T$_3$ induces both markers of maturation and aging in pancreatic β-cells

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ABSTRACT 200 words

Previously we showed thyroid hormone (T₃) enhanced β-cell functional maturation through induction of Mafa. High levels of T₃ have been linked to decreased lifespan in mammals, and low levels to lengthened lifespan, suggesting a relationship between thyroid hormone and aging. Here we show T₃ increased p16<sup>Ink4a</sup> (a β-cell senescence marker and effector) mRNA in rodent and human β-cells. The kinetics of Mafa and p16<sup>Ink4a</sup> induction suggested both genes as targets of thyroid hormone via TH receptor (THR) binding to specific response elements (TRE). Using specific agonists CO23 and GC1, we show p16<sup>Ink4a</sup> expression was controlled by THRA and Mafa by THRβ. Using chromatin immunoprecipitation and a transient transfection yielding biotinylated THRβ1 or THRA isoforms to achieve specificity, we determined that THRA isoform bound to p16<sup>Ink4a</sup> whereas THRβ1 bound to Mafa but not to p16<sup>Ink4a</sup>. On a cellular level, T₃ treatment accelerated cell senescence as shown by increased number of β-cells with acidic β-galactosidase activity. Our data show T₃ can simultaneously induce both maturation (Mafa) and aging (p16<sup>Ink4a</sup>) effectors and 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.
INTRODUCTION
Thyroid hormones (TH) 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 thyroid hormones also affect aging: high levels of T\textsubscript{3} are associated with a shortened lifespan, 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\textsubscript{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 (see review 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\textsubscript{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, thyroid hormone receptors are key players determining the specificity of T\textsubscript{3} effects with the main isoforms, THRA1, THRA2, THRBI and THRIB2, differentially expressed across tissues and developmental stages.

The role of thyroid hormone in the endocrine pancreas is only partially known. Thyroid hormone 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 factor \textit{Mafa} increases in expression in parallel to acquisition of glucose-responsiveness [10] and that MAFA drove this maturation. Over this same time span, systemic thyroid hormone increases and there is a switch in expression of its receptor isoforms THRA and THRIB within the islet [11]. We further showed that T\textsubscript{3} was a physiological inducer of MAFA [11] and stimulated \textit{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 (hESC) to glucose-responsive β-cells \textit{in vitro} [13-15]. A recent paper showing that thyroid hormone 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 \textit{p16\textsuperscript{Ink4a}}, an alternative splice product of the \textit{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 $p16^{\text{ink4a}}$ [20]. However, correlations between T$_3$ and $p16^{\text{ink4a}}$ 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 thyroid hormones 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 $p16^{\text{ink4a}}$ as effector and marker of senescence [18]. Herein, we show that T$_3$ induces both maturation and aging markers by increasing Mafa and $p16^{\text{ink4a}}$ in rodent primary islets, rodent β-cell-derived cell lines, and insulin positive-cells derived from human embryonic stem cells. We show this dual activation by T$_3$ is dependent upon different isoforms of the thyroid hormone receptor with THRA mediating $Cdkn2a(p16^{\text{ink4a}})$ gene transcription while THRβ induces that of the Mafa gene. Thus, selective THRβ agonists may further improve current stem cell differentiation protocols to produce functional β-cells for replacement therapies in diabetes.

METHODS

Animals: Adult Sprague-Dawley rats (some with litters) from Taconic Farms (Germantown, NY) and adult C57Bl/6J mice from Jackson Labs are 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 +10% FBS, followed by handpicking to ensure purity. For each P7 sample, islets from 10 pups were pooled; each adult sample of either mouse or rats was isolated from 1 animal; with 3-6 samples per age. Gender of animals was not recorded nor 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 4d in RPMI 1640 (20 mM glucose and 10% charcoal-stripped (CS) fetal bovine serum) with/without tri-iodothyronine (150 pM T$_3$, equivalent to 7.5 pM free T$_3$ in 10% CS-FBS [23].
Concentrations of triiodothyronine. T3 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 modifications of: 1) Matrigel-coated silicon rubber as the surface on which hESC (CyT49) were plated; 2) Stage 3 lengthened to 7 d; 3) a 3 day stage 5 with DMEM-B27 media and 100 pM T3 or 1 μM GC1. At the end of each stage, cells were harvested and RNA extracted for qPCR analysis [12].

Quantitative real-time PCR. Total RNA was isolated with PicoRNA extraction kit (Arcturus) or a QIAGEN kit and reverse transcribed (SuperScript reverse transcriptase, Invitrogen). Real-time quantitative (q)PCR 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 hESC), and the comparative CT (threshold cycle) method used to calculate gene expression levels.

Cell line culture. INS1 cells maintained in RPMI 1640 (11mM glucose + 10% FCS, 10 mM HEPES, 2mM L-glutamine, penicillin/streptomycin, 1mM sodium pyruvate and 20 mM β-mercaptoethanol) were switched to RPMI 1640 (1.6 mM glucose + 10% charcoal-stripped (CS)-FBS) 24h before treatment and then for 14h with T3 (150 pM). MIN6 cells, maintained in DMEM-high glucose (DMEM-H) supplemented with 15% FBS, were switched to DMEM-H with 15% charcoal stripped (CS)-FBS and T3 (10 nM, 24h; a dose proven to be effective in a dose response curve) and then harvested for chromatin immunoprecipitation (ChIP) or RNA.

Prediction of TREs in the Mafa and Cdkn2a genes. We previously reported TRE motifs in mouse Mafa proximal gene and coding sequence [11]. For Cdkn2a gene, we used the TRANSFAC/Match database (BIOBASE) to analyze the 21kb mouse Cdkn2a region including 1.5kb 3’ and 5’ to the coding sequence. The primary criterion by which TREs were filtered was the Matrix Similarity Score (MSS). Candidates were optimized by considering 1) proximity to a second putative TR or RXR 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).

Chromatin immunoprecipitation assay (ChIP). Antibodies listed in Supplementary Table 3 were used with Imprint Chromatin immunoprecipitation kit (Sigma Aldrich, CHP1) following
manufacturer’s instructions. DNA from 250,000 cells was used for each condition in 4 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 hTHRA.** The human THRA (hTHRA) transcript variant 1 was obtained from OriGene, and the insert was amplified with PCR and cloned into the XhoI and Pme I sites of the Blrp-TEV vector (kindly provided by Dr. Christopher Glass). T4 DNA ligase (NEB) was used for ligation and the ligation product was transferred to E.coli DH5α competent cells (NEB). Recombinant colonies were cultured overnight and plasmid extraction was done by miniprep. Successful integration and direction was confirmed by sequencing.

**Tagged THRA and THRBI Double Transfection protocol for ChIP.** MIN6 cells cultured in DMEM-H with 15% FBS and 5uL/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 hTHRBI [26] or Blrp-TEV hTHRA. For selection of the transfected cells, they were cultured 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 d15 after the first transfection, biotinylated THRBI or biotinylated THRA was precipitated using streptavidin-agarose beads as 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 siRNA against mouse Thra, Thrb or nonspecific (scrambled) siRNA (ThermoScientific/Dharmacon (Lafayette, CO) using DharmaFECT following manufacturer’s instructions (100nM of siRNA, 2.5uL of 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 in respect to MIN6 cells treated with nonspecific siRNA.

**Acidic β-galactosidase activity.** We used activity of acidic β-galactosidase for in vivo evaluation of cellular senescence. We used a fluorescent substrate (Enzo Life Sciences enz-kit 130-0010) 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 1h at 37°C. 

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

RESULTS

T₃ induces p16ᵢnkd expression in rodent islets and cell lines as well as in hESC differentiated toward islets. Similar to the T₃ induction of Mafa mRNA in rodent islets and cell lines [11], T₃ increased p16ᵢnkd mRNA in cultured rat and mouse primary islets and rodent β-cell lines. p16ᵢnkd expression increased 40% in mouse islets treated with T₃ compared to untreated islets and had a similar tendency in murine insulinoma cell line MIN6 (Figure 1A). In both rat islets and the rat insulinoma cell line INS1, p16ᵢnkd mRNA was 4-fold increased after T₃ treatment (Figure 1B). p19Arf, the alternative splicing product of the Cdkn2a gene, was not increased in either mouse or rat islets after T₃ treatment (Supplementary Figure 1A). 

Expression of p21, another important regulator of β-cell senescence, was tested in adult mouse islets treated with T₃ and its levels were unchanged by the hormone (Supplementary Figure 1B). The expression of additional beta cell aging markers, Bambi and Igf1r, in response to T₃ were also unchanged (Supplementary Figure 1C and 1D). The translational aspect of these findings was tested in human ES cells differentiated to insulin-positive cells. The addition of 100 pM T₃ during the last step of differentiation (3 days), increased MAFA expression 6-fold (Figure 1C) with respect to stem cells differentiated without T₃, suggesting the acceleration of maturation. Similarly, as in the rodent models, in these human cells T₃ induced highly variable increases of P16ᵢNK4A, (average 3.3 fold increase compared to cells without T₃) (Figure 1D) and there was a direct correlation between the induction of MAFA and P16ᵢNK4A (Figure 1E) in the same samples. Thus, T₃ activates both β-cell maturation and senescence pathways in all three species.

As with Mafa, p16ᵢnkd was induced by T₃, suggesting it a target of TH. TH exerts its effects through binding to thyroid hormone receptors (THR) that belong to the family of nuclear
receptors and bind to thyroid response elements (TRE) 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 Figure 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 mice (S18) and rat (S25) tissues, it is difficult to compare the absolute values between species. When the time courses of T₃-induction of Mafa and p16⁰ᴷα were studied in INS-1 cells, Mafa mRNA expression was significantly upregulated at 6h, 14h and 24h after the addition of a low dose of T₃ (0.1 nM) (Figure 2A) whereas p16⁰ᴷα mRNA was significantly increased (4.3 to 5.8-fold) only after 24h of exposure to higher doses of T₃ (1 and 10 nM) (Figure 2B). The induction of both Mafa and p16⁰ᴷα suggest they both contain functional TREs. We previously reported Mafa a direct target of TH with 2 TREs (sites 2 and 3) in the mouse Mafa gene [11], but p16⁰ᴷα having a TRE is novel. The Cdkn2a (which encodes the p16⁰ᴷα gene) had several potential THR binding sites with a 70-80% stringency (Figure 3A, 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 to IgG negative control (Figure 3B), supporting p16⁰ᴷα as a direct target of TH.

**T₃ induction of Mafa and p16⁰ᴷα is differentially mediated by specific THR agonists in P7 rat islets.** Activation of Mafa and p16⁰ᴷα by different thyroid hormone receptor isoforms may provide the basis for simultaneous activation by T₃ 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 THRβ specific agonist [29]. P7 rat islets, previously shown to express both THRA and THRβ [11], were cultured 4 days in the presence of 150 pM of either agonist and then analyzed for mRNA expression of maturation (Mafa) and senescence (p16⁰ᴷα) effectors. Mafa expression was significantly upregulated (60%) in the presence of GC1 (Figure 4A) and p16⁰ᴷα was significantly upregulated (3-fold) by CO23 (Figure 4B) in the same set of samples. T₃ treatment of adult islets did not cause a further increase of the transcription factor since physiological functional levels have already been reached (levels of T3 adult rat islets were 0.92±0.2 those of the control levels) therefore P7 rat islets were used since their Mafa levels are still lower than in
the adult, and so the effects of T₃ upon Mafa transcription could be evaluated. Similar effects of CO23 and GC1 upon Mafa transcription were observed in INS1 cells (Supplementary Figure 3), validating these results in both primary tissue and cell lines.

**Biotinylated THRA binds to S5 in Cdkn2a while biotinylated THRBI binds to S2 TRE in Mafa.** The two main isoforms of the thyroid hormone receptor, Thra and Thrb, share the same TRE motif but have different target genes based mainly on differential temporal and spatial expression; however it is not known whether specific TR isoforms have gene-specific effects on transcription [30]. Since the IP antibodies available are not specific to either isoform, we used a transient double transfection system of the biotinylated THRA and THRBI [26] in MIN6 cells to identify which THR isoform bound to Mafa and Cdkn2a. In this system, biotinylated THRA or THRBI 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 using streptavidin-agarose resin, which binds biotin with high affinity, allowing identification of TREs that bind specifically to it in ChIP assay. Using this system, site 5 of the Cdkn2a gene was significantly enriched with Blrp-TEV-THRA (Figure 5A) but not with Blrp-TEV-THRBI (Figure 5B). Conversely, site 2 of the Mafa gene was significantly enriched (4-fold) with Blrp-TEV-THRBI but not with Blrp-TEV-THRA (Figure 5C). Site 3 of the Mafa gene was not enriched with either (Figure 5D). These results indicate that THRA binds to the Cdkn2a gene while THRBI binds to the Mafa gene. The lack of enrichment of site 5 in the Cdkn2a gene by biotinylated THRBI in the same pull-down samples that showed enrichment of Mafa site 2 demonstrates that the THRA isoform is the potential regulator of p16⁰⁵⁴⁴ transcription via site 5. These ChIP studies are consistent with the agonist studies (Figure 4) indicating that THR activates Mafa transcription and THRA, p16⁰⁵⁴⁴ transcription.

**Knockdown experiments further support the differential effect of T₃ on Mafa and p16⁰⁵⁴⁴.** The differential regulation of the THR isoforms was confirmed using a siRNA-based knockdown system in MIN6 cells. Both THRA (Figure 6A) and THRBI (Figure 6B) expression were
reduced by 40-50%. Under basal conditions, $p16^{\text{ink4a}}$ mRNA was decreased 10% with siThra (Figure 6C) but $Mafa$ mRNA levels were unchanged with either siThra or siThrb (Figure 6D). $Igf1r$ mRNA, a β-cell aging marker that we had previously shown is downregulated after $p16^{\text{ink4a}}$ knock down [20], was significantly decreased with both siThra and siThrb (Figure 6E).

**Physiological and translational implications of T₃-induced upregulation of $p16^{\text{ink4a}}$ in mouse and human cells.** Since T₃ increased expression of the senescence effector $p16^{\text{ink4a}}$ in islets, we evaluated whether T₃ upregulation of $p16^{\text{ink4a}}$ expression induced the cellular senescence phenotype using senescence-associated acidic β-galactosidase (β-Gal) activity to identify and quantify senescent β-cells. After islets from 5 m old mice were treated with T₃ for 72h, the percentage of β-Gal positive β-cells significantly increased (Figure 7A). This increase in senescent β-cells underscores the physiological importance of T₃-induced $p16^{\text{ink4a}}$ expression and is in line with our previous observation of 4-7% β-Gal⁺ Ins⁺ cells in islets obtained from 3-6 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 to T₃ in the final stages of differentiation of hESC. We have previously published [12] that this differentiation protocol resulted in 17% of C-Peptide⁺ and 9% Glucagon⁺ 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 THRβ. At the end of stage 5 under control conditions, 16% expressed C-peptide whereas 9% expressed glucagon. At the end of stage 5, T₃ treatment resulted in a 20-fold increase in Insulin mRNA and a 5-fold increase in transcription factors MAFA and NKX6.1, 2-fold increase in CPE and a tendency for increase PCSK1 and PCSK2 mRNA. Scattered double hormone positive cells were observed rarely. HESC cells treated with GC1 had significantly decreased $P16^{\text{INK4A}}$ mRNA levels (Figure 7B) with no consistent increase in MAFA. However, 4
out of 5 samples had induction of MAFA and/or MAFB after treatment with GC1 (Supplementary Figure 4). From a functional point of view, GC1 treatment of differentiated hESC increased insulin secretion at 16.8 mM glucose (Figure 7C) and significantly increased insulin content (Figure 7D) than those treated with T3, which we believe resulted from an increase in the amount of insulin per cell as the β–cell matured as we previously showed [11, 12]. These findings underline the translational potential of our findings.

In summary, our results indicate that in pancreatic β-cells, T3 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 thyroid hormone was a physiological driver of β-cell maturation via its induction of the β-cell transcription factor Mafa, the present study suggests that TH is directly involved in β-cell aging via the induction of the senescence- β-cell marker p16^{ink4a}. Further we showed here that these dichotomous effects can occur simultaneously and that these opposing effects on β-cell physiology are due to T3 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 THR 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 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 have failed to demonstrate differences in T3-binding properties [31]. A switch of THR isoforms occurs in postnatal rat β-cell development[11], with predominance of *THRA* mRNA from postnatal day 2 (P2) to P9, then similar levels of *THRA* and *THRB* until P15 and then by a 2:1 dominance of *THRβ* expression through adulthood. At the protein level, as reflected by immunostaining, β-cell nuclei were predominantly stained for THRA through P15 after which nuclear THRβ 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 TH receptor isoform usually expressed in neonatal tissues, is expressed in β-cells from neonatal rodents. However, circulating T3 levels are very low and do not induce *p16Ink4a* at this early age. As circulating and local T3 levels begin to increase as induced by changes in dediodinases expression (increase in Dio2 and a decrease in Dio3 expression), expression of THRβ is induced [11] leading to increased β-cell *Mafa* and acquisition of glucose-responsive insulin secretion. In adult mature β-cells THRβ expression dominates and maintains the high levels of *Mafa* while low levels of THRA allow the senescence effector and mediator *p16Ink4a* expression to be minimal. However, increasing T3 further in adult β-cells does not further increase *Mafa* expression but can induce *p16Ink4a* 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 THRβ1 binding to site 2 TRE of *Mafa* and of THRA to site 5 of *Cdkn2a*. The
specificity of effect on these genes by receptor isoform was consistent with our findings of $p16^{\text{ink4a}}$ induction by a THRA functional agonist CO23, decreased $p16^{\text{ink4a}}$ with Thra siRNA, and induction of $Mafa$ mRNA both by THRβ1 binding to its Site 2 TRE and the functional THRβ 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 THRβ were minimal, we should point out that that only 40-50% of receptor knockdown was achieved in spite of using maximal doses of both DharmaFECT and siRNA. Difficulty to transfect 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 (KD) $p16$ with siRNA in Min6 cells significantly reduced $Igf1r$ levels but $Igf1r$ KD 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 $p16^{\text{ink4a}}$ mRNA, these authors focused on the role of THRβ inducing the $Cdkn2a$ pathway in the tissues with strong THRβ expression. It is important to remember that the $Cdkn2a$ encodes two different senescence effectors $p16^{\text{ink4a}}$ and $p19^{\text{Arf}}$; however, we saw no increased expression of $p19^{\text{Arf}}$ in adult rodent islets.

The induction of $p16^{\text{ink4a}}$ for 10 days in β-cells of 3-4 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 5-6 week old mice may not yet all be fully mature; islets from 3 wk old rats are glucose responsive but without the robustness of a 2-3 month rat [35]. Additionally, in contrast to this recent report, we showed that β-cells that had high levels of $p16^{\text{ink4a}}$ expression had decreased glucose-induced insulin response [20]. By understanding the roles of THRA and THRβ in maturation and senescence of β-cells, we may now be able to discriminate between processes that are regulated during maturation and those of
The dichotomous role of T₃ 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, T₃ accelerates this process, impairing repair mechanisms and limiting longevity. Caloric restriction, which prolongs lifespan, has been shown to decrease circulating T₃ levels, providing additional evidence of the important role of T₃ on aging. The molecular mechanisms behind the aging effects of T₃ are thought to involve the IGF-1/insulin pathway since low T₃ 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 T₃ upon growth hormone are direct since a TRE has been identified in the promoter region of GH [36].

In conclusion, we have shown that T₃ induces both maturation and aging effectors through direct binding of different isoforms of thyroid hormone receptor to the key regulators of these processes, Mafa and p16INK4a. Since the effects are conserved across species and experimental models including humans, our results provide a molecular basis to develop specific highly targeted THRB-based strategies to promote β-cell maturity without accelerating aging.

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CA-M and SB-W conceived the project and wrote the manuscript. CA-M, MM, AD, TBLJ, HB and PR researched data. AMZ, PRL and TS provided THR agonists; AH, CC and AS provided critical discussions and edited the manuscript. All authors reviewed the manuscript.
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**Figure legends**

**Figure 1.** T₃ 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 24-48h of T₃ exposures, respectively. C57Bl/6 16 week old mice, n=4 ; MIN6: n=5 independent samples in duplicate. **B.** Rat islets and INS1 cells had T₃-induced p16_Ink4a mRNA expression after 4 days and 24-48h culture in the presence of T₃. Adult rat islets, n=3 independent samples. INS1 n=4 independent samples. Mean ± SEM; p value respect to baseline. Differentiated hESCs exposed to T₃ during the last step of differentiation were analyzed by qPCR for expression of maturation and senescence markers. T₃ 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 2-4 individual experiments. Mean ± SEM; *p <0.05 with respect to baseline. R and R² values are shown for linear correlations.

**Figure 2.** Kinetics of T₃-induction of Mafa and p16_Ink4a suggest they are both targets of TH. INS1 were incubated in the presence of T₃ for different durations and different doses of T₃ at 24h. **A.** Kinetics of T₃-Mafa induction **B.** Kinetics of T₃-induced p16_Ink4a transcription. INS1 cells, n=3-10 individual samples. Mean ± SEM; *p < 0.05 with respect to control.

**Figure 3.** Potential Thyroid Response Elements (TRE) were identified in the Cdkn2a gene and experimentally tested with chromatin immunoprecipitation (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 respect to IgG. n=13 immunoprecipitation reactions from 4 individual experiments. RPII- RNA Polymerase II.

**Figure 4.** T₃-induction of Mafa and p16_Ink4a are differentially mediated by specific Thr agonists in P7 rat islets. P7 rat islets were treated in vitro with 150pM of either
THRA agonist CO23 or THRB agonist GC1 in CS-FBS RPMI 11.1 mM glucose. 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 value respect to control.

**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 chromatin immunoprecipitation, tested specific binding of THRA and THRBI1 to potential TREs in the *Cdkn2a* and *Mafa* genes. Site 5 in the *Cdkn2a* was enriched with THRA (A) but not with THRBI1 (B), indicating the binding of the THRA isoform. Neither Site 2 or Site 3 in the *Mafa* gene was enriched with THRA (C) but Site 2 was with THRBI1 (D), indicating the specific binding of THRBI1 to Site 2 and not site 3; n= 6-8 samples from 3-4 independent experiments. MIN6 cells. Mean ± SEM; *p<0.05 respect to control.

**Figure 6.** Differential THR isoform induction of *Mafa* and *p16\(^{Ink4a}\)* shown by siRNA. siRNA knockdown of THR receptor isoform transcription was specific and 40% (A. *siThra*; B. *siThrb*) C. Basal levels of *p16\(^{Ink4a}\)* transcription were significantly decreased with *siThra*. D. Basal *Mafa* RNA levels were unchanged with either *siThra* or *siThrb*. E. β-cell aging marker *Igf1r* levels were significantly decreased with both *siThra* and *siThrb*. MIN6 cells. n= 3 experiments, each in triplicate. Mean ± SEM; *p < 0.05 respect to scrambled siRNA (siSc).

**Figure 7.** T\(_3\) induced cellular senescence in mouse islets and hESC treated with GC1 increased insulin secretion at 16.8 mM glucose, 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-galactosidase activity, a marker of cellular senescence. Data presented for FACs sorted β-Gal +cells from individual experiments of islets pooled from 10 mice. THRBI 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), increased insulin secretion in response to 16.8 mM glucose.
glucose (C) and insulin content (D). Absolute values of insulin secretion per experiment (averaged 2-3 samples): 0.02, 0.04 and 0.06 pg Ins/ng DNA for controls and 0.04, 0.04, 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); treated- average 13.7 pg Ins/ng DNA (4, 15.47, 18.32) n=8-9 samples, 3 independent experiments.
Figure 1

43x10mm (300 x 300 DPI)
Figure 2

88x54mm (300 x 300 DPI)

A. Mafa induction

B. p16^{ink4a} induction
Figure 3

69x54mm (300 x 300 DPI)
Figure 4

50x28mm (300 x 300 DPI)
Figure 5

90x45mm (300 x 300 DPI)
Figure 6

41x10mm (300 x 300 DPI)
Figure 7

56x18mm (300 x 300 DPI)
Supplemental table 1. Sequence of primers for real time PCR

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\textbf{Cdkn2a (p16\textsuperscript{\textit{Ink4a}}) potential TREs}

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\textbf{Mafa potential TREs}

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**Supplemental Table 2. Sequence of potential TRE sites in the Cdkn2a gene**

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<td>T3R α</td>
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**Supplemental Table 3. Antibodies for ChIP**

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<tr>
<td>TRb1</td>
<td>Santa Cruz sc-738</td>
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Supplemental figure 1. T₃ did not affect transcription levels of Cdkn2a alternate isoform splicing p19Arf (A), senescence marker p21 (B), or aging markers Bambi (C) and Igf1r (D). Data from same independent samples as Fig 1A and B. C57Bl/6 16 week old mice; n=4; Adult rat islets, n=3 for A. For figures B,C and D samples are from Mouse islets after 4 days and 24-48h of T₃ exposures, respectively. C57Bl/6 16 week old mice, n=3-4

Supplemental figure 2. Comparison of Thra and Thrb mRNA levels in mouse islets and MIN6 cells (A) and between rat islets and INS1 cells (B). Individual independent samples shown. Different housekeeping genes were used for the different species.

Supplemental figure 3. Similar to primary islets, induction of Mafa is selectively mediated by GC1 in INS1 cells. INS1 cells were treated in vitro with 10nM of either THRA agonist CO23 or THRB agonist GC1 in 1.6 mM glucose for 14-24h. n=5 experiments, *p<0.05 respect to control.

Supplemental figure 4. GC1 treatment of hESC induced either MAFA or MAFB. MAFA and MAFB mRNA were measured in hESC subjected to a differentiation protocol that included GC1 instead of T3. In 4 out of the 5 individual samples from 2 independent experiments, either MAFA or MAFB expression levels were higher than in samples differentiated without GC1. This is interesting since it has been shown that unlike rodent adult β-cells that express only MAFA, adult human β-cells (donors aged 17-58 years of age) express both MAFA and MAFB [36]. Lines connect MAFA and MAFB mRNA levels for the same samples.
Mafa

Fold change to control

Control  CO23  GC1 (10 nM)

*