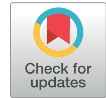

T_3 Induces Both Markers of Maturation and Aging in Pancreatic β-Cells

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ISLET STUDIES

Previously, we showed that thyroid hormone (TH) triiodothyronine (T_3) enhanced β-cell functional maturation through induction of *Mafa*. High levels of T_3 have been linked to decreased life span in mammals and low levels to lengthened life span, suggesting a relationship between TH and aging. Here, we show that T_3 increased $p16^{Ink4a}$ (a β-cell senescence marker and effector) mRNA in rodent and human β-cells. The kinetics of *Mafa* and $p16^{Ink4a}$ induction suggested both genes as targets of TH via TH receptors (THRs) binding to specific response elements. Using specific agonists CO23 and GC1, we showed that $p16^{Ink4a}$ expression was controlled by THRA and *Mafa* by THRB. Using chromatin immunoprecipitation and a transient transfection yielding biotinylated THRB1 or THRA isoforms to achieve specificity, we determined that THRA isoform bound to $p16^{Ink4a}$, whereas THRB1 bound to *Mafa* but not to $p16^{Ink4a}$. On a cellular level, T_3 treatment accelerated cell senescence as shown by increased number of β-cells with acidic β-galactosidase activity. Our data show that T_3 can simultaneously induce both maturation (*Mafa*) and aging ($p16^{Ink4a}$) effectors and that these dichotomous effects are mediated through different THR isoforms. These findings may be important for further improving stem cell differentiation protocols to produce functional β-cells for replacement therapies in diabetes.

Thyroid hormones (THs) mediate development and aging processes in different tissues. They are known to be important in postnatal maturation of many tissues, including the central nervous system, gastrointestinal system, and inner ear (1–3). Yet, THs also affect aging: high levels of triiodothyronine (T_3) are associated with a shortened life span, and lowering the level can increase longevity in mammals and humans (4–8). Moreover, caloric restriction, a model known to delay aging across species, decreases T_3 levels. The mechanism behind this association is not completely understood but is thought a result from interaction between TH and metabolism, growth, and inflammation (rev. in 9). Understanding the molecular mechanisms underlying these opposing effects is crucial to predict and manipulate the various effects of this ubiquitous hormone and to effectively apply them translationally. Given the diverse T_3 effects on different tissues and even within the same tissue at different stages of development, a tight regulatory system that determines the specificity of these effects must be present. In addition to deiodinases and membrane transporters, TH receptors (THRs) are key players determining the specificity of T_3 effects, with the main isoforms, THRA1, THRA2, THRB1, and THRB2, differentially expressed across tissues and developmental stages.

The role of TH in the endocrine pancreas is only partially known. TH plays a part in the functional maturation of the pancreatic β-cells. At birth, rodent β-cells lack glucose-responsive insulin secretion and thus are functionally immature. Previously, we have shown that during postnatal development before weaning, the mRNA of β-cell transcription

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factor *Mafa* increases in expression in parallel to acquisition of glucose responsiveness (10) and that MAFA drove this maturation. Over this same time span, systemic TH increases and there is a switch in expression of its receptor isoforms THRA and THRB within the islet (11). We further showed that T_3 was a physiological inducer of MAFA (11) and stimulated *MAFA* expression and functional maturation in immature human islet tissue (12). These findings have been key to improvements in protocols used to differentiate human embryonic stem cells (hESCs) to glucose-responsive β-cells in vitro (13–15). A recent study showing that TH coordinates pancreatic islet maturation during zebrafish development suggest that the role of TH in the functional maturation of β-cells is preserved across species (16). Aging in pancreatic β-cells is not well understood; yet, $p16^{Ink4a}$, an alternative splice product of the *Cdkn2a* gene (17), is both a marker and effector of β-cell senescence (18,19). We recently showed a decline in function as β-cells age that is accompanied by increased expression of $p16^{Ink4a}$ (20). However, correlations between T_3 and $p16^{Ink4a}$ or any other aging marker have not been studied in pancreatic β-cells. It could be problematic for β-cell–replacement therapy if T_3 induced senescence in hESC-derived β-cells.

Given the translation potential of using TH during differentiation of human stem cells to β-cells for replacement therapy, our aims were to determine whether THs mediate aging of β-cells and which THR isoform was involved in the processes of maturation using *Mafa* as the effector and marker of glucose-induced insulin secretion (10,21) and $p16^{Ink4a}$ as effector and marker of senescence (18). Herein, we show that T_3 induces both maturation and aging markers by increasing *Mafa* and $p16^{Ink4a}$ in rodent primary islets, rodent β-cell–derived cell lines, and insulin-positive cells derived from hESCs. We show that this dual activation by T_3 is dependent on different isoforms of the THR with THRA mediating *Cdkn2a* ($p16^{Ink4a}$) gene transcription while THRB induces that of the *Mafa* gene. Thus, selective THRB agonists may further improve current stem cell differentiation protocols to produce functional β-cells for replacement therapies in diabetes.

RESEARCH DESIGN AND METHODS

Animals

Adult Sprague-Dawley rats (some with litters) from Taconic Farms (Germantown, NY) and adult C57Bl/6J mice from The Jackson Laboratory were kept under conventional conditions with access to water and food ad libitum. Pancreas was excised from anesthetized animals for islet isolation (22). Islets were cultured overnight in RPMI 1640 plus 10% FBS, followed by handpicking to ensure purity. For each postnatal day 7 (P7) sample, islets from 10 pups were pooled; each adult sample of either mice or rats was isolated from one animal, with three to six samples per age. Sex of animals was not recorded or taken into account in the analysis of results. The Joslin Institutional Animal Care and Use Committee approved all animal procedures.

Islet Culture

Islets were cultured for 4 days in RPMI 1640 (11 mmol/L glucose and 10% charcoal-stripped [CS]-FBS) with/without T_3 (150 pmol/L T_3, equivalent to 7.5 pmol/L free T_3 in 10% CS-FBS) (23).

Concentrations of T_3

T_3 concentrations and incubation times were optimized to the specific model being used as well as the readout in which we were interested and vary across experimental models. The concentration used is specified in each model.

hESC Differentiation Protocol

We followed the Viacyte surface-attached β-cell differentiation protocol (24,25) with the following modifications: *1*) Matrigel-coated silicon rubber as the surface on which hESCs (CyT49) were plated, *2*) stage 3 lengthened to 7 days, and *3*) a 3-day stage 5 with DMEM-B27 media and 100 pmol/L T_3 or 1 μmol/L GC1. At the end of each stage, cells were harvested and RNA was extracted for real-time quantitative PCR (qPCR) analysis (12).

Real-time qPCR

Total RNA was isolated with a PicoRNA extraction kit (Arcturus) or a QIAGEN kit and reverse transcribed (SuperScript reverse transcriptase; Invitrogen). Real-time qPCR used SYBR Green detection and specific primers (Supplementary Table 1). Samples were normalized to a control gene (S25 for rats, S18 for mice, and TATA binding protein [TBP] for hESCs), and the comparative CT (threshold cycle) method was used to calculate gene expression levels.

Cell Line Culture

INS1 cells maintained in RPMI 1640 (11 mmol/L glucose plus 10% FCS, 10 mmol/L HEPES, 2 mmol/L L-glutamine, penicillin/streptomycin, 1 mmol/L sodium pyruvate, and 20 mmol/L β-mercaptoethanol) were switched to RPMI 1640 (1.6 mmol/L glucose plus 10% CS-FBS) 24 h before treatment and then incubated for 14 h with T_3 (150 pmol/L). Murine insulinoma (MIN)6 cells, maintained in DMEM (high glucose) (DMEM-H) supplemented with 15% FBS, were switched to DMEM-H with 15% CS-FBS and T_3 (10 nmol/L for 24 h, a dose proven to be effective in a dose response curve) and then harvested for chromatin immunoprecipitation (ChIP) or RNA.

Prediction of Thyroid Response Elements in the *Mafa* and *Cdkn2a* Genes

We previously reported thyroid response element (TRE) motifs in mouse *Mafa* proximal gene and coding sequences (11). For the *Cdkn2a* gene, we used the TRANSFAC/MATCH database (BIOBASE) to analyze the 21 kb mouse *Cdkn2a* region including 1.5 kb 3′ and 5′ to the coding sequence. The primary criterion by which TREs were filtered was the matrix similarity score. Candidates were optimized by considering *1*) proximity to a second putative TR- or retinoid X receptor–binding site, *2*) position in the genome, *3*) sequence homology to rat and human, and *4*) presence in similar TREs using AliBaba. Primers were designed for five putative TREs (Supplementary Table 2).

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ChIP

Antibodies listed in Supplementary Table 3 were used with the Imprint chromatin immunoprecipitation kit (CHP1) (Sigma-Aldrich) following the manufacturer's instructions. DNA from 250,000 cells was used for each condition in four independent experiments. Samples were analyzed by qPCR using specific primers for three putative TREs, S1, S2, and S3 in the *Mafa* gene as previously reported (11) and five putative TREs, S1, S2, S3, S4, and S5 for the *Cdkn2a* gene. The same ChIP samples were analyzed for both *Cdkn2a* and *Mafa* TREs.

Generation of Blrp-TEV Human THRA

The human THRA (hTHRA) transcript variant 1 was obtained from OriGene, and the insert was amplified with PCR and cloned into the XhoI and PmeI sites of the BirA recognition peptide-TEV cleavage site (Blrp-TEV) vector (kindly provided by Dr. Christopher Glass, University of California, San Diego). T4 DNA ligase (NEB) was used for ligation, and the ligation product was transferred to *Escherichia coli* DH5α-competent cells (NEB). Recombinant colonies were cultured overnight, and plasmid extraction was done by miniprep. Successful integration and direction were confirmed by sequencing.

Tagged THRA and THRB1 Double Transfection Protocol for ChIP

MIN6 cells cultured in DMEM-H with 15% FBS and 5 μL/L β-mercaptoethanol were transiently transfected via lipofectamine with either empty Blrp-TEV vector (kindly provided along with the BirA-expressing plasmid by Dr. Christopher Glass), Blrp-TEV hTHRB1 (26), or Blrp-TEV hTHRA. For selection of the transfected cells, they were cultured for 8 days in regular MIN6 media supplemented with puromycin. Selected MIN6 cells were then transiently transfected with BirA plasmid and further selected with geneticin. At day 15 after the first transfection, biotinylated THRB1 or biotinylated THRA was precipitated using streptavidin-agarose beads as previously described (26). DNA was used in qPCRs with SYBR chemistry to detect enrichment of different potential TREs sites.

Knockdown Experiments

MIN6 cells were transfected with small interfering (si)RNA against mouse *Thra*, *Thrb*, or nonspecific (scrambled) siRNA (Thermo Scientific/Dharmacon, Lafayette, CO) using DharmaFECT following the manufacturer's instructions (100 nmol/L siRNA and 2.5 μL DharmaFECT in 200,000 cells plated in 24-well plates). After 48 h culture in basal maintenance media, the cells were harvested for RNA and qPCR analysis. Knockdown was confirmed by real-time PCR. Results are presented as fold changes with respect to MIN6 cells treated with nonspecific siRNA.

Acidic β-Galactosidase Activity

We used activity of acidic β-galactosidase (β-gal) for in vivo evaluation of cellular senescence. We used a fluorescent substrate (ENZ-KIT 130-0010; Enzo Life Sciences) following the manufacturer's instructions and quantified β-gal$^+$ β-cells by FACS sorting for β-cells on the basis of higher endogenous fluorescence (27) and for β-gal activity as previously described (20). Substrate incubation time was optimized to 1 h at 37°C.

Data Analysis

Data are shown as mean ± SEM. For statistical analysis, unpaired Student *t* tests were used to compare two groups and one-way ANOVA followed by Bonferroni post hoc test was used to compare more than two groups. When samples did not have normal distribution as assessed by a normality test, nonparametric statistics were used. A P value <0.05 was considered significant.

RESULTS

T_3 Induces $p16^{Ink4a}$ Expression in Rodent Islets and Cell Lines as Well as in hESCs Differentiated Toward Islets

Similar to the T_3 induction of *Mafa* mRNA in rodent islets and cell lines (11), T_3 increased $p16^{Ink4a}$ mRNA in cultured rat and mouse primary islets and rodent β-cell lines. $p16^{Ink4a}$ expression increased 40% in mouse islets treated with T_3 compared with untreated islets and had a similar tendency in MIN6 cells (Fig. 1*A*). In both rat islets and the rat insulinoma cell line INS1, $p16^{Ink4a}$ mRNA was fourfold increased after T_3 treatment (Fig. 1*B*). $p19^{Arf}$, the alternative splicing product of the *Cdkn2a* gene, was not increased in either mouse or rat islets after T_3 treatment (Supplementary Fig. 1*A*). Expression of *p21*, another important regulator of β-cell senescence, was tested in adult mouse islets treated with T_3, and its levels were unchanged by the hormone (Supplementary Fig. 1*B*). The expression of additional β-cell aging markers, *Bambi* and *Igf1r*, in response to T_3 was also unchanged (Supplementary Fig. 1*C* and *D*). The translational aspect of these findings was tested in hESCs differentiated to insulin-positive cells. The addition of 100 pmol/L T_3 during the last step of differentiation (3 days) increased *MAFA* expression sixfold (Fig. 1*C*) with respect to stem cells differentiated without T_3, suggesting the acceleration of maturation. Similarly, as in the rodent models, in these human cells T_3 induced highly variable increases of $P16^{INK4A}$ (average 3.3-fold increase compared with cells without T_3) (Fig. 1*D*) and there was a direct correlation between the induction of *MAFA* and $P16^{INK4A}$ (Fig. 1*E*) in the same samples. Thus, T_3 activates both β-cell maturation and senescence pathways in all three species.

As With *Mafa*, $p16^{Ink4a}$ Was Induced by T_3, Suggesting It as a Target of TH

TH exerts its effects through binding to THRs that belong to the family of nuclear receptors and bind to TREs in target genes. Upon ligand binding, these receptors upregulate or downregulate transcription of their targets. The presence of both isoforms of THR was verified by qPCR in both mice and rat primary islets (Supplementary Fig. 2) and in the species-specific β-cell lines: MIN6 for mice and INS1 for rats. Both cell lines had higher receptor levels than the primary islets of the same species. However, since different housekeeping genes were used for mouse (*S18*) and rat (*S25*) tissues, it is difficult to compare the absolute values between species.

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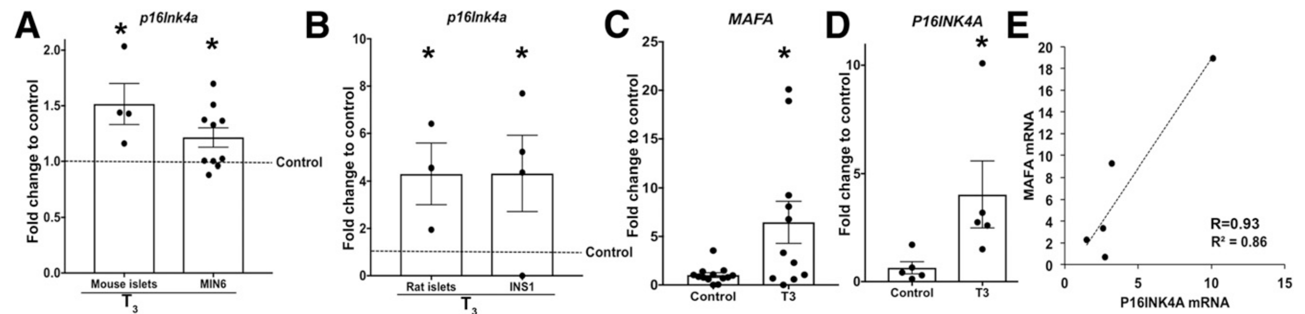

Figure 1—T_3 induces *p16^Ink4a^* expression in rodent islets/cell lines and hESCs differentiated toward islets. *A*: Mouse islets and MIN6 cells had increased *p16^Ink4a^* mRNA after 4 days and 24–48 h of T_3 exposures, respectively. C57Bl/6J 16-week-old mice, $n = 4$; MIN6, $n = 5$ independent samples in duplicate. *B*: Rat islets and INS1 cells had T_3-induced *p16^Ink4a^* mRNA expression after 4 days and 24–48 h culture in the presence of T_3. Adult rat islets, $n = 3$ independent samples; INS1, $n = 4$ independent samples. Differentiated hESCs exposed to T_3 during the last step of differentiation were analyzed by qPCR for expression of maturation and senescence markers. T_3 increased both *MAFA* mRNA (*C*) and senescence marker *P16^INK4A^* mRNA (*D*). *E*: There was a linear correlation between the induction of *MAFA* and *P16^INK4A^* in matched samples from hESCs. $n = 5$–11 samples from two to four individual experiments. Mean ± SEM. $^*P < 0.05$ with respect to baseline. R and R^2 values are shown for linear correlations.

When the time courses of T_3 induction of *Mafa* and *p16^Ink4a^* were studied in INS1 cells, *Mafa* mRNA expression was significantly upregulated at 6, 14, and 24 h after the addition of a low dose of T_3 (0.1 nmol/L) (Fig. 2*A*), whereas *p16^Ink4a^* mRNA was significantly increased (4.3- to 5.8-fold) only after 24 h of exposure to higher doses of T_3 (1 and 10 nmol/L) (Fig. 2*B*). The induction of both *Mafa* and *p16^Ink4a^* suggests they both contain functional TREs. We previously reported *Mafa* to be a direct target of TH with two TREs (sites 2 and 3) in the mouse *Mafa* gene (11), but *p16^Ink4a^* having a TRE is novel. *Cdkn2a* (which encodes the *p16^Ink4a^* gene) had several potential THR-binding sites with a 70–80% stringency (Fig. 3*A* and Supplementary Table 2). Using an antibody that recognizes both THR isoforms in ChIP assay, one (site 5) of the five potential TREs in *Cdkn2a* was significantly enriched (10-fold) compared with IgG negative control (Fig. 3*B*), supporting *p16^Ink4a^* as a direct target of TH.

T_3 Induction of *Mafa* and *p16^Ink4a^* Is Differentially Mediated by Specific THR Agonists in P7 Rat Islets

Activation of *Mafa* and *p16^Ink4a^* by different TH receptor isoforms may provide the basis for simultaneous activation by T_3 of both maturation and senescence markers. To test the functionality of the identified THR binding to the *Mafa* and *Cdkn2a* TREs, we used THR isoform-specific agonists: CO23, a THRA1-specific agonist (28), and GC1, a THRB-specific agonist (29). P7 rat islets, previously shown to express both THRA and THRB (11), were cultured for 4 days in the presence of 150 pmol/L of either agonist and then analyzed for mRNA expression of maturation (*Mafa*) and senescence (*p16^Ink4a^*) effectors. *Mafa* expression

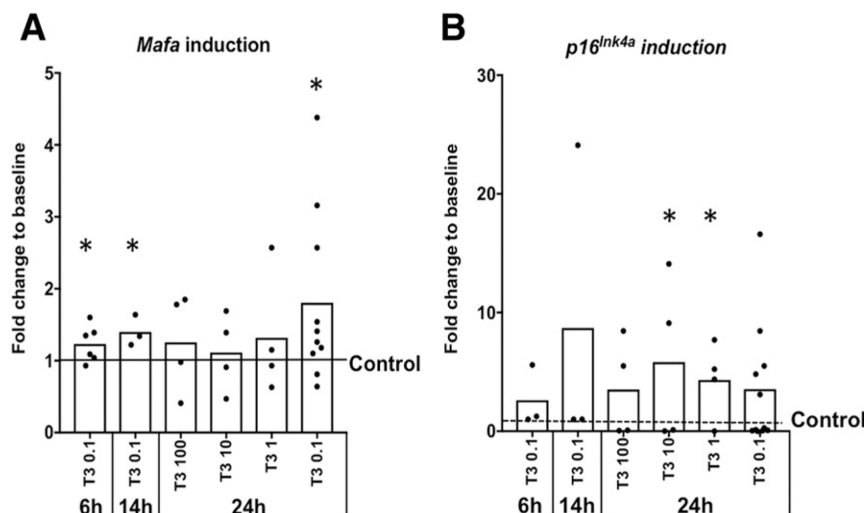

Figure 2—Kinetics of T_3 induction of *Mafa* and *p16^Ink4a^* suggest that they are both targets of TH. INS1 were incubated in the presence of T_3 for different durations and different doses of T_3 at 24 h. *A*: Kinetics of T_3-induced *Mafa* induction. *B*: Kinetics of T_3-induced *p16^Ink4a^* transcription. INS1 cells, $n = 3$–10 individual samples. Mean ± SEM. $^*P < 0.05$ with respect to control.

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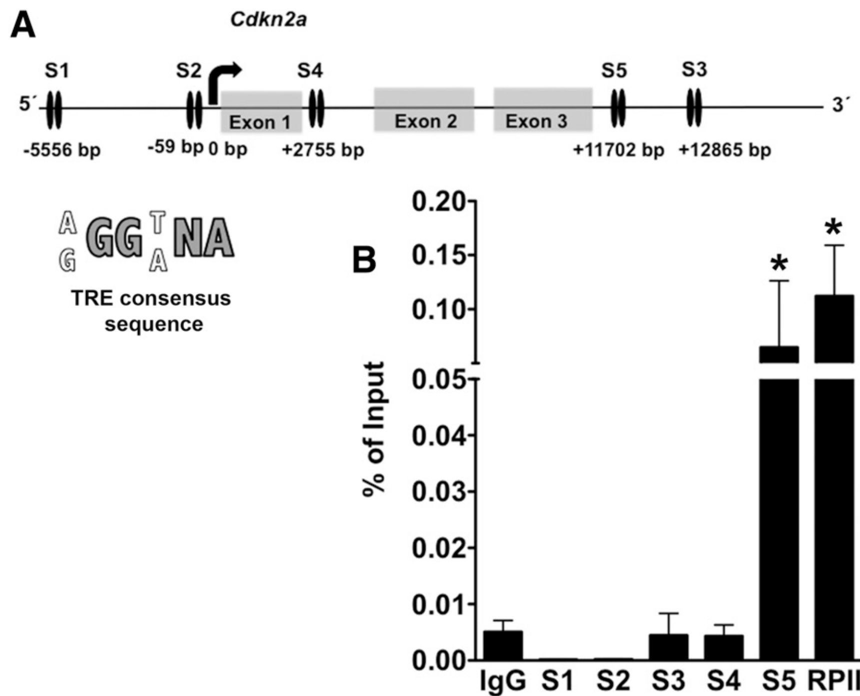

Figure 3—Potential TREs were identified in the *Cdkn2a* gene and experimentally tested with ChIP. *A*: Potential TREs based on shown consensus sequence were identified in the *Cdkn2a (p16*^{Ink4a}*)* gene using AliBaba and TRANSFAC/MATCH. *B*: ChIP confirmed the binding of THR to site 5 in the *Cdkn2a (p16*^{Ink4a}*)* gene (MIN6 cells). *$P < 0.03$ with respect to IgG. n = 13 immunoprecipitation reactions from four individual experiments. bp, base pairs; RPII, RNA polymerase II.

was significantly upregulated (60%) in the presence of GC1 (Fig. 4*A*), and *p16*^{Ink4a} was significantly upregulated (threefold) by CO23 (Fig. 4*B*) in the same set of samples. T_3 treatment of adult islets did not cause a further increase of the transcription factor, since physiological functional levels had already been reached (levels of T_3 adult rat islets were 0.92 ± 0.2 those of the control levels); therefore, P7 rat islets were used, since their *Mafa* levels were still lower than in the adult, and so the effects of T_3 on *Mafa* transcription could be evaluated. Similar effects of CO23 and GC1 on *Mafa* transcription were observed in INS1 cells (Supplementary Fig. 3), validating these results in both primary tissue and cell lines.

Biotinylated THRA Binds to S5 in *Cdkn2a*, While Biotinylated THRB1 Binds to S2 TRE in *Mafa*

The two main isoforms of the THR, *Thra* and *Thrb,* share the same TRE motif but have different target genes based mainly

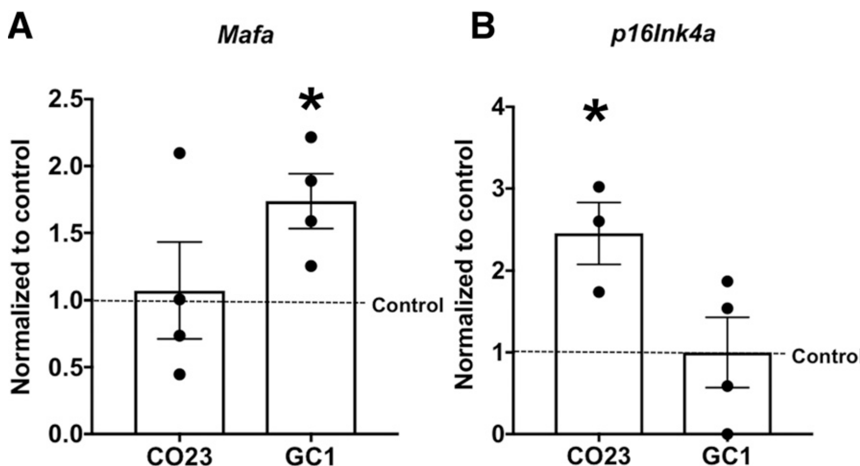

Figure 4—T_3 induction of *Mafa* and *p16*^{Ink4a} is differentially mediated by specific Thr agonists in P7 rat islets. P7 rat islets were treated in vitro with 150 pmol/L of either THRA agonist CO23 or THRB agonist GC1 in RPMI 1640 (11.1 mmol/L glucose plus 10% CS-FBS). *A*: *Mafa* transcription was significantly increased by THRB agonist GC1. *B*: *p16*^{Ink4a} transcription was upregulated by THRA agonist CO23. n = 3 independent experiments. Mean ± SEM. *$P < 0.05$ with respect to control.

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on differential temporal and spatial expression; however, it is not known whether specific TR isoforms have gene-specific effects on transcription (30). Since the immunoprecipitation antibodies available are not specific to either isoform, we used a transient double transfection system of the biotinylated THRA and THRB1 (26) in MIN6 cells to identify which THR isoform bound to *Mafa* and *Cdkn2a*. In this system, biotinylated THRA or THRB1 is expressed after transfection of plasmids carrying *1*) BirA construct that expresses the BirA enzyme that is able to specifically biotinylate the Blrp sequence and *2*) the Blrp-TEV-THRA/B1 construct expressing THRA or B1 with a Blrp peptide sequence 5′ to the N-terminus, which is recognized and biotinylated by the BirA enzyme; the biotinylated receptor is expressed only when both plasmids are present. The receptor is pulled down with streptavidin-agarose resin, which binds biotin with high affinity, allowing identification of TREs that bind specifically to it in ChIP assay. With use of this system, site 5 of the *Cdkn2a* gene was significantly enriched with Blrp-TEV-THRA (Fig. 5*A*) but not with Blrp-TEV-THRB1 (Fig. 5*B*). Conversely, site 2 of the *Mafa* gene was significantly enriched (fourfold) with Blrp-TEV-THRB1 but not with Blrp-TEV-THRA (Fig. 5*C*). Site 3 of the *Mafa* gene was not enriched with either (Fig. 5*D*). These results indicate that THRA binds to the *Cdkn2a* gene, while THRB1 binds to the *Mafa* gene. The lack of enrichment of site 5 in the *Cdkn2a* gene by biotinylated THRB1 in the same pull-down samples that showed enrichment of *Mafa* site 2 demonstrates that the THRA isoform is the potential regulator of $p16^{Ink4a}$ transcription via site 5. These ChIP studies are consistent with the agonist studies (Fig. 4) indicating that THRB activates *Mafa* transcription and THRA $p16^{Ink4a}$ transcription.

Knockdown Experiments Further Support the Differential Effect of T_3 on *Mafa* and $p16^{Ink4a}$

The differential regulation of the THR isoforms was confirmed using a siRNA-based knockdown system in MIN6 cells. Both *Thra* (Fig. 6*A*) and *Thrb* (Fig. 6*B*) expression were reduced by 40–50%. Under basal conditions, $p16^{Ink4a}$ mRNA was decreased 10% with si*Thra* (Fig. 6*C*), but *Mafa* mRNA levels were unchanged with either si*Thra* or si*Thrb* (Fig. 6*D*). *Igf1r* mRNA, a β-cell aging marker that we have previously shown to be downregulated after $p16^{Ink4a}$ knockdown (20), was significantly decreased with both si*Thra* and si*Thrb* (Fig. 6*E*).

Physiological and Translational Implications of T_3-Induced Upregulation of $p16^{Ink4a}$ in Mouse and Human Cells

Since T_3 increased expression of the senescence effector $p16^{Ink4a}$ in islets, we evaluated whether T_3 upregulation of $p16^{ink4a}$ expression induced the cellular senescence phenotype using senescence-associated acidic β-gal activity to identify and quantify senescent β-cells. After islets from 5-month-old mice were treated with T_3 for 72 h, the percentage of β-gal$^+$ β-cells significantly increased (Fig. 7*A*). This increase in senescent β-cells underscores the physiological importance of T_3-induced $p16^{Ink4a}$ expression and is

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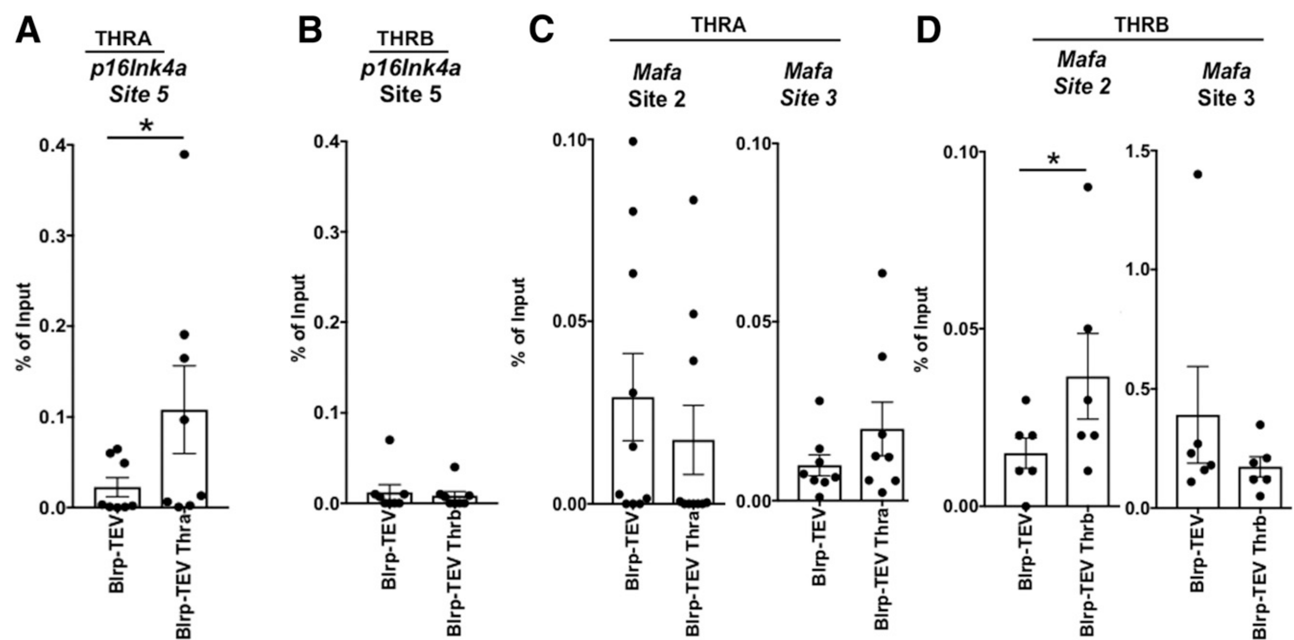

Figure 5—Biotinylated receptor isoforms show specificity of binding to *Mafa* and *Cdkn2a* TREs. Double transfection of MIN6 cells with BirA and either Blrp-TEV-THRA or Blrp-TEV-THRB1 plasmids, followed by streptavidin ChIP, tested specific binding of THRA and THRB1 to potential TREs in the *Cdkn2a* and *Mafa* genes. Site 5 in the *Cdkn2a* gene was enriched with THRA (*A*) but not with THRB1 (*B*), indicating the binding of the THRA isoform. Neither site 2 nor site 3 in in the *Mafa* gene was enriched with THRA (*C*) but site 2 was with THRB1 (*D*), indicating the specific binding of THRB1 to site 2 and not site 3. n = 6–8 samples from three to four independent experiments (MIN6 cells). Mean ± SEM. $^*P < 0.05$ with respect to control.

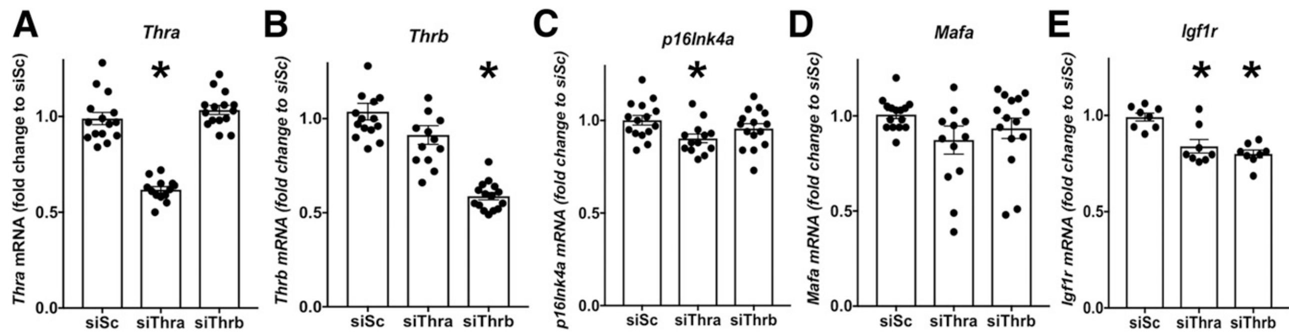

Figure 6—Differential THR isoform induction of *Mafa* and *$p16^{Ink4a}$* shown by siRNA. siRNA knockdown of THR isoform transcription (40%) was specific (si*Thra* [*A*] and si*Thrb* [*B*].) *C*: Basal levels of *p16Ink4a* transcription were significantly decreased with si*Thra*. *D*: Basal *Mafa* RNA levels were unchanged with either si*Thra* or si*Thrb*. *E*: β-Cell aging marker *Igf1r* levels were significantly decreased with both si*Thra* and si*Thrb*. Results in MIN6 cells are shown. *n* = 3 experiments, each in triplicate. Mean ± SEM. **P* < 0.05 respect to scrambled siRNA (siSc).

in line with our previous observation of 4–7% β-gal$^+$ Ins$^+$ cells in islets obtained from 3 to 6 months of age (20).

To evaluate the translational value of the differential regulation by THR in human β-cells, we tested the effects of GC1 compared with T_3 in the final stages of differentiation of hESC. We previously published (12) that this differentiation protocol resulted in 17% of C-peptide–positive and 9% glucagon-positive cells under control conditions. Moreover, at the end of stage 4, 70% of cells expressed PDX1 protein and 24% expressed NKX6.1. By qPCR at this stage, the cells expressed *insulin*, *PDX1*, *NKX6.1*, and *NEUROD1* mRNA but lacked *MAFA* expression. They also expressed *THRA* and *THRB*. At the end of stage 5 under control conditions, 16% expressed C-peptide, whereas 9% expressed glucagon. At the end of stage 5, T_3 treatment resulted in a 20-fold increase in *Insulin* mRNA and a fivefold increase in transcription factors *MAFA* and *NKX6.1*, twofold increase in *CPE*, and a tendency for increase in *PCSK1* and *PCSK2* mRNA. Scattered double hormone-positive cells were observed rarely. HESC cells treated with GC1 had significantly decreased *$P16^{INK4A}$* mRNA levels (Fig. 7*B*) with no consistent increase in *MAFA*. However, four of five samples had induction of *MAFA* or *MAFB* after treatment with GC1 (Supplementary Fig. 4). From a functional point of view, GC1 treatment of differentiated hESCs increased insulin secretion at 16.8 mmol/L glucose (Fig. 7*C*) and significantly increased insulin content (Fig. 7*D*) compared with those treated with T_3, which we believe resulted from an increase in the amount of insulin per cell as the β-cell matured, as we have previously shown (11,12). These findings underline the translational potential of our findings.

In summary, our results indicate that in pancreatic β-cells, T_3 upregulates the transcription of *Mafa* and *$p16^{Ink4a}$* genes through its interaction with different THR isoforms that bind directly to TREs. Through the two THR isoforms, TH simultaneously regulates both maturation and cellular senescence markers/effectors in β-cells, but a selective effect can be achieved using specific concentrations and timing or receptor isoform agonists.

DISCUSSION

While we had previously shown that TH was a physiological driver of β-cell maturation via its induction of the β-cell

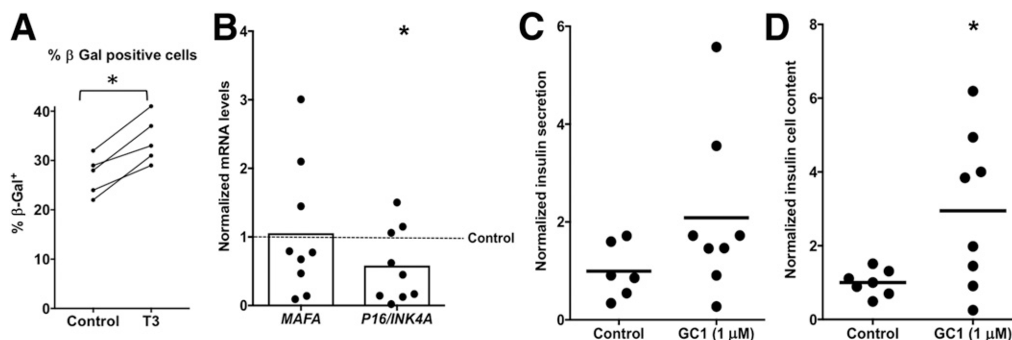

Figure 7—T_3 induced cellular senescence in mouse islets and hESC treated with GC1 increased insulin secretion at 16.8 mmol/L glucose and absolute insulin content and decreased *$P16^{INK4A}$* levels. *A*: In vitro T_3 treatment of islets isolated from 5-month-old C57Bl/6J mice increased their senescence-associated β-gal activity, a marker of cellular senescence. Data presented for FACS sorted β-gal$^+$ cells from individual experiments of islets pooled from 10 mice. THRB agonist GC1 substituted for T_3 in stage 5 of the differentiation protocol of hESC cells resulted in decreased *$P16^{INK4A}$* mRNA with no change of *MAFA* mRNA (*B*) and increased insulin secretion in response to 16.8 mmol/L glucose (*C*) and insulin content (*D*). Absolute values of insulin secretion per experiment (averaged two to three samples): 0.02, 0.04, and 0.06 pg Ins/ng DNA for controls and 0.04, 0.04, and 0.06 pg Ins/ng DNA for GC1-treated cells. Absolute insulin content: controls, average 5.4 pg Ins/ng DNA (1.02, 4.84, 10.70); GC1-treated, average 13.7 pg Ins/ng DNA (4, 15.47, 18.32). *n* = 8–9 samples, three independent experiments. **P* < 0.003 in *A*; **P* < 0.04 in *B* and *D*.

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transcription factor *Mafa*, the current study suggests that TH is directly involved in β-cell aging via the induction of the senescence β-cell marker $p16^{Ink4a}$. Further, we have shown here that these dichotomous effects can occur simultaneously and that these opposing effects on β-cell physiology are due to T_3 binding to different thyroid receptor isoforms that selectively induce *Mafa* and $p16^{Ink4a}$ gene expression. THRB1 binds to *Mafa* at site 2 TRE, while THRA binds to site 5 TRE of the *Cdkn2a* gene. These findings have increased importance, since the recent protocols for hESC-derived insulin-positive cells (13–15) have included TH to induce functional maturation. The simultaneous induction of the aging marker $p16^{Ink4a}$ and potential acceleration of cellular senescence could be detrimental for the therapeutic use of hESC-derived β-cells. It would be ideal to induce the positive effects of MAFA upon β-cell function without inducing accelerated aging that might hamper the overall function or proliferative capacity of β-cells. Our studies provide a molecular basis to circumvent the increased $p16^{Ink4a}$ expression by using specific THRB agonists that are able to selectively induce *Mafa* expression without inducing $p16^{Ink4a}$. Even though further studies are needed to fully translate these findings into hESC differentiation protocols, our data suggest that the use of specific THR agonists could optimize the final functional maturation step without compromising the ability of these cells to respond to growth factors. Additionally, different incubation times and concentrations might also favor one transcript over the other.

In vivo the differential effects of the THR isoforms are enhanced by the temporal differences in their expression, since a direct comparison of both isoforms from a single species has failed to demonstrate differences in T_3 binding properties (31). A switch of THR isoforms occurs in postnatal rat β-cell development (11), with predominance of *THRA* mRNA from P2 to P9, then similar levels of *THRA* and *THRB* until P15, and then a 2:1 dominance of *THRB* expression through adulthood. At the protein level, as reflected by immunostaining, β-cell nuclei were predominantly stained for THRA through P15, after which point nuclear THRB protein dominates, with lower levels of THRA. Although this isoform switch might initially seem contradictory to the proposed effects of each isoform, it is consistent with the physiological changes that accompany β-cell maturation. THRA, the THR isoform usually expressed in neonatal tissues, is expressed in β-cells from neonatal rodents. However, circulating T_3 levels are very low and do not induce $p16^{Ink4a}$ at this early age. As circulating and local T_3 levels begin to increase as induced by changes in dediodinases expression (increase in Dio2 and a decrease in Dio3 expression), expression of THRB is induced (11) leading to increased β-cell *Mafa* and acquisition of glucose-responsive insulin secretion. In adult mature β-cells THRB expression dominates and maintains the high levels of *Mafa*, while low levels of THRA allow the senescence effector and mediator $p16^{Ink4a}$ expression to be minimal. However, increasing T_3 further in adult β-cells does not further increase *Mafa* expression but can induce $p16^{Ink4a}$ leading to senescence.

The protein biotinylation for ChIP was particularly useful for testing the isoform binding to the different TREs, since the commercial THR antibodies for immunoprecipitation are limited in their specificity for THR isoforms. This system has the advantage of providing an antibody-independent means to detect direct interaction between the receptors and TREs. Moreover, the high affinity of biotin-avidin interaction provided greater enrichment of target DNA, improved signal-to-noise ratio, and a more robust ChIP assay (32). With this system, we were able to identify specific THRB1 binding to site 2 TRE of *Mafa* and THRA to site 5 of *Cdkn2a*. The specificity of effect on these genes by receptor isoform was consistent with our findings of $p16^{Ink4a}$ induction by a THRA functional agonist, CO23; decreased $p16^{Ink4a}$ with Thra siRNA; and induction of *Mafa* mRNA both by THRB1 binding to its site 2 TRE and the functional THRB agonist GC1. A second TRE (site 3) within the *Mafa* gene that was immunoprecipitated with antibodies against both isoforms of THR (11) was not precipitated using the biotinylated system.

Even though changes induced by the siRNA knockdown of *Thra* and *Thrb* were minimal, we should point out that that only 40–50% of receptor knockdown was achieved in spite of our use of maximal doses of both DharmaFECT and siRNA. Difficulty with transfection is an inherent characteristic of these cells. Furthermore, it is difficult to directly translate these results from a cell line into a physiological setting, since immortalized cells may have changed the regulation of cell cycle inhibitors such as *p16*. Interestingly, *Igf1r* is likely a downstream target of *p16*, since knocking down *p16* with siRNA in Min6 cells significantly reduced *Igf1r* levels but *Igf1r* knockdown did not influence *p16* mRNA (20). Thus, a longer period of treatment with T_3 may be needed to see effects on these other aging markers.

There may be further complexity of the effects of T_3. T_3 was shown to mediate senescence and DNA damage in cultured mouse embryonic fibroblasts and in liver and kidney, but not heart, of hyperthyroid mice (33). In contrast to our finding that THRA directly induces $p16^{Ink4a}$ mRNA, these authors focused on the role of THRB inducing the *Cdkn2a* pathway in the tissues with strong THRB expression. It is important to remember that the *Cdkn2a* encodes two different senescence effectors $p16^{Ink4a}$ and $p19^{Arf}$; however, we saw no increased expression of $p19^{Arf}$ in adult rodent islets.

The induction of $p16^{Ink4a}$ for 10 days in β-cells of 3- to 4-week-old transgenic mice has been reported to result in increased function of β-cells (34), leading to the conclusion of a novel functional benefit of senescent β-cells. However, the β-cells of 5- to 6-week-old mice may not yet all be fully mature; islets from 3-week-old rats are glucose responsive but without the robustness of a 2- to 3-month-old rat (35). Additionally, in contrast to this recent report, we showed that β-cells that had high levels of $p16^{Ink4a}$ expression had decreased glucose-induced insulin response (20). By

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understanding the roles of THRA and THRB in maturation and senescence of β-cells, we may now be able to discriminate between processes that are regulated during maturation and those of senescence.

The dichotomous role of T_3 upon maturation and aging is not exclusive to β-cells; it has been observed in other tissues and in different organisms (9). From an evolutionary point of view, it has been proposed that during early life, TH mediates growth and maturation, optimizing the health and reproductive chances of different organisms (9), partly by decreasing proliferation. However, as the organism ages, T_3 accelerates this process, impairing repair mechanisms and limiting longevity. Caloric restriction, which prolongs life span, has been shown to decrease circulating T_3 levels, providing additional evidence of the important role of T_3 on aging. The molecular mechanisms behind the aging effects of T_3 are thought to involve the IGF-1/insulin pathway, since low T_3 levels, such as those induced by caloric restriction, decrease circulating growth hormone levels with resultant decreased IGF-1 and insulin levels (9). In rats, the effects of T_3 on growth hormone are direct, since a TRE has been identified in the promoter region of growth hormone (36).

In conclusion, we have shown that T_3 induces both maturation and aging effectors through direct binding of different isoforms of THR to the key regulators of these processes, *Mafa* and *p16^{Ink4a}*. Since the effects are conserved across species and experimental models including humans, our results provide a molecular basis for development of specific highly targeted THRB-based strategies to promote β-cell maturity without accelerating aging.

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Author Contributions. C.A.-M. and S.B.-W. conceived the project and wrote the manuscript. C.A.-M., T.B.L., M.M., A.D., H.R., and P.R. researched data. T.S., A.M.Z., and P.R.L. provided THR agonists. A.H., C.C., and A.S. provided critical discussions and edited the manuscript. All authors reviewed the manuscript. S.B.-W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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