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Thyroid Hormone Promotes Postnatal Rat Pancreatic β-Cell Development and Glucose-Responsive Insulin Secretion Through MAFA

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Neonatal β cells do not secrete glucose-responsive insulin and are considered immature. We previously showed the transcription factor MAFA is key for the functional maturation of β cells, but the physiological regulators of this process are unknown. Here we show that postnatal rat β cells express thyroid hormone (TH) receptor isoforms and deiodinases in an age-dependent pattern as glucose responsiveness develops. In vivo neonatal triiodothyronine supplementation and TH inhibition, respectively, accelerated and delayed metabolic development. In vitro exposure of immature islets to triiodothyronine enhanced the expression of *Mafa*, the secretion of glucose-responsive insulin, and the proportion of responsive cells, all of which are effects that were abolished in the presence of dominant-negative *Mafa*. Using chromatin immunoprecipitation and electrophoretic mobility shift assay, we show that TH has a direct receptor-ligand interaction with the *Maf*a promoter and, using a luciferase reporter, that this interaction was functional. Thus, TH can be considered a physiological regulator of functional maturation of β cells via its induction of *Mafa*. ***Diabetes*** **62:1569–1580, 2013**

β-Cell replacement therapy is a major goal of diabetes research. Insulin-positive cells have been successfully derived from stem cells (1–4), but these cells have not been responsive to glucose in vitro and must be considered functionally immature. Fetal and neonatal rodent β cells also lack glucose responsiveness (5) and therefore provide a good model to study the acquisition of glucose responsiveness and β-cell maturation.

Neonatal β cells are characterized by low expression of many key genes of the specialized β-cell phenotype (6). We previously showed that in neonatal islets, *Mafa* is expressed at ~10% of the adult level and that its adenoviral-mediated reconstitution to adult levels in P2 islets induced secretion of glucose-responsive insulin (7). But what regulates *Mafa* in vivo? To identify physiological regulators, we considered the changes that normally occur in the physiological milieu during the neonatal period (8). During the second postnatal week, serum thyroxine (T_4) (9) and corticosterone (10) surge and prolactin levels rise by postnatal day 20 (11).

We hypothesized that thyroid hormone (TH) could physiologically regulate *Mafa*, enhancing its expression and driving the maturation of the insulin secretory response to glucose in neonatal β cells. TH serum levels start increasing in parallel with *Mafa* expression increases, and TH is important in the postnatal development of the central nervous system and the digestive track (8). Inhibition of TH synthesis prevents or delays maturation of these systems, and TH administration results in precocious development. Triiodothyronine (T_3) has been shown to enhance the differentiation of a human pancreatic duct cell line toward a β-cell phenotype (12). Moreover, thyrotoxicosis leads to hyperinsulinemia with increased hepatic glucose production and insulin resistance (13); hypothyroidism reduces production of hepatic glucose and insulin resistance (14).

The effects of TH are mediated by TH receptors (THRs), which are members of the nuclear receptor superfamily. Three major isoforms—THRA1, THRB1, and THRB2—exhibit similar ligand-dependent regulation of gene activity, whereas a fourth isoform, THRA2, lacks the ligand-binding and transactivation domains (15). Different isoforms have been identified in whole pancreas during development (16), and THRA1 has been identified in adult islet cells (17); however, little is known about their expression in β cells during the postnatal period.

The active TH bound to receptors is 3,5,3′-T_3, and available T_3 is derived from the thyroid gland or from conversion from thyroxine (T_4) by type 1 or type 2 iodothyronine deiodinases (D1 and D2). A third deiodinase, type 3 (or D3), inactivates T_3. D3-null animals, which had higher levels of TH during development, were glucose intolerant with impaired secretion of glucose-stimulated insulin, suggesting that early exposure to high amounts of T_3 might be deleterious to developing β cells (18). However, the role of TH in β-cell development under physiological conditions as well as the mechanisms involved are still unclear.

Herein we show that postnatal rat β cells express THR isoforms and deiodinases in an age-dependent pattern and therefore have the ability to respond to the rapidly rising T_4 concentration that peaks at about postnatal day 15. In vivo neonatal T_3 supplementation and TH inhibition, respectively, accelerated and delayed metabolic development. In vitro exposure of immature islets to T_3 enhanced *Mafa* expression and increased glucose responsiveness, effects that were abolished in the presence of dominant-negative (DN) *Mafa*. Using chromatin immunoprecipitation

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Received 27 June 2012 and accepted 27 November 2012.
DOI: 10.2337/db12-0849
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-0849/-/DC1.

(ChIP) and electrophoretic mobility shift assay (EMSA) we show that TH has a direct receptor-ligand interaction with the *Mafa* promoter; using a luciferase reporter, we then showed that this interaction is functional. Thus, TH is a physiological stimulus for the postnatal maturation of functional β cells.

RESEARCH DESIGN AND METHODS

Animals. Female Sprague-Dawley rats with litters of various ages (P0 is day of birth) were purchased from Taconic Farms (Germantown, NY) and kept under conventional conditions with free access to water and food. Animals were killed under anesthesia at postnatal days 2–28 or as an adult; blood was collected by cardiac puncture, and pancreas was excised for histology or islet isolation. Islets were isolated (19), cultured overnight in RPMI-1640 medium, and were handpicked to ensure purity. For each sample from postnatal days 2 or 7, islets from 10 pups were pooled; for samples from postnatal days 9 to 28, islets from 2 or 3 pups were pooled; and for the adult sample, islets from one animal were used. Three to six samples per age group were included. For immunostaining, excised pancreas was either fixed for 2 h in 4% paraformaldehyde for paraffin embedding or embedded in optimal cutting temperature medium (Tissue Tek) and was frozen in chilled isopentane. The Joslin Institutional Animal Care and Use Committee approved all animal procedures.

Plasma insulin and T_4 levels. Plasma insulin and T_4 levels were measured using enzyme-linked immunoassay (ALPCO, Windham NH) and COAT-A-COUNT total T_4 kit (DPC, Los Angeles, CA), as previously described (20).

Neonatal models of T_3 supplementation and inhibition of TH synthesis. Timed-pregnant Sprague-Dawley rats were randomized into one of three groups:

1) Control pups received subcutaneous injections of 0.9% NaCl daily for 7 days, starting at postnatal day 1.
2) For inhibition of TH synthesis, rats were given tap water ad libitum with 20 mg methimazole (MMI)/100 mL water (21) from birth to postnatal days 15 or 21, when the pups were killed.
3) For T_3 supplementation, pups received subcutaneous injections of T_3 (0.05 μg/g body weight) daily for 7 days starting on postnatal day 1 (22).

Body weight and fed glucose levels were measured weekly. For intraperitoneal glucose tolerance tests, glucose solution was injected intraperitoneally (2 g/kg) after fasting for 4 h, and blood glucose levels were determined at 0, 30, and 120 min. Islets were isolated from T_3-treated and control rats at postnatal day 7 and reported as in vivo.

The effectiveness of treatments was evaluated by growth parameters, T_4 levels, and deiodinase activity when the animal was killed (23) (Table 1). Hepatic D1 activity was measured in 5- to 20-mg liver homogenate with 10 mmol/L dithiothreitol and 1 μmol/L $^{125}IrT_3$ for 60 min. Background levels were determined by the addition of 1 mmol/L propylthiouracil (20).

Islet culture: Hormone treatment and adenoviral infection. At postnatal day 7 or 9, islets were cultured for 4 days in RPMI-1640 medium (20 mmol/L glucose and 10% charcoal-stripped [CS] FBS) with T_3 (150 pmol/L, equivalent to 7.5 pmol/L free T_3 in 10% CS-FBS [24]) for RNA or insulin secretion assays. As an alternative, islets at postnatal day 9 were cultured for 2 days in RPMI-1640 medium in 10% FBS with dexamethasone (50 pmol/L) for RNA.

To test the specificity of MAFA as the mechanism of T_3 effect, islets at postnatal day 7 were totally dispersed and cultured in RPMI-1640 medium (20 mmol/L glucose, 10% CS-FBS) with or without T_3 (7.5 pmol/L free T_3) and with or without adeno-*CMV*-DN *Mafa-Ires-Gfp* (DN-*Mafa*) (25,26) (MOI 2) or control adeno-*CMV-Ires-Gfp* (Ad-*Gfp*). Reaggregated islets were cultured for 4 days for secretion or RNA.

Insulin secretion in vitro. Insulin secretion was measured by static sequential incubation in 2.6 mmol/L and 16.8 mmol/L glucose in Krebs-Ringer bicarbonate buffer (16 mmol/L HEPES and 0.1% BSA; pH 7.4), as previously described (27). Supernatants and cells were frozen until assayed. As an alternative, we measured insulin secretion of single β cells using the reverse hemolytic plaque assay (7,28) in which secreted insulin is revealed by the presence of hemolytic plaques around secreting cells. The percentage of insulin-secreting cells forming plaques and the area of the plaques were measured and multiplied to calculate the secretion index, a measure of the overall secretory activity of β cells.

Quantitative real-time PCR. Total RNA was isolated with a PicoRNA extraction kit (Arcturus) and reverse transcribed (SuperScript reverse transcriptase, Invitrogen, Grand Island, NY). Real-time quantitative PCR used SYBR green detection and specific primers (Supplementary Table 1). Samples were normalized to a control gene (S25), and the comparative threshold cycle method used to calculate levels of gene expression.

Immunostaining. Paraffin sections were incubated at 4°C overnight with primary antibodies listed in Supplementary Table 2. Apoptosis was assessed by TUNEL staining using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Indianapolis, IN) after retrieval of microwave antigen. DAPI was used for nuclear staining.

For each antigen, all images were taken with the same settings in confocal mode using a Zeiss LSM 410 or LSM 710 microscope and were handled similarly in Adobe Photoshop; at least two animals per age were examined.

Morphometric evaluation. Images covering entire whole pancreatic sections were collected using the LSM 710 tile-scan system. For MAFA nuclear staining, insulin-positive cells were scored as high, low, or undetected nuclear MAFA. Data from four or five individual animals were averaged; between 36 and 58 islets were sampled for each age. For proliferation and apoptosis, insulin-positive and Ki-67-positive or TUNEL-positive cells were counted in 160–327 islets and 2,764–6,189 cells per group; data from individual animals (n = 3–5) were averaged. β-Cell mass was calculated by multiplying the relative area of β cells by the pancreatic weight (29). The densitometric mean of the intensity of MAFA staining was calculated using the AxioVision Measure feature and were considered a reflection of protein levels (n = 6–7 animals per group). Cross-sectional area of the β cell was used as an indicator for cell size and was determined by dividing the number of nuclei in a given insulin-positive area; 7,000–8,794 nuclei were counted among four animals in each group.

Cell line culture. INS-1 cells maintained in RPMI-1640 medium containing 11 mmol/L glucose + 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 medium containing 1.6 mmol/L glucose + 10%

TABLE 1
Effects of thyroid supplementation or diminished TH synthesis with or without partial replacement on body and pancreatic weight and an index of thyroid status

	Thyroid supplementation (n = 9–32)			
	Control at P7	Treated with T_3 at P7*	Control at P11	Treated with T_3 at P11†
Body weight (g)	15 ± 0.3	17 ± 0.4‡	21 ± 0.3	23 ± 1§
Pancreas weight (mg)	50 ± 10	80 ± 2‡	70 ± 4	100 ± 7§
D1 liver activity (pmol/min/mg)	21 ± 3	36 ± 4‡	41 ± 6	73 ± 15§
T_4 serum levels (mg/dL)	2.0 ± 0.4	0.3 ± 0.2‡	2.12 ± 0.5	0.2 ± 0.09‡
	Diminished TH synthesis (n = 4–5)			
	Control at P15	P15 + MMI	Control at P21	P21 + MMI
Body weight (g)	33 ± 1.3	27 ± 1¶	46 ± 2.5	36 ± 3.1§
Pancreas weight (mg)	140 ± 2	120 ± 5¶	410 ± 2	110 ± 3¶
D1 liver activity (pmol/min/mg)	62 ± 5	48 ± 6	176 ± 15	48 ± 9¶
T_4 serum levels (mg/dL)	6 ± 0.3	ND	3.0 ± 0.3	0.8 ± 0.3¶

Values are presented as means ± SEM. * Thyroid supplementation model (P1–P7). †Thyroid supplementation model (P1–P11). §$P \leq 0.04$ with respect to age-matched controls. ‡$P \leq 0.004$ with respect to age-matched controls. ¶$P \leq 0.00001$ with respect to age-matched controls. ND, no data; P, postnatal day.

CS-FBS 24 h before treatment and then were kept for 14 h in T_3 (150 pmol/L) and actinomycin D (5 mg/mL). MIN-6 cells, maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS, were switched to high-glucose DMEM with 15% CS-FBS and T_3 (10 nmol/L) for 24 h; they then were harvested for ChIP or to assess the effect of MMI (1.5 mmol/L, 0.15 mmol/L, and 0.015 mmol/L) on gene transcription.

For the luciferase reporter construct, a 10-Kb Nhe1-Fse1 fragment from the BAC mouse genomic library clone 128L24 spanning the mouse *Mafa* promoter to downstream of the transcription start site was cloned into *Hind*III-*Bgl*II cut –238 wild-type luciferase plasmid (30). *Renilla* luciferase in a *SacI* backbone was used as a transfection control. For transient transfections, MIN-6 cells were transfected using lipofectamine and 7 μg of final DNA and grown in high-glucose DMEM with/without 100 nmol/L T_3 for 24 h.

ChIP assay. Rabbit anti-THR (TRa/b, Santa Cruz FL-408×) was used with the Imprint ChIP kit (Sigma Aldrich, St. Louis, MO; CHP1) by following manufacturer's instructions. DNA from 250,000 cells was used for each condition in four independent experiments. Samples were analyzed by quantitative PCR using specific primers for three putative thyroid response elements (TREs; S1, S2, and S3) (Supplementary Table 1) and were run on gels.

Gel-mobility shift assay (EMSA). Nuclear extracts were obtained from HEK1 cells transfected with *Thrb*, or untransfected cells as controls, using the NucBuster Protein Extraction Kit (Novagen, EMD Biosciences, San Diego, CA) following the manufacturer's instructions. Twenty micrograms of nuclear extract were incubated with 80 pmol/L of double-stranded oligonucleotides, reproducing the potential TRE S3 in the rat *Mafa* gene or the reported TRE of D1 (31) at room temperature for 30 min. The reaction buffer for binding was 75 mmol/L Tris (pH 7.8), 264 mmol/L potassium chloride, 1.5 mmol/L EDTA, 30 mmol/L β-mercaptoethanol, 30% glycerol, and 1.2 mg/mL BSA. In some of the binding assays, anti-THRB antiserum (Affinity Bioreagents, Golden, CO) or a random antibody were added 1 h before addition of the DNA probes. After the binding reactions, samples were analyzed by separation on 10% polyacrylamide gel in 1× Tris-acetate-EDTA buffer followed by 40 min of staining with SYBR green EMSA nucleic acid gel stain (Molecular Probes).

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 a Bonferroni post hoc test were used to compare more than two groups. A P value <0.05 was considered statistically significant.

RESULTS

Postnatal glucose and insulin levels change concurrently with expression of TH, deiodinases, and THR. Blood glucose levels at postnatal day 2 were significantly lower than levels in adults (Fig. 1*A*), but they increased, peaking at postnatal day 21 at a level 20% higher than that of adults. Perhaps as a result of rising plasma glucose levels and as part of the functional maturation process of β cells, plasma insulin levels peaked at postnatal day 11, with levels threefold higher than in newborns or adults (Fig. 1*B*). During the first postnatal month (Fig. 1*C*), the rate of glucose disposal evaluated by the area under the curve (AUC) of intraperitoneal glucose tolerance test was progressively enhanced; that at postnatal days 7 and 11 were slower (high AUC values) than at later ages. Adult values were not yet achieved by postnatal day 21.

For TH to physiologically regulate the functional development of β cells, its serum level and the tissue regulation of biologically active T_3 must be sufficient and its receptors must be expressed by β cells. Serum T_4, the principal circulating TH, steadily increased over the first 2 weeks, peaking at about twice adult levels at postnatal day 15 before falling to adult levels by postnatal day 21 (Fig. 1*D*). Because tissue concentrations of T_3 are determined by deiodinases as well as by serum T_3, we measured the islet expression of deiodinases *Dio1*, *Dio2*, and *Dio3* transcripts during postnatal development (Fig. 1*E*). *Dio1* mRNA in islets increased throughout the postnatal period; its positive regulation by TH provides an increased paracrine supply of T_3 as the islet develops. In contrast, expression of both *Dio2* and *Dio3* fell during the postnatal period.

D1 and D3 proteins were examined by immunostaining (Supplementary Fig. 1) in pancreas from animals at postnatal day 7 and adult animals. The small number of islets obtained from rats at postnatal day 7 precludes protein quantification using Western blots. However, careful titration of antibody, parallel staining, and confocal imaging allowed assessment of protein levels by differences in intensity. Islet D1 protein was much lower at postnatal day 7 than in adults (Supplementary Fig. 1*A*), although its mRNA level did not differ. This discrepancy might reflect developmental differences in the synthesis of selenoproteins that occur downstream of transcription (32). In contrast, D3 (Supplementary Fig. 1*B*) had very strong cytoplasmic localization in islets at postnatal day 7 and became almost undetectable in adult islets.

As serum T_4 increased, islet expression of thyroid receptors increased at both mRNA and protein levels. *Thra* mRNA increased sharply between postnatal days 7 and 9; they were sixfold higher in islets at postnatal day 9 than in adults (Fig. 1*F*) and decreased to adult levels by postnatal day 15. By immunostaining (Fig. 2*A*), nuclear THRA protein (both the T_3-binding THRA1 and the non-T_3-binding THRA2 isoforms are recognized by the antibody) increased from postnatal day 7 to day 10 but became much lower by adulthood (Fig. 2*C*). Similarly, *Thrb* mRNA increased between postnatal days 7 and 9 and decreased to adult levels by postnatal day 15 (Fig. 1*F*). THRB protein also increased by postnatal day 7 but was mainly cytoplasmic; nuclear THRB localization was seen only by postnatal day 15 and increased in the adult (Fig. 2*B* and *C*). Comparison of mRNA expression of *Thra* and *Thrb* in islets (Supplementary Fig. 2) shows a change in predominant receptor isoform through development: *Thra* predominates at early ages, *Thra* and *Thrb* are equal from postnatal day 9 to 15, after which *Thrb* becomes the predominant isoform in islets.

T_3 supplementation until postnatal day 7 accelerated metabolic development. To analyze the direct effects of TH, newborn rats were injected with T_3 from postnatal days 1 to 7. As expected, this supplementation increased body and pancreatic weights, reduced T_4 levels (because of suppression of thyrotropin by T_3), and increased D1 activity in the liver (Table 1). The pancreas of animals treated with T_3 had greater density of both acinar and islet cells (Fig. 3*A*), greater β-cell proliferation (20% vs. 8% Ki-67$^+$insulin$^+$ cells in untreated animals) (Fig. 3*B* and *C*), and no change in the frequency of apoptotic β cells (Fig. 3*D*). Their β-cell mass was unchanged (Fig. 3*E*), but their β cells were smaller (Fig. 3*F*), with no significant change in the number of cells (Fig. 3*G*). Fasting glucose levels were lower (Fig. 3*H*) and fed plasma insulin levels elevated (Fig. 3*I*), but their response to intraperitoneal glucose tolerance tests were not different (data not shown). These results show that T_3 supplementation enhanced the functional development of β cells.

Inhibition of TH synthesis delayed pancreatic development. Animals treated with MMI from birth until postnatal day 15 or 21 were confirmed as having hypothyroidism because of delayed growth with lower body and pancreatic weights, decreased circulating T_4 (due to inhibition of TH synthesis from the thyroid gland), and decreased activity of D1 in the liver (Table 1). At postnatal day 15 there was no change in pancreatic cell density (Supplementary Fig. 3*A*), β-cell proliferation (Supplementary

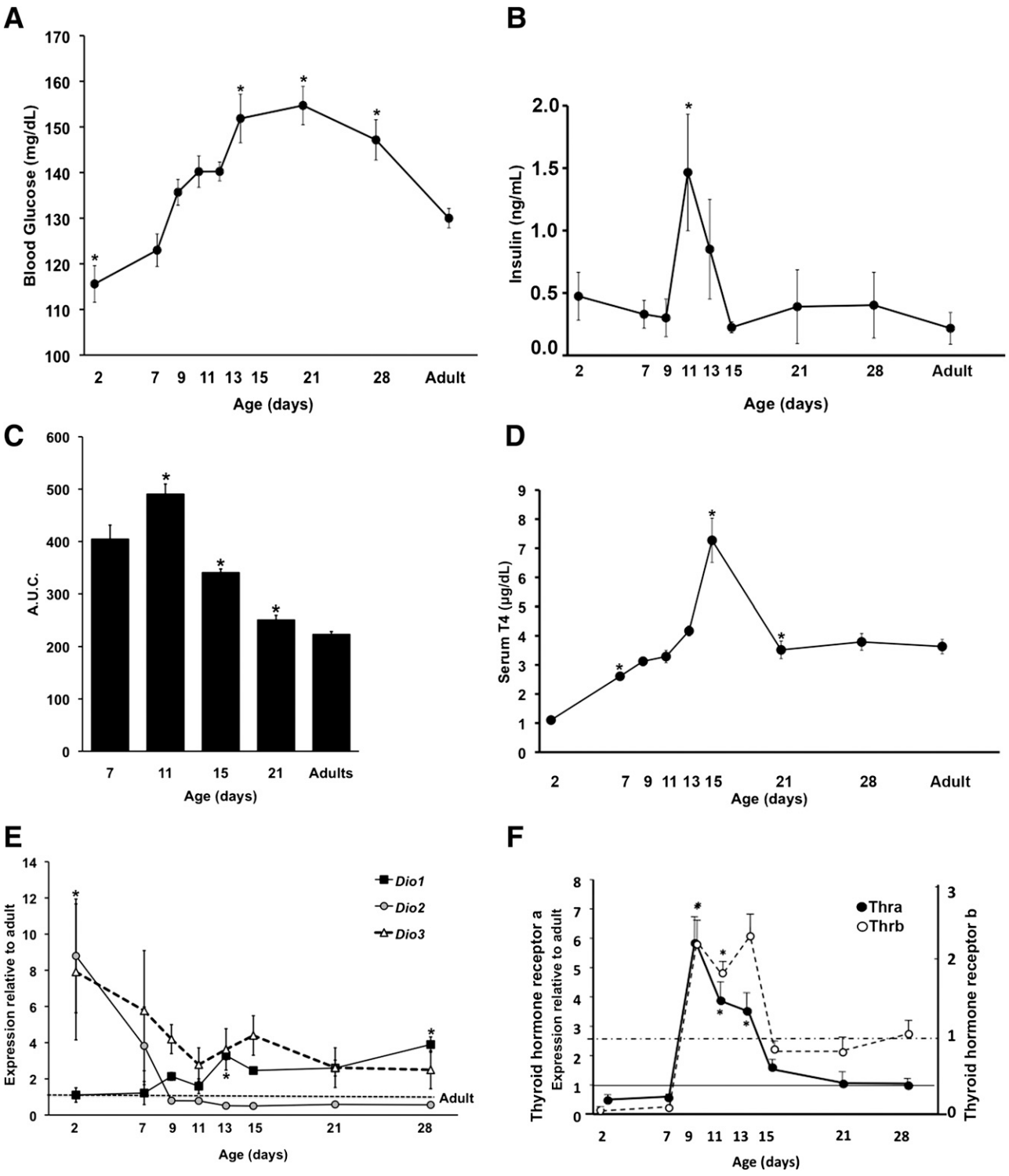

FIG. 1. Metabolism and thyroid status change over the first postnatal month. Values shown as means ± SEM. *A*: Fed blood glucose (n = 5–27 animals per age; *P < 0.0001); *B*: plasma insulin (n = 4 animals per age; *P < 0.04). AUC for intraperitoneal glucose tolerance test (*C*) and serum T_4 (*D*) seen at different ages; n = 5–27 animals per age, *P < 0.0001 with respect to the previous age in *C* and *D*. Deiodinase (*Dio1*, type 1; *Dio2*, type 2; *Dio3*, type 3) (*E*) and thyroid receptor isoform mRNA (*F*) over the same time course by quantitative PCR; the same samples are used for both. Data are expressed as -fold change with respect to adult levels (10 weeks old) using *S25* as the internal control gene (n = 4–6 samples per age, each pooled from 3–10 animals; *$P \leq 0.0001$).

Fig. 3*C*) or β-cell mass (Supplementary Fig. 3*B*). However, animals treated with MMI at postnatal days 15 and 21 had lower fasting blood glucose levels than their untreated age-matched controls (Supplementary Figs. 3*D* and 4*A*), similar to that of younger animals (Fig. 1*A*). Compared with untreated controls, plasma insulin levels of animals treated with MMI were lower at postnatal day 15 (Supplementary Fig. 3*E*) but were fivefold higher at postnatal day 21 (Supplementary Fig. 4*B*); values were similar to those at postnatal day 11, when plasma insulin levels peak (Fig. 1*B*). The

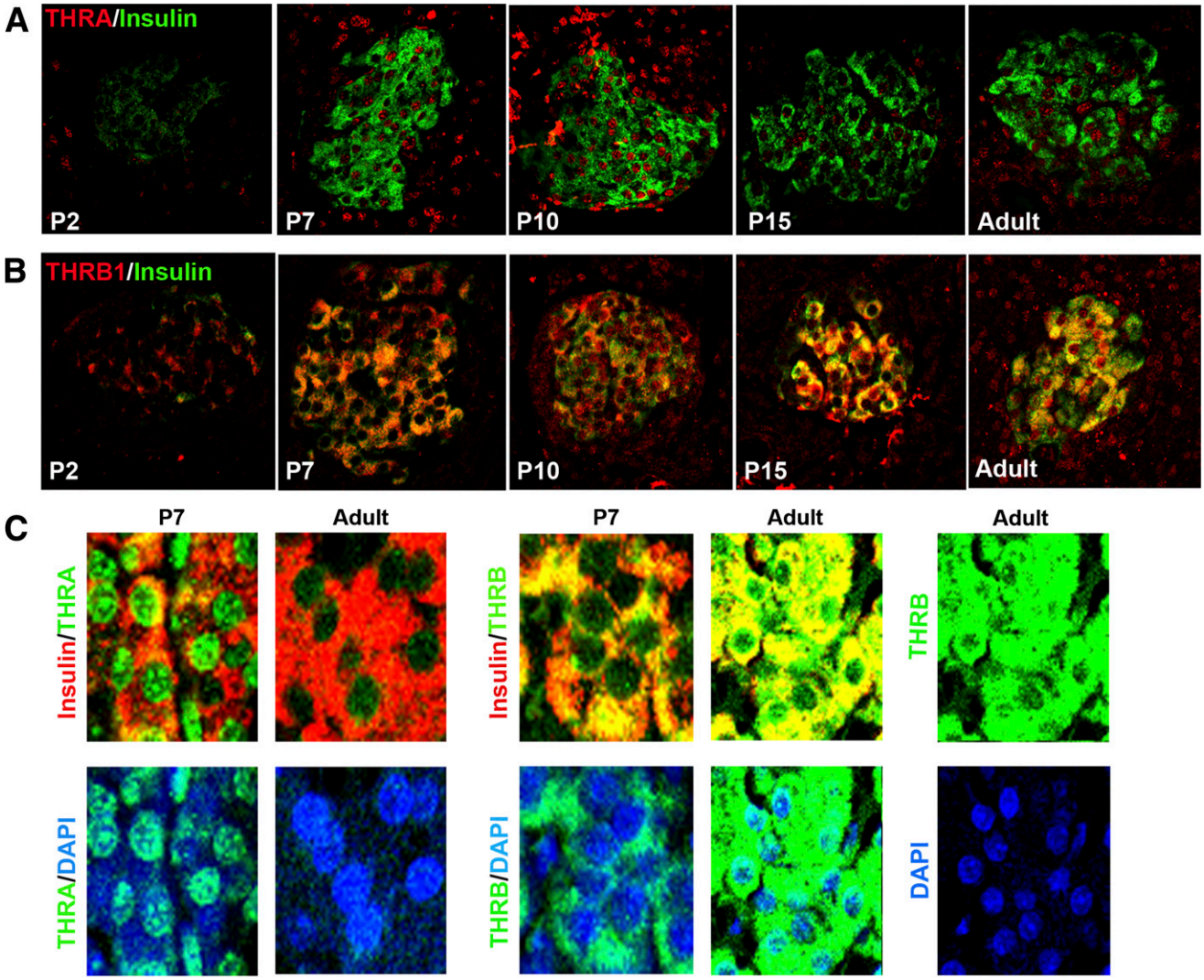

FIG. 2. Changing pattern of THR isoform proteins over the neonatal period shown by immunostaining. THRA protein (*A*, red) and THRB1 protein (*B*, red) in insulin-expressing (green) β cells change in intensity and location from postnatal day (P) 2 to adult. *C*: (*top*) Nuclear localization of THRA or THRB (green) in insulin-positive cells (red) shown at a higher magnification and costained with nuclear stain DAPI (blue); *C*: (*bottom*) red channel deleted for visualization of THR. Representative confocal images taken in parallel at the same settings for each protein so the differences in intensity reflect the differences in protein. *n* = at least three animals per group.

gradual postnatal development of glucose clearance was delayed by inhibiting T_4 synthesis (Supplementary Fig. 3*G* and Fig. 1*C*). Animals treated with MMI at postnatal day 21 had glucose clearance similar to untreated animals at postnatal day 7 (Fig. 1*C*). Animals treated with MMI at postnatal day 15 were not as affected, possibly because of shorter treatment and differences in T_3 sensitivity of other glucose-disposing tissues. Overall, the lack of TH during the postnatal period delayed the development of efficient glucose disposal.

Local regulation of TH action in postnatal rat islets by T_3 or MMI treatment. Systemic changes in TH status can be regulated by local changes in T_3 concentrations by deiodinases in target tissues. Changing levels and isoforms of thyroid receptors also potentially regulate local TH effects. Islets from animals treated with T_3 at postnatal day 7 had increased nuclear THRA (Fig. 4*A*) and cytoplasmic THRB protein (Fig. 4*C*), suggesting more functional receptors. In animals treated with MMI at postnatal day 21 (Fig. 4*B* and *D*), both THRA and THRB had the nonfunctional cytoplasmic localization typical of younger ages. Deiodinase changes also reflected the hormonal status, with increased *Dio1* and *Dio3* mRNAs in islets after T_3 treatment (Fig. 4*E*) and reduced *Dio1* and *Dio3* and elevated *Dio2* mRNA levels (Supplementary Fig. 3*F*) after MMI treatment until postnatal day 21. This confirmation that normal neonatal changes in deiodinases and thyroid receptors were replicated by external manipulation of TH status suggests that the endogenous events are causally related to the TH status.

In vivo T_3 modulation affected *Mafa* gene expression and its protein nuclear translocation. Islet transcriptional profile changed with in vivo T_3 treatment (Fig. 4*E* and *F*): *Mafa*, *Mafb*, *Pgc1a* (peroxisome proliferator–activated receptor γ coactivator-1 α), and *Thrb* mRNA increased; *Pdx1* and *Neurod1* mRNA were unchanged; and *Rest* (RE1-silencing transcription factor) mRNA decreased. In contrast, *Mafa* decreased at postnatal day 15 with MMI treatment (Supplementary Fig. 5*D* and Supplementary Table 3); partial T_3 supplementation reversed this decrease. *Mafb*, preproinsulin, *Ins2*, *Glp1r*, and *Thra* mRNAs increased and *Rest* mRNA decreased in P15 islets (Supplementary Table 3). *Rest*, *Thra*, and *Thrb* mRNAs

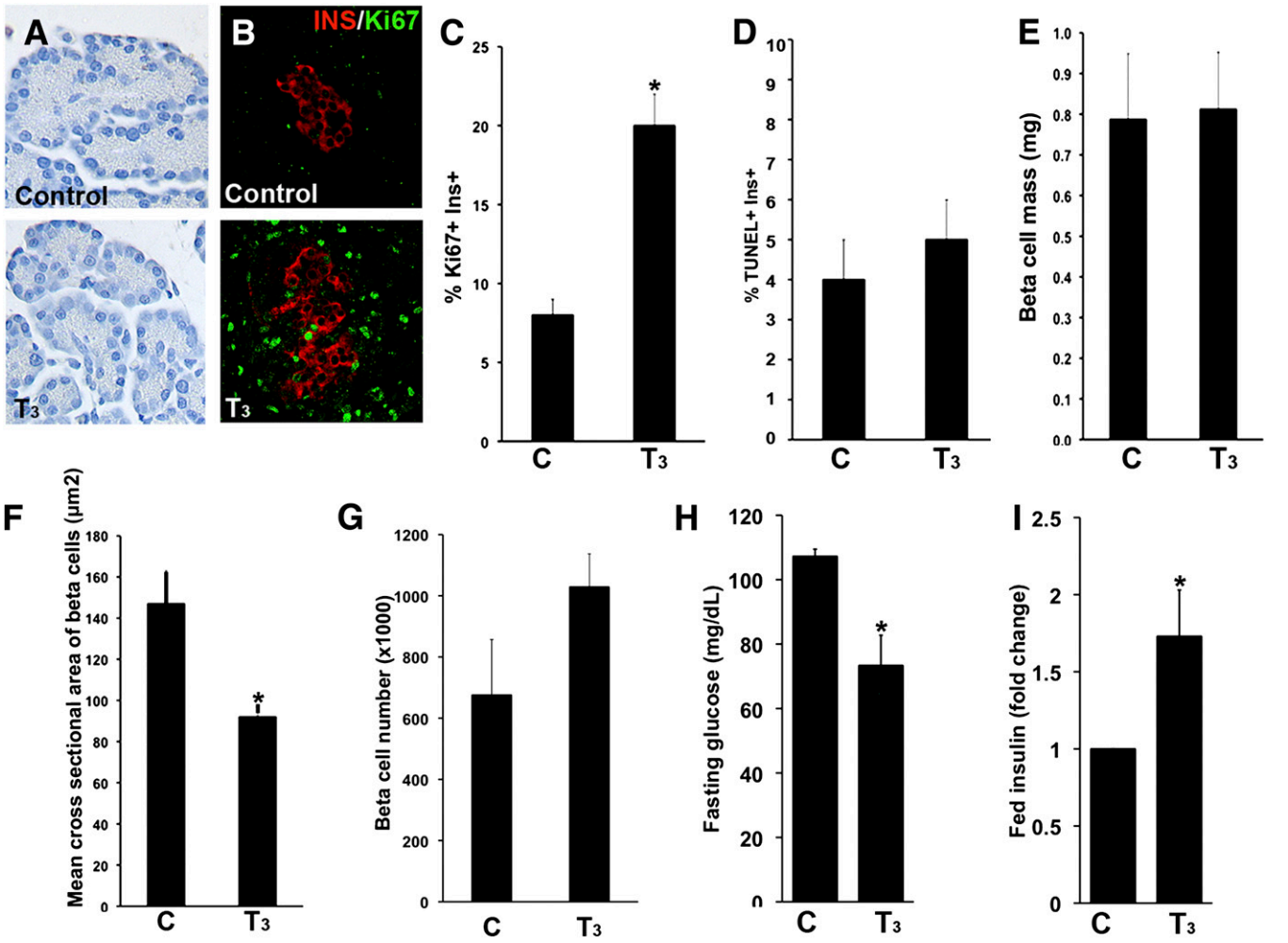

FIG. 3. In vivo T_3 treatment from birth until postnatal day (P) 7 affects pancreatic structure, β-cell dynamics, and glucose homeostasis. Representative pictures of acinar cell density (*A*, hematoxylin stained) and replicating β cells (*B*, insulin [red] and Ki-67 [green]) in pancreatic sections from T_3-treated and control rats at P7. β-Cell proliferation (Ki-67$^+$; n = 160–327 islets) (*C*); apoptosis (TUNEL staining; n = 160–178 islets) (*D*); β-cell mass (n = 6–7 animals) (*E*); mean cross-sectional area (cell size; n = 7,000–8,794 cells per condition) (*F*); calculated number of β cells per pancreas (n = 3–4 animals) (*G*); fasting blood glucose (n = 8–9 animals) (*H*); and fed plasma insulin (n = 21–22 animals; controls: 0.4 ± 0.03 vs. T_3 treated: 0.5 ± 0.04 ng/mL) (*I*) levels for T_3-treated and control animals at P7. Values shown as mean ± SEM; *P < 0.01 with respect to untreated animals.

increased at postnatal day 21 (Supplementary Table 3). Insulin staining was more intense in the T_3-treated group and less intense in MMI-treated animals (Fig. 5*A*).

T_3 supplementation increased MAFA protein both in nuclear location (Fig. 5*A*) and amount (Fig. 5*B*). In pups treated with T_3 at postnatal day 7, 93% of β cells had nuclear MAFA compared with only 62% in untreated pups (Figs. 5*A* and *C*); 51% of T_3-treated pups had MAFA^{high} nuclear staining compared with 10% of untreated animals. The observed effect of T_3 on MAFA translocation could be an indirect effect mediated by altering the redox state of the cell (33) through enhancement of mitochondrial function following increased expression of *Pgc1a* (Fig. 4*C*). It is surprising that even with the increased nuclear expression of MAFA, glucose-stimulated insulin secretion was not increased in islets from T_3-treated animals (Fig. 5*D*). In contrast, hypothyroidism at postnatal day 15 decreased *Mafa* mRNA levels (Supplementary Fig. 5*D*) and protein (Supplementary Fig. 5*A* and *B*) without changes in location (Supplementary Fig. 5*C*). MMI treatment also decreased β-cell function as estimated by homeostasis model assessment-B index (Supplementary Fig. 5*E*) (18). The direct effect of MMI on transcription was ruled out because no significant changes were found in the above genes after culturing MIN-6 cells in the presence of MMI (Supplementary Fig. 5*F*).

In vitro T_3 specifically increased *Mafa* mRNA and improved glucose-stimulated insulin secretion. Because the in vivo data may be confounded by the effects on insulin sensitivity by thyrotoxicosis or hypothyroidism (14), we developed an in vitro system to directly test whether increasing *Mafa* was the mechanism mediating the T_3-maturation effect. DN MAFA lacks an *N*-terminal transactivation domain and can form heterodimers with endogenous MAFA, impairing its ability to activate target genes (25). In T_3-treated Ad-*Gfp* transduced islets, *Mafa* mRNA increased significantly (Fig. 6*A*), confirming the in vivo T_3 results (Fig. 4*F*); glucokinase mRNA also increased. It is important to note that these increases were absent in T_3-treated islets infected with Ad-DN-*Mafa*, indicating a specific role of MAFA in T_3-mediated effects. Interestingly, the T_3-induced *Mafa* increase was inhibited in the presence of DN-*Mafa*, suggesting a positive feedback mechanism of MAFA upon its own expression.

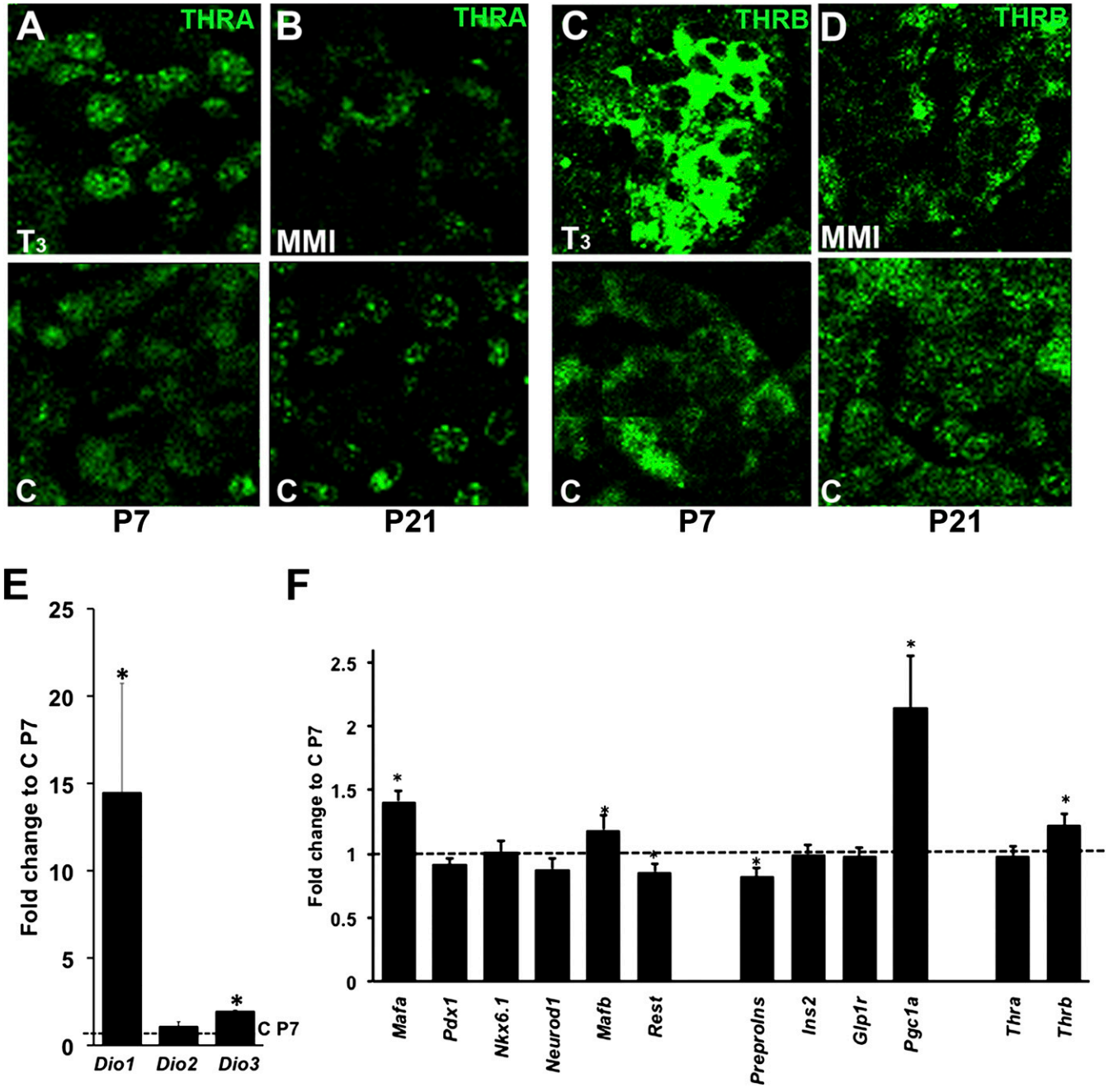

FIG. 4. T_3 treatment results in differential changes of thyroid receptor proteins and islet gene expression. Islets from T_3-treated and untreated animals at postnatal day (P) 7 immunostained for THRA (*A*) and THRB (*C*) or from MMI-treated rats at P21 stained for THRA (*B*) and THRB (*D*). Representative confocal images were taken at the same settings for each protein so differences in intensity reflect differences in protein. At least three animals each group. Changes in *Dio1*, *Dio2*, and *Dio3* ($n = 3$) (*E*) and key islet gene ($n = 18$) (*F*) mRNA in islets isolated from T_3-treated and untreated control pups by quantitative PCR at P7. Values shown as mean ± SEM; *$P < 0.05$ with respect to control animals at P7.

In vitro T_3-treatment induced glucose-stimulated insulin secretion. In static incubations at postnatal day 7, islets, whether untreated or Ad-*Gfp* infected, had little response to 16.8 mmol/L glucose (Fig. 6*B*), whereas T_3-treated islets, both uninfected and Ad-*Gfp* infected, had insulin secretion increased 5.5-fold (Fig. 6*B*). This effect also was abolished in the presence of Ad-DN-*Mafa*. Using the reverse hemolytic plaque assay to evaluate insulin secretion from individual β cells (Fig. 6*C*), the effect of T_3 on glucose responsiveness was shown to result from an increased proportion of insulin-secreting cells (Fig. 6*D*).

Glucocorticoids inhibit the effects of T_3 on glucose-stimulated insulin secretion. Paradoxically, glucose-stimulated insulin secretion was not increased in islets from animals treated with T_3 in vivo (Fig. 5*D*), as was expected from our in vitro studies (7) and those described earlier. However, T_3 affects the maturation of other tissues, including the adrenal gland (34), so the blunted in vivo glucose responsiveness may have resulted from increased glucocorticoids. In untreated animals, circulating corticosterone was quite low after birth and increased only modestly by postnatal day 15 and then surged to adult levels (Supplementary Fig. 6*A*). Glucocorticoid receptor

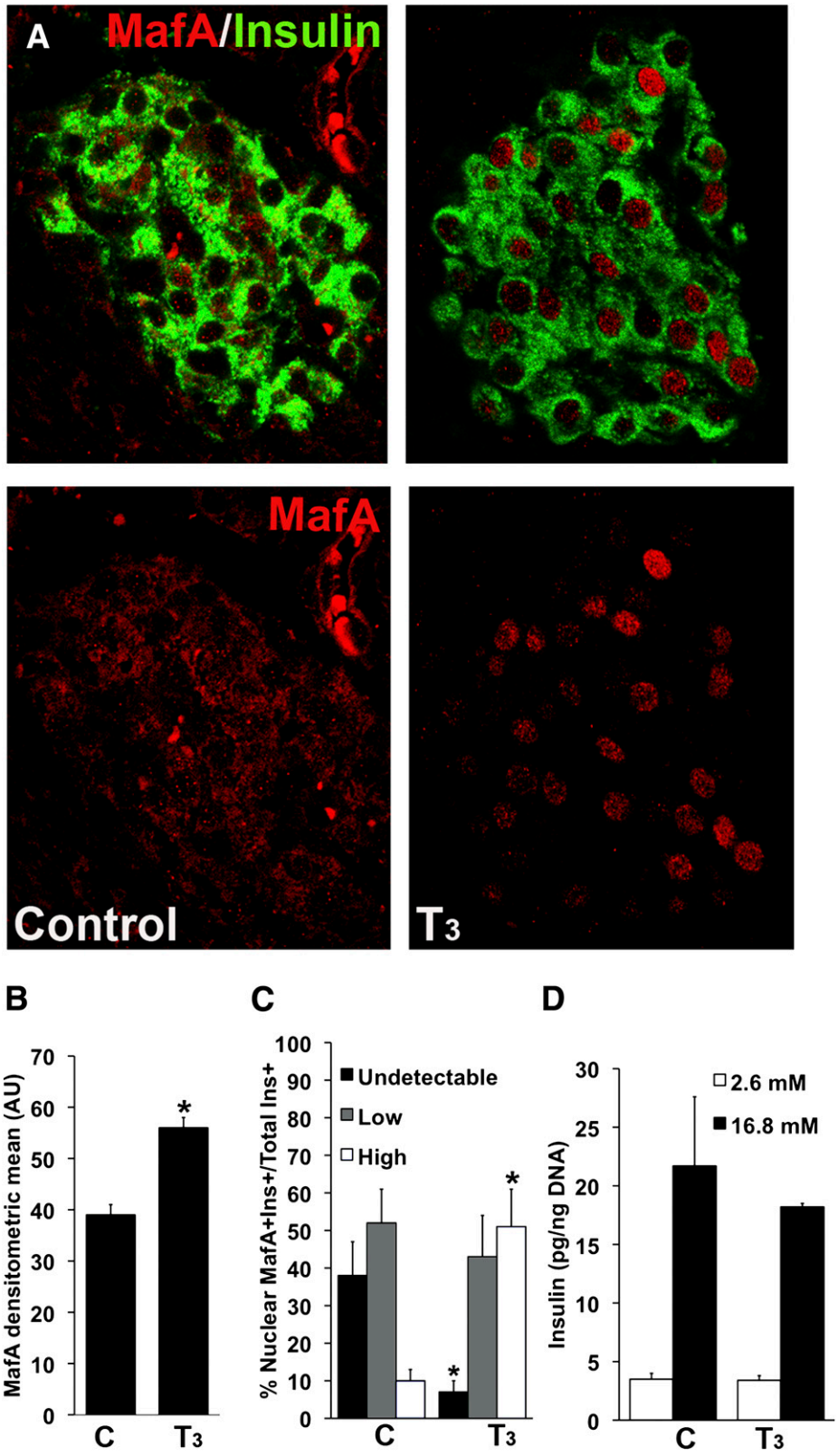

FIG. 5. In vivo T_3 treatment increased *Mafa* expression and enhanced its nuclear localization but did not increase glucose-stimulated insulin secretion. *A*: Representative images from T_3-treated and control animals at postnatal day (P) 7 immunostained for insulin (green) and MAFA (red); bottom panels show only the red channel for MAFA visualization. *B*: Intensity was quantified as densitometric mean of MAFA staining from at least three animals per group. *C*: Nuclear localization of MAFA in islets of animals treated with T_3 increased compared with untreated controls at P7. Quantification of 600–2,200 insulin$^+$ cells from four or five animals for each group. *D*: Glucose-stimulated insulin secretion in static incubations of islets freshly isolated from in vivo T_3-treated animals at P7 or control animals (n = 5–6 experiments). Values shown as mean ± SEM; *$P < 0.02$ with respect to controls.

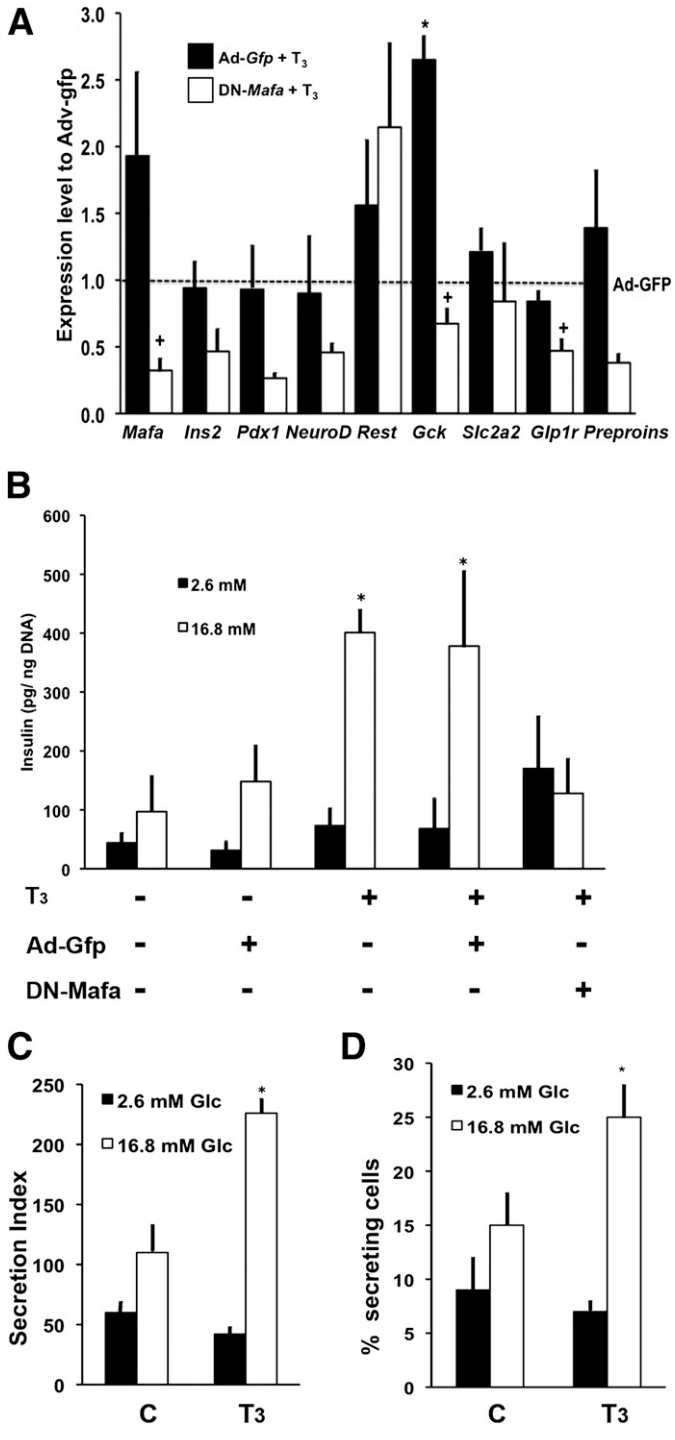

FIG. 6. In vitro T_3 selectively increased *Mafa* mRNA and enhanced glucose-stimulated insulin secretion. *A*: Culture for 4 days with T_3 (7.5 pmol/L free T_3) induced changes in key islet mRNA levels in islets isolated from animals at postnatal day (P) 7 and infected with either control Ad-*Gfp* (black bars) or DN *Mafa* (white bars) as normalized to those of Ad-*Gfp* without T_3 treatment. (*$P < 0.04$ respect to Ad-*Gfp*; +$P < 0.05$ respect to Ad-*Gfp*+T_3; $n = 4–7$). *B*: Insulin secretion from similarly infected and cultured islets at P7 in response to 2.6 mmol/L glucose (black bars) and 16.8 mmol/L glucose (white bars) in sequential static incubations (seven experiments for Ad-*Gfp* +T_3 and four for Ad-*Gfp* +T_3 +DN-*Mafa*; *$P < 0.04$ with respect to Ad-*Gfp*). *C*: T_3-enhanced glucose-responsive insulin secretion was confirmed by reverse hemolytic plaque assay for individual cell secretion, showing an increased percentage of insulin-secreting cells (*D*) (three experiments; *$P \leq 0.006$ with respect to 2.6 mmol/L glucose). Values shown as mean ± SEM.

mRNA in islets remained unchanged from embryonic day 20.5 to postnatal day 28 (Supplementary Fig. 6*B*). However, with T_3 supplementation, corticosterone levels at postnatal day 7 were twice the normal values and were comparable with those at postnatal day 11 (Supplementary Fig. 6*C*). In contrast, after MMI treatment, corticosterone levels were reduced at postnatal day 21 (Supplementary Fig. 6*D*). These data indicate a rapid T_3-dependent change in adrenal function that could affect in vivo islet function over the neonatal period.

To assess whether increased glucocorticoids explained the difference in functional maturation of in vitro and in vivo T_3 treatments, islets at postnatal day 9 were cultured for 48 h with T_3, dexamethasone, or both. In islets cultured with T_3 alone, *Mafa* mRNA was increased significantly (Supplementary Fig. 6*E*), consistent with islets from animals treated with T_3 at postnatal day 7 (Figs. 4*F* and 6*A*). However, in islets cultured in the presence of dexamethasone, with or without T_3, *Mafa* levels remained unchanged and *Pdx1* mRNA was significantly suppressed compared with untreated controls (Supplementary Fig. 6*E*). Insulin secretion was blunted in similarly cultured islets at postnatal day 9 (Supplementary Fig. 6*F*). These results suggest that islets from animals treated with T_3 in vivo lacked enhanced glucose-stimulated insulin secretion due to the counter effects of increased glucocorticoids.

T_3 directly enhanced *Mafa* transcription. To analyze the mechanism by which T_3 regulates *Mafa* expression, we used β-cell lines (INS-1 and MIN-6), both of which express THRA and THRB (data not shown). T_3 increased *Mafa* mRNA transcriptionally rather than by enhancing its stability, as shown by a 90% inhibition of *Mafa* mRNA in the presence of transcription inhibitor actinomycin D (Fig. 7*A*).

For T_3 to have direct effect on *Mafa* transcription, THR must bind to TREs. Using AliBaba 2.1 software, we found potential TRE motifs in the proximal promoter and coding sequence of mouse *Mafa* and designed quantitative PCR primers for them (Supplementary Table 1). In ChIP assay with MIN-6 cells (Fig. 7*B–D*), S2 had 2-fold and S3 had 10-fold enrichment with respect to IgG, indicating both were TREs. Further evidence of binding of THR to S3 is provided by EMSA, in which a band observed in the presence of HEK1 nuclear extract from cells transfected with *Thr* expression plasmid was inhibited upon incubation with antibody against THR (Fig. 7*E*), suggesting a specific disruption of the THR:S3 interaction. In addition, in a luciferase assay using a reporter construct containing the *Mafa* gene 5′-promoter region (S3 was not included) (Fig. 7*F*), T_3 induced a significant increase of luciferase activity, indicating S2 is active in the presence of T_3 and increases *Mafa* transcription. The modest size of the increase in luciferase activity is primarily the result of the lack of the S3 site in the construct and the high basal *Mafa/luciferase* expression in the MIN-6 cells, limiting the impact of T_3 on the transcription of *Mafa/luc* gene. Overall, these results indicate that T_3 regulates *Mafa* transcription through direct receptor-ligand interaction.

DISCUSSION

Previously we showed that overexpression of *Mafa* in neonatal islets to approximately adult *Mafa* mRNA levels induced glucose-responsive insulin secretion and thus the functional maturation of β cells (7). The data presented here support TH as a physiological regulator of *Mafa*

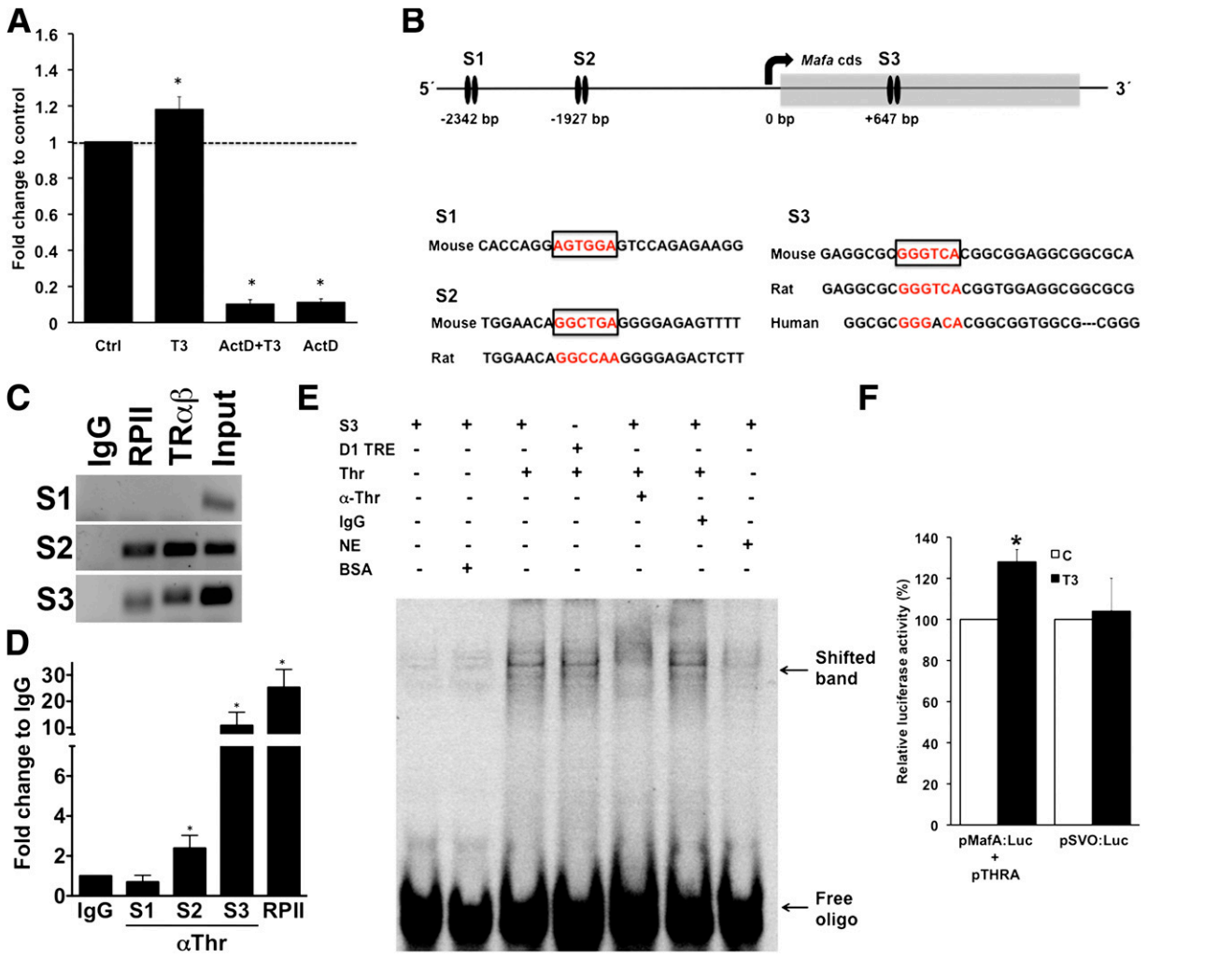

FIG. 7. T_3 directly enhanced *Mafa* transcription. *A*: Increase of *Mafa* mRNA induced in INS-1 cells by T_3 was ablated by incubation with actinomycin D (ActD). Culture with T_3 in 1.6 mmol/L glucose for 6 h (values shown as mean ± SEM; *$P < 0.03$; $n = 3$–6 independent experiments). *B*: Schema of the murine *Mafa* promoter and coding sequence. The top arrow indicates the transcription start site. The black ovals indicate the experimentally tested TRE sites by ChIP: site 1 (S1), site 2 (S2), and site 3 (S3). The TREs are localized at −2,342/–2,354, −1,927/–1,946 and +647/+659. The amplified sequences are shown as is their conservation in other species. S1 is not conserved in humans or rats, whereas S2 is conserved in rats but not in humans. *C*: In ChIP studies of MIN-6 cells using an antibody against THR, putative TREs from S2 and S3 showed direct binding of the THR, but no binding was evident at S1. Gel representative of three or four experiments. *D*: Quantitative PCR products from ChIP-amplified DNA with corresponding IgG, RPII, and input. (values shown as mean ± SEM; *$P < 0.03$; $n = 3$–4 controls). *E*: Electrophoretic mobility shift assay showing a band observed in the presence of HEK1 nuclear extract from cells transfected with *Thr* expression plasmid that was inhibited upon incubation with antibody against. S3, potential TRE Site 3 in *Mafa* cds; NE, nontransfected nuclear extract; D1 TRE, known TRE in *Dio1* promoter; IgG, unspecific antibody; α-THR, antibody against Thr. *F*: A dual luciferase reporter assay using a firefly luciferase reporter construct with the 5′ *Mafa* promoter region in MIN-6 cells grown in high-glucose DMEM $^{+/-}$ 100 nmol/L T3 for 24h. *Renilla* luciferase in a *SacI* backbone was used as a transfection control (values shown as mean ± SEM; *$P < 0.01$; $n = 3$). (A high-quality color representation of this figure is available in the online issue.)

expression and postnatal functional maturation of β cells. Increased expression of THR and increased serum T_4 levels accompanied the increased *Mafa* mRNA between postnatal days 7 and 9. Islets treated with T_3 in vitro at postnatal day 7 showed increased glucose responsiveness with a greater proportion of responsive β cells. Importantly, expression of DN-MAFA blocked secretion of glucose-responsive insulin and the increased *Mafa* mRNA. These data, along with our previous work (7), indicate that T_3 induction of glucose responsiveness was dependent on MAFA. Moreover, the thyroid receptor directly interacts with two putative TREs in the *Mafa* gene. In vivo manipulation of TH (treatment with T_3 or MMI) resulted in the expected change in *Mafa* expression, demonstrating the importance of this mechanism during normal physiological development.

TH is a known regulator of development in different tissues, yet its developmental effects on β-cell function have not been described. Previous studies showed that the T_3-THRA complex enhanced proliferation of RINm5f cells (35) and that THRA knockout mice had greater whole-body insulin sensitivity (36). We now show that *Mafa* is a direct target of TH in β cells. The physiological importance of T_3 regulation of islet development is underscored by our in vivo models of T_3 modulation.

Although a postnatal switch of thyroid receptor isoforms has been described in the heart, dorsal root ganglia, and sciatic nerve with *Thra* characteristic of immature tissues and *Thrb* characteristic of functionally mature tissues (37,38), such an isoform switch had not been previously described for β cells. Comparison of the relative mRNA expression of the isoforms shows that *Thra* predominates

at early ages, that both *Thra* and *Thrb* are equal from postnatal days 9 to 15, and that *Thrb* becomes the predominant isoform in adult islets. At the protein level, β cells expressed both THRA and THRB at postnatal day 7, but the "immature" isoform THRA had nuclear localization between postnatal days 7 and 15, whereas the THRB isoform was mainly cytoplasmic, suggesting that THRA mediates TH effects on β cells during the early postnatal period. THRB showed nuclear translocation only at postnatal day 15, which was when T_3 plasma levels peaked. Nucleocytoplasmic shuttling of both THRA and THRB have been described in other cell types (39–41) and can be mediated by T_3 in an energy-dependent process (39); their nuclear export is considered passive (40), but it is unclear why there is differential shuttling of the two isoforms.

We also showed that the effects T_3 has on other tissues could influence the observed phenotype. The surprising absence of glucose-stimulated insulin secretion in islets from pups treated with T_3 until postnatal day 7 was likely due to the accelerated maturation of the adrenal gland induced by T_3. At birth the adrenal lacks full function, and circulating corticosterone increases only gradually after postnatal day 7 until about postnatal day 15, when a surge occurs (10). Size of the adrenal gland and circulating corticosterone levels previously have been directly related to circulating T_3 levels (42). In our studies, corticosterone levels doubled in animals that received T_3 supplementation and significantly decreased in those in which T_4 synthesis was inhibited. Even low concentrations of dexamethasone decreased *Pdx1* mRNA and blocked the stimulatory effects of T_3 on *Mafa* mRNA and insulin secretion.

In conclusion, we have shown that TH is a physiological regulator of β-cell maturation, a process mediated through direct interaction of THR and the *Mafa* promoter. In vitro, the active hormone T_3 increased glucose-stimulated insulin secretion and potentially could have a similar maturation role for in vitro stem cell–derived β cells. Identification of additional physiological regulators that drive β-cell maturation and glucose responsiveness should lead to effective strategies for developing fully mature in vitro–derived β cells for replacement therapy for diabetes.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health (NIH R01 DK 66056 and DK 93909 [S.B.-W.], R01 DK 60127 [A.S.], DK 36256 [P.R.L.], DK 076117 [A.M.Z.], P30 DK36836 Joslin Diabetes and Endocrinology Research Center (DERC) Advanced Microscopy Core) and by JDRF Grant 1-2011-591 (S.B.-W.). This work was also supported by the Diabetes Research and Wellness Foundation, the Graetz Fund, and an important group of private donors. C.A.-M. was partially supported by the Sheenan Family Fellowship and the Mary Iaccoca Fellowship; A.M. was supported by Harvard College Research Program and the National Institute of Diabetes and Digestive and Kidney Diseases Short-Term Education Program for Underrepresented Persons (STEP-UP).

No potential conflicts of interest relevant to this article were reported.

C.A.-M. and S.B.-W. conceived the project and wrote the manuscript. C.A.-M., A.M.Z., A.Mari., J.H.-L., I.E.K., and A.Mars. researched data. A.M.Z., G.C.W., A.S., and P.R.L., 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 of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Parts of this study were presented in abstract format (as posters and oral presentations) at the 70th Scientific Sessions of the American Diabetes Association, Orlando, Florida, 25–29 June 2010; 71st Scientific Sessions of the American Diabetes Association, San Diego, California, 24–28 June 2011; and the 72nd Scientific Sessions of the American Diabetes Association, Philadelphia, Pennsylvania, 8–12 June 2012.

The authors thank Christopher Cahill (Joslin Diabetes Center) for his expert technical assistance.

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