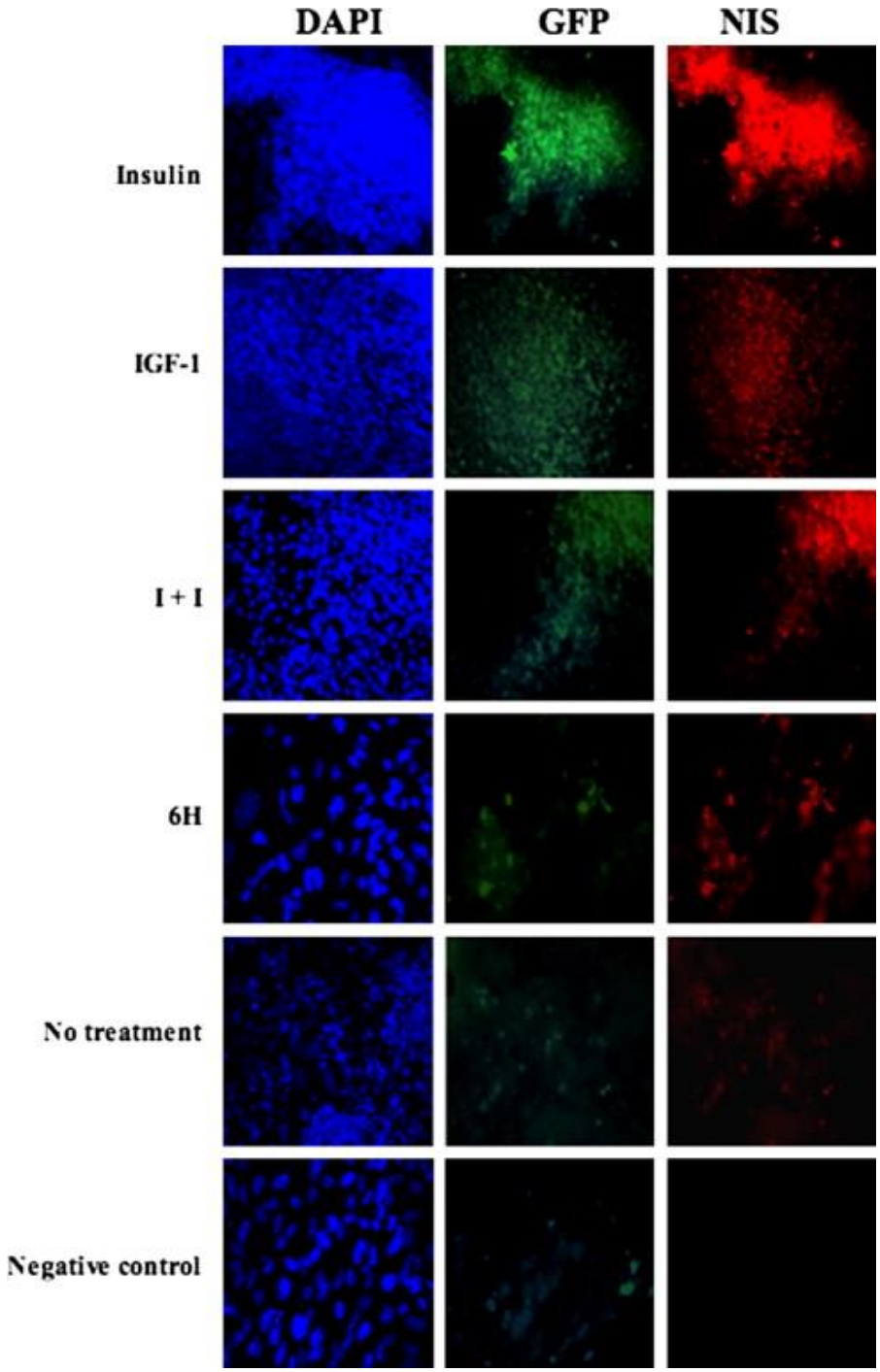

Fig. 3. Characterization of thyrocytes derived from mouse ES cells. Two-color immunofluorescent analysis was performed on day 11 of differentiation. Cells were stained with an antibody to NIS (red). TSHR expression is indicated by GFP (green). DAPI staining was used to identify the nucleus (blue). Note that immunofluorescence is not detected with the isotype control antibody. *Abbreviations:* I + I, insulin and IGF-1; 6H, 6 hormone medium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

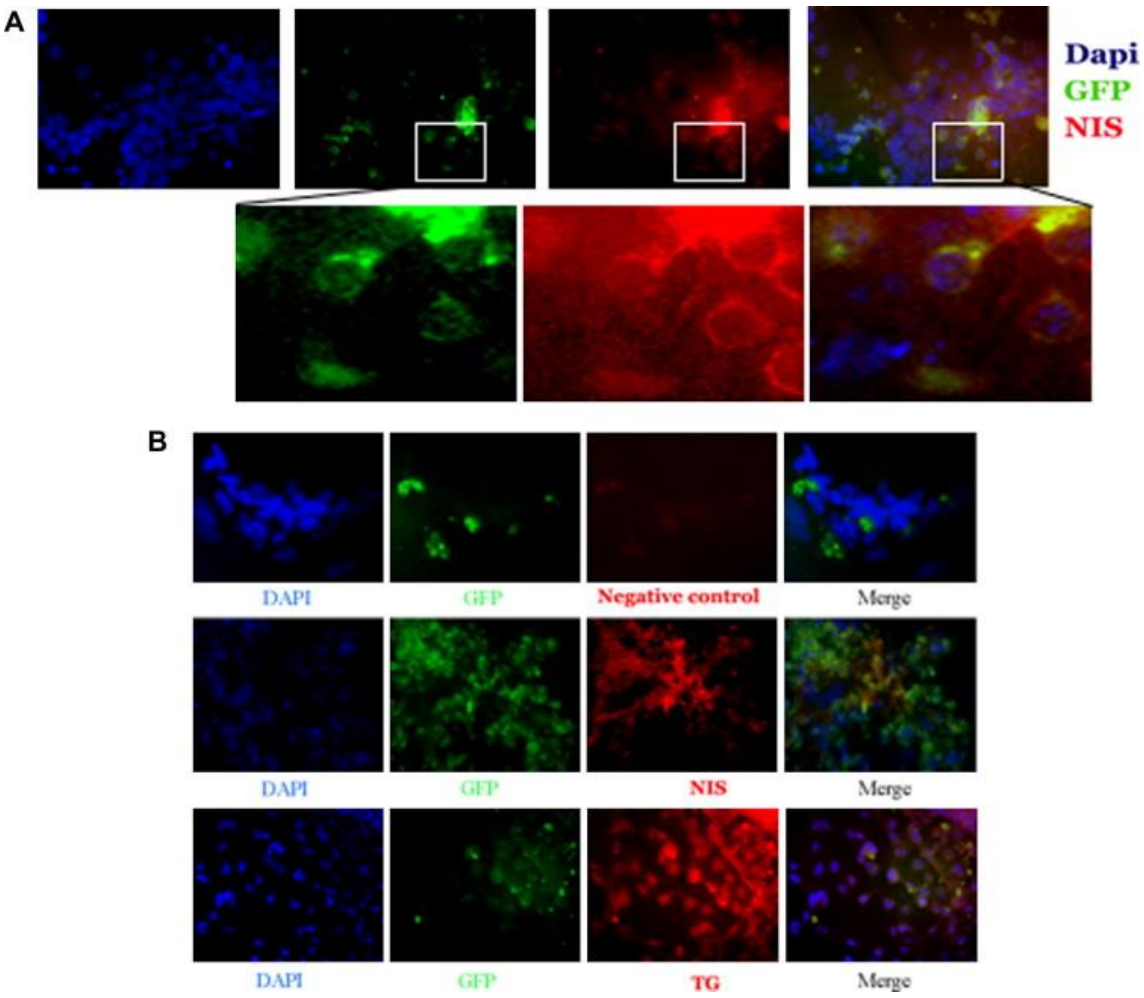

Fig. 4. Further characterization of thyrocytes derived from mouse ES cells. (A) Microscopic analysis of cell aggregates derived from mouse *TSHR*$^{+/-}$ ES cells after 11 days of differentiation and stained with an antibody to NIS (red). TSHR expression is indicated by the GFP signal (green). An overlaid image shows the co-localization of NIS with TSHR (yellow). DAPI staining was used to identify the nucleus (blue). Note that high magnification demonstrates NIS expression in the plasma membrane. (B) Immunofluorescent images of several cell clusters derived from mouse ES cells after 16 days of differentiation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Discussion

We have previously shown that mouse ES cells can differentiate into thyrocyte-like cells if they are cultured during differentiation with TSH [2], [3] and [4]. This finding substantiates the severe congenital hypothyroidism that occurs in TSH- and TSHR-knockout mice [9] and [14]. Additional analyses of the expression of thyroid genes in differentiating ES cells suggest that the cells encompass the entire spectrum of thyroid development *in vivo*, offering an attractive model of otherwise inaccessible early embryonic events. Here we describe a new protocol for directing mouse ES cells to differentiate into mature thyrocytes within 16 days. This newly developed method is based on our previous studies, which demonstrated that TSH efficiently induces the differentiation of thyrocyte progenitors from mouse ES cells. However, this approach is not sufficient to induce the terminal differentiation of thyrocytes [4]. In the present study, we applied a combination of activin A, TSH, insulin and IGF-1, to differentiating ES cells in a step-wise approach. We clearly demonstrate through evaluation of the transcriptional status of known early-stage, thyroid-specific genes, FACS analysis and immunochemistry that mouse ES cells can differentiate into mature, Tg-expressing thyrocytes.

A main goal of this work was to determine the molecular and cellular characteristics of thyrocyte-fated populations derived from ES cells. In order to achieve this, it was crucial to devise a method to identify and monitor thyrocyte progenitors during differentiation. Although there is no single specific marker of a thyrocyte, a combination of specific markers can be used, including TSHR, NIS, and Tg. We found that a step-wise combination of activin A, TSH, insulin and IGF-1 enabled the differentiation of Tg-expressing thyrocyte populations. It is important to note that EB cell differentiation after day 2, activin A treatment and late thyrocyte differentiation were all carried out in the absence of serum. This probably contributed significantly to the high reproducibility of the terminal differentiation of thyrocyte cultures. Such a highly reliable and simple system will be essential for the development of therapeutic applications for human thyroid disease.

In summary, the work describe here demonstrates for the first time that mouse ES cells can be induced by the presence of specific growth factors and signaling molecules to generate cell populations with characteristics similar to those of thyrocytes. Although many questions remain to be answered regarding the biochemical characteristics of these thyrocytes, this protocol represents an important step toward generating thyroid-hormone-producing cells from mouse ES cells. Our system will permit *in vitro* "gain-of-function" or "loss-of-function" genetic studies that will help identify the genes downstream of *TSHR* that contribute to thyrocyte development. Studies of pluripotent ES cells will lead to *in vitro* models of thyroid development, which may ultimately provide new insights into the underlying causes of human thyroid disease.

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