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Thyroid hormone negatively regulates CDX2 and SOAT2 mRNA expression via induction of miRNA-181d in hepatic cells

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ABSTRACT

Thyroid hormones (THs) regulate transcription of many metabolic genes in the liver through its nuclear receptors (TRs). Although the molecular mechanisms for positive regulation of hepatic genes by TH are well understood, much less is known about TH-mediated negative regulation. Recently, several nuclear hormone receptors were shown to downregulate gene expression via miRNAs. To further examine the potential role of miRNAs in TH-mediated negative regulation, we used a miRNA microarray to identify miRNAs that were directly regulated by TH in a human hepatic cell line. In our screen, we discovered that miRNA-181d is a novel hepatic miRNA that was regulated by TH in hepatic cell culture and *in vivo*. Furthermore, we identified and characterized two novel TH-regulated target genes that were downstream of miR-181d signaling: caudal type homeobox 2 (*CDX2*) and sterol *O*-acyltransferase 2 (*SOAT2* or *ACAT2*). CDX2, a known positive regulator of hepatocyte differentiation, was regulated by miR-181d and directly activated *SOAT2* gene expression. Since SOAT2 is an enzyme that generates cholesteryl esters that are packaged into lipoproteins, our results suggest miR-181d plays a significant role in the negative regulation of key metabolic genes by TH in the liver.

1. Introduction

Thyroid hormones (THs) regulate many important biological processes during the lifetimes of most organisms such as fetal development, post-natal growth, and metabolic homeostasis in adulthood [1]. The major forms of THs are thyroxine (T_4) and triiodothyronine (T_3). They bind to nuclear thyroid hormone receptors (TRs), which recognize and bind to thyroid hormone response elements (TREs) in the promoters of target genes to regulate their transcription. The molecular mechanisms for positive gene regulation by TH are well understood and the consensus sequences and arrangements of positive TREs in the promoters have been characterized [1]. However, little is known about the mechanism(s) for negative transcriptional regulation by TH. Moreover, the consensus sequence for negative TREs remains elusive and controversial despite the identification of a large number of negatively-regulated target genes. Thus, it is possible that negative transcriptional regulation by TH may involve other processes besides TR-TRE binding [2]. One possible mechanism involves decreased transcript levels or translation of TH-regulated target genes by miRNAs. MiR-208, which is encoded in an intron of α-myosin heavy chain, was induced by TH and found to downregulate mRNA expression of several TR-binding cofactors [3]. MiRNAs are also involved in negative transcriptional regulation by other nuclear hormone receptors [4].

In this study, we used a miRNA microarray to screen for TH-regulated miRNAs in the liver. We found that TH stimulates miR-181d in hepatic cells and *in vivo*, leading to contingent downregulation of two novel TH targets, *CDX2* and *SOAT2*. Together, miR181d/CDX2/SOAT2 form a TH-regulated signaling cascade that may decrease cholesterol secretion in hepatic cells.

2. Materials and methods

2.1. Reagents

T_3 was from Sigma–Aldrich. Antibodies recognizing CDX2 and tubulin were from Cell Signaling Technology, while antibodies recognizing β-actin and secondary antibodies were from Santa Cruz. Culture medium and serum were from Invitrogen.

2.2. Cell culture

HepG2 and the transformed HepG2 expressing ectopic TR isoforms (HepG2-TRα and HepG2-TRβ), which were previously described [5], were maintained in DMEM supplemented with 10%

Abbreviations: LDL, low density lipoprotein; TH, thyroid hormone; T_3, triiodothyronine; T_4, thyroxine; TR, thyroid hormone receptor; TRE, thyroid hormone response element; VLDL, very low density lipoprotein.

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FBS in an atmosphere of 5% CO_2 and 37 °C. For T_3 treatment, cells were cultured for at least 3 days in DMEM containing 10% Dowex-treated (Sigma–Aldrich) FBS before the addition of T_3 at 100 nM.

2.3. RNA isolation and quantitative PCR

Total RNA was isolated using miRNeasy Mini Kit (Qiagen) for quantitation of both miRNAs and mRNAs or Invitek Mini Kit (Invitek) for quantitation of mRNAs. Messenger RNAs were quantified by reverse transcription using iScript Select cDNA Synthesis Kit (Bio-Rad), followed by real-time PCR using QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturers' instructions. MicroRNAs were quantified using miScript II RT Kit, followed by QuantiTect SYBR Green PCR Kit (Qiagen). Actin and RNU6b levels were respectively used for normalizing mRNAs and miRNAs of interest. Sequences of mRNA primers are provided in Supplemental Table 1; while miScript Primer Assays (Qiagen) were used for miRNA qPCRs.

2.4. MiRNA microarray

Total RNA from HepG2-TRα treated with T_3 or control for 24 h were sent to Origen Labs for miRNA microarray analysis on GeneChip™ miRNA Array (Affymetrix). The samples were checked using an Agilent Bioanalyzer and labeled using FlashTag Biotin HAS RNA Labeling Kit (Genisphere). Raw data was processed using Affymetrix GeneChip Command Console. T_3-regulated miRNAs were identified using Mann–Whitney test.

2.5. Short RNAs and transfection

Control short RNA, siRNA cocktail targeting Cdx2 (siCdx2; Invitrogen), miR-181d mimic and inhibitor (Qiagen) were transfected using Lipofectamine RNAiMax (Invitrogen), following the instructions for reverse transfection of HepG2 cells. MiR-181d inhibitor was transfected at 50 nM, while the rest were transfected at 10 nM.

2.6. Western blotting

Cells were lysed using CelLytic™ reagent (Sigma–Aldrich) and the protein concentration measured using BCA Kit (Bio-Rad). Equal amounts of protein were heated to 100 °C for 5 min with Laemmli sample buffer, then separated by SDS–PAGE and transferred onto PVDF membranes. The entire process was performed using Bio-Rad equipment and following the instructions provided. Blots were blocked in 5% milk in PBS-T (0.1% Tween) before incubation with specific primary antibodies in 1% bovine serum albumin in PBS-T overnight at 4 °C. Blots were washed 3 times in PBS-T before incubation with species-appropriate, peroxidase-conjugated secondary antibodies for 1 h. Blots were washed 3 times in PBS-T and developed using ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences). Densitometry was done using ImageJ software (NIH).

Table 1
MicroRNAs potentially regulated by T_3 in human liver cells.

miRNA	*p*-Value[a]	Mean fold diff.	Extreme fold diff.[b]
Positively regulated miRNAs			
miR-125b-2*	0.121	2.23	1.98
miR-134	0.121	2.50	1.03
miR-150*	0.121	2.42	1.25
miR-181d	0.121	2.10	1.67
miR-373*	0.121	2.27	1.78
miR-1266	0.121	2.22	1.68
Negatively regulated miRNAs			
miR-337	0.121	0.47	0.68
miR-767	0.121	0.47	0.58
miR-1201	0.121	0.41	0.57

Only miRNAs that showed more than a 50% fold difference (i.e., less than 0.50 or more than 2.00) and differences in the same direction for mean fold difference and extreme difference are included in this table.

[a] A *p*-value of '0.121' is the lowest possible value when using the Mann–Whitney test on 2 sets of data. In this case, each set consists of the microarray analysis results of a control-treated and a T_3-treated HepG2-TRα RNA sample.

[b] Extreme difference measures the smallest possible difference between a value from each of the treatment group.

2.7. Cholesterol quantitation

HepG2-TRβ cells were cultured in T_3-free media before transfection with the miR-181d mimic or a control oligo in triplicates. The media was changed to serum-free DMEM after 24 h. After another 24 h in culture, the media from each well was collected and cell debris removed by centrifugation. The total cholesterol, including free cholesterol and cholesteryl esters, in the media was quantified using the Cholesterol Fluorometric Assay Kit (Cayman Chemical).

3. Results

HepG2-TRα and HepG2-TRβ cells are human liver cell lines that were transformed to express exogenous TRα or TRβ isoforms, respectively. We used HepG2-TRα cells for miRNA microarray screening since HepG2 cells express low amounts of endogenous TRs [5]. HepG2-TRα cells were treated with or without T_3 for 24 h, and total RNA was isolated. This procedure was repeated to obtain 2 sets of paired samples, which were then analyzed by miRNA microarray. We identified nine miRNAs regulated by T_3 in our miRNA microarray analysis; 6 were upregulated, while 3 were downregulated (Table 1). We validated the T_3 induction of positively-regulated miRNAs in HepG2-TRα and HepG2-TRβ cells by qRT-PCR. Only miR-181d consistently showed significant upregulation by T_3. MiR-181d also displayed similar fold induction in both cell lines. T_3 increased miR-181d expression as early as 3 h after treatment and miR-181d expression remained higher than untreated cells 48 h after treatment (Fig. 1A).

We next used qRT-PCR to study the expression of twelve genes (ADAM metallopeptidase with thrombospondin type 1, motif 5; brain-specific angiogenesis inhibitor 3; carboxypeptidase D; DEAD (Asp-Glu-Ala-Asp) box helicase 3, X-linked; karyopherin alpha 1; AP2 associated kinase 1; activin A receptor, type IIB; chromodomain helicase DNA binding protein 7; glutaminase; integrin, beta 8; polyhomeotic homolog 3 and SRY (sex determining region Y)-box 5) that were previously shown to be regulated by miR-181d and could potentially reduce lipid droplet levels in another human hepatic cell line, HuH7 [6]. However, we did not observe downregulation of any of these genes after transfection of a miR-181d mimic in our HepG2-TRβ cells (data not shown). Further literature search identified several other target genes regulated by miR-181d including caudal type homeobox 2 (CDX2), GATA binding protein 6 (GATA6), nemo-like kinase (NLK), B-cell CLL/lymphoma 2 (BCL-2) and K rat sarcoma viral oncogene homolog (K-RAS). Moreover, these genes all had miR-181d binding sites in their 3′-UTRs [7,8]. Accordingly, we examined the gene expression of these genes, and found that only *CDX2* was consistently downregulated by both miR-181d mimic and T_3 (Fig. 1B and C). Additionally, a time course study showed that T_3 induced miR-181d before *CDX2* mRNA was downregulated (Supplementary Fig. 1). The downregulation of *CDX2* mRNA by T_3 was partially rescued when an inhibitor of miR-181d was transfected into the cells during T_3 treatment (Fig. 1D). Western blotting also confirmed that CDX2 protein level was reduced by both T_3 and the miR-181d mimic (Fig. 1E). Thus, T_3

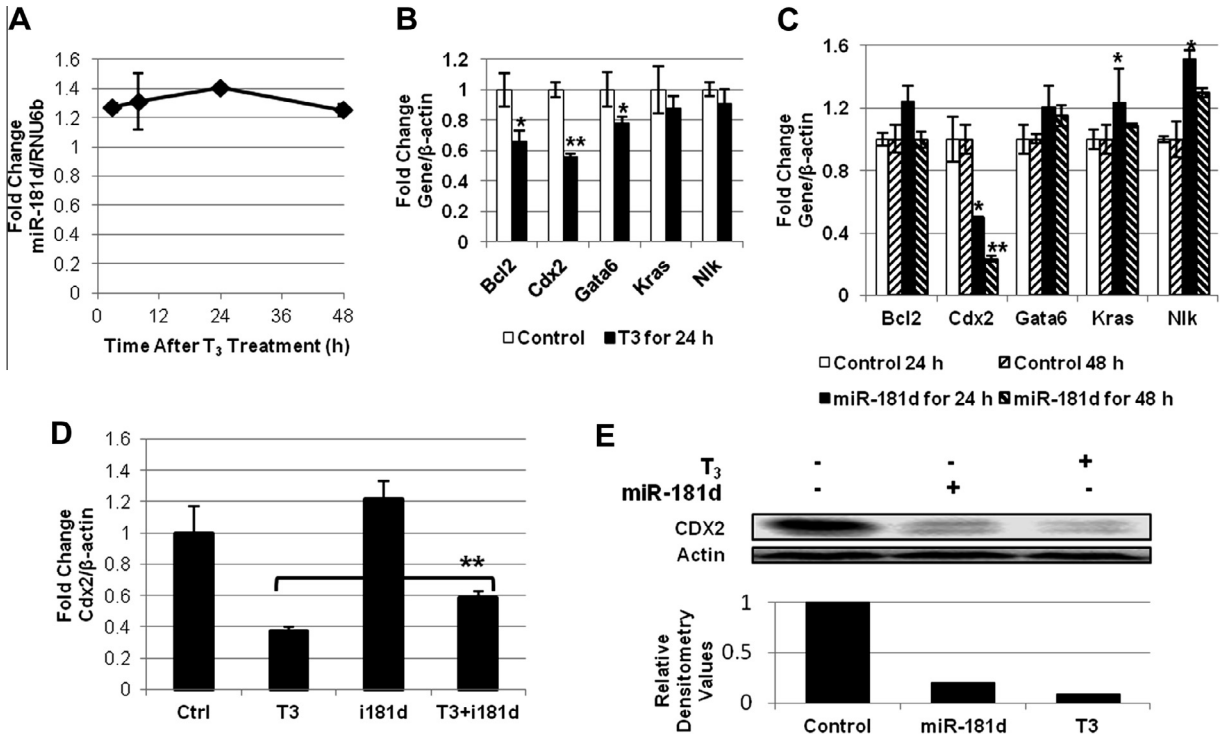

Fig. 1. T_3 downregulated CDX2 by upregulating a miRNA, miR-181d. (A) MiR-181d expression was induced as early as 3 h after T_3 treatment in a human liver cell line, HepG2 that was transformed to stably express exogenous thyroid hormone receptor β (TRβ). mRNA was harvested from the cells 3, 8, 24 and 48 h after T_3 treatment. Expression was quantified by qPCR. (B) Of known miR-181d target genes, *BCL2*, *CDX2* and *GATA6* expression were reduced by T_3 in human hepatic cells. (C) *CDX2* expression was also reduced by transfection of an exogenous miR-181d mimic into the HepG2-TRβ cells. (D) The T_3-mediated downregulation of *CDX2* was significantly derepressed when an inhibitor of miR-181d (i181d) was transfected into the cells. (E) The decrease in CDX2 protein levels reflected the decrease in *CDX2* mRNA levels in HepG2-TRβ cells treated with T_3 or transfected with miR-181d mimic. * significant difference with $p < 0.05$; **Significant difference with $p < 0.005$.

downregulated *CDX2* mRNA and protein expression by inducing miR-181d expression.

CDX2 is a transcription factor that directly binds to promoter elements in the sterol *O*-acyltransferase 2 (SOAT2; also known as acyl-coenzyme A: cholesterol *O*-acyl transferase 2 or ACAT2) gene promoter, and together with hepatocyte nuclear factor 1α (HNF1α), synergistically induce *SOAT2* expression [9]. We thus examined whether *SOAT2* expression was reduced by T_3 treatment and miR-181d since they downregulate *CDX2*. Both T_3 and miR-181d mimic decreased *SOAT2* expression but had no effect on the expression of *SOAT1*, another member of the SOAT family (Fig. 2A and B). To demonstrate that CDX2 served as an essential link between T_3/miR-181d, and SOAT2, we knocked down *CDX2* using a siRNA cocktail, and showed that *SOAT2* expression in HepG2-TRβ cells was dependent upon *CDX2* expression (Fig. 2C).

SOATs catalyze the esterification of cholesterol and long-chain or medium-chain acyl-CoA to form cholesteryl esters that are subsequently packed into low density lipoprotein (LDL) and very low density lipoprotein (VLDL) complexes. *SOAT2* knockout mice had lower serum cholesterol levels that were independent of LDL receptor expression levels [10]. Thus, we examined the effect of miR-181d on cholesterol secretion in T_3-deprived hepatic cells and found that miR-181d alone reduced the concentration of cholesterol in the culture medium of HepG2-TRβ cells over a 24 h period (Fig. 3D). These findings show that the miR-181d/CDX2/SOAT2 cascade can decrease cholesterol secretion by hepatic cells in culture.

To determine whether T_3/miR-181d/CDX2/SOAT2 signaling cascade occurred *in vivo*, we studied the effects of T_3 on hepatic miR181d, Cdx2, and Soat2 expression in mice rendered hyperthyroid by daily T_3 injections for 3 days. MiR-181d was significantly upregulated in the livers of the hyperthyroid mice, while *Cdx2* and *Soat2* were significantly downregulated (Fig. 3). Thus, the *in vivo* results are consistent with results from our *in vitro* studies using HepG2-TR cells.

4. Discussion

We have identified 3 novel targets of TH: miR-181d, *CDX2* and *SOAT2* that together form a signaling cascade, which regulates hepatic cholesterol secretion. MiR-181d expression was rapidly induced by T_3 (within 3 h) and remained elevated for 48 h. TRs were required for the expression of miR-181d since T_3 did not induce miR181d in wild-type HepG2 cells, which contain insignificant levels of TR, whereas it increased miR-181d expression in HepG2-TR cells which express exogenous TRs [5] (Supplemental Fig. 2). These findings suggest that miR-181d expression is TR-dependent. The miR-181d gene is encoded in an intergenic region in the human chromosome 19 with no definable promoter region. Analyses of the region 2 kb upstream or 1 kb downstream from the gene did not reveal any TRE consensus hexamer binding sites (AGGTCA) that were arranged as direct repeats, palindromes, or inverted palindromes 2 kb upstream or 1 kb downstream from the coding sequence (using NUBIScan; http://www.nubiscan.unibas.ch/) [11].

Based on our current data, T_3 most likely induces miR-181d expression via TR binding to distant TREs that exert long-range effects or it interacts directly with another unidentified transcription factor that co-regulates miR-181d expression. Thus, while miR181d induction requires TRs and is rapid, the precise mode of induction requires further elucidation.

The miR181d binding site for human *CDX2* mRNA was previously mapped to the 3′-UTR (697-715) and was shown to regulate

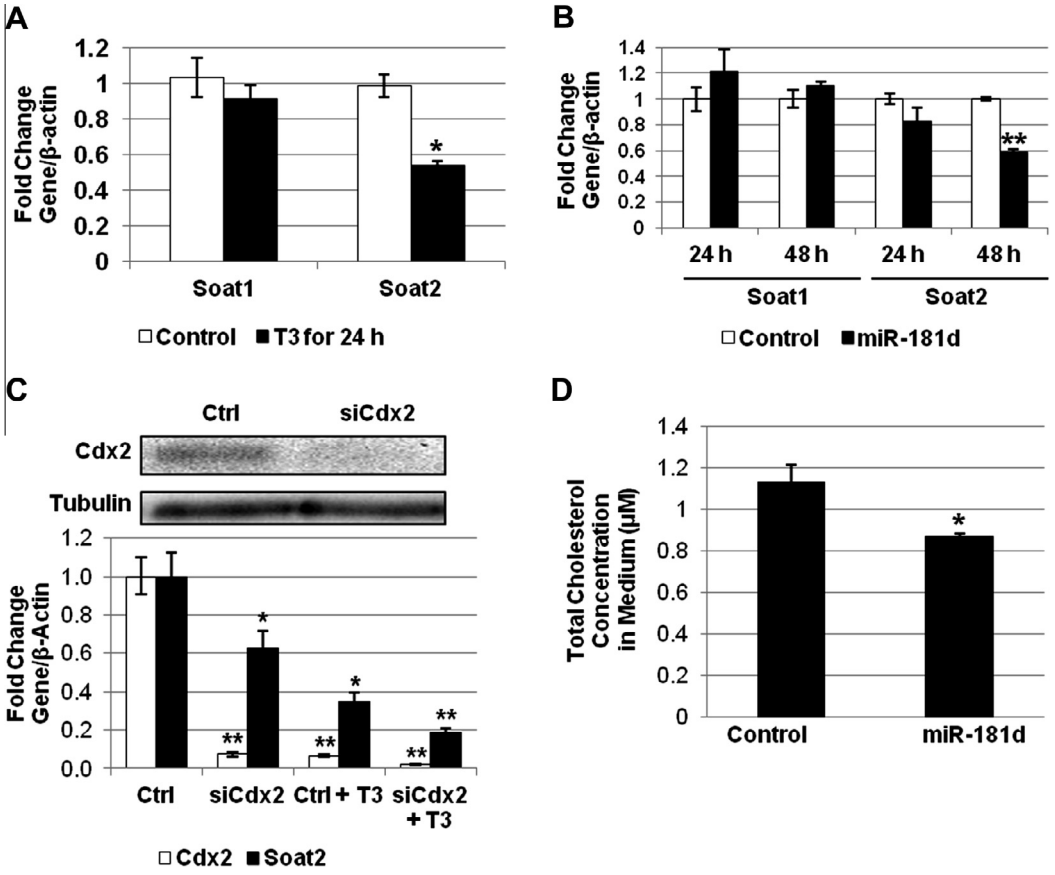

Fig. 2. T_3 downregulated *SOAT2* (or *ACAT2*), potentially by reducing CDX2 with miR-181d. (A) T_3 specifically downregulated *SOAT2*. (B) Exogenous miR-181d also specifically downregulated *SOAT2*, after 48 h. (C) *CDX2* knockdown, with siRNA, decreased *SOAT2* transcript levels independently of T_3. (D) MiR-181d reduced the total cholesterol output of human hepatic cells. Total cholesterol, which includes free cholesterol and cholesterol esters, were measured after 24 h incubation in serum-free media. HepG2-TRβ cells transfected with miR-181d mimic secreted less total cholesterol compared to mock transfected cells. *Significant difference with $p < 0.05$; ** Significant difference with $p < 0.005$.

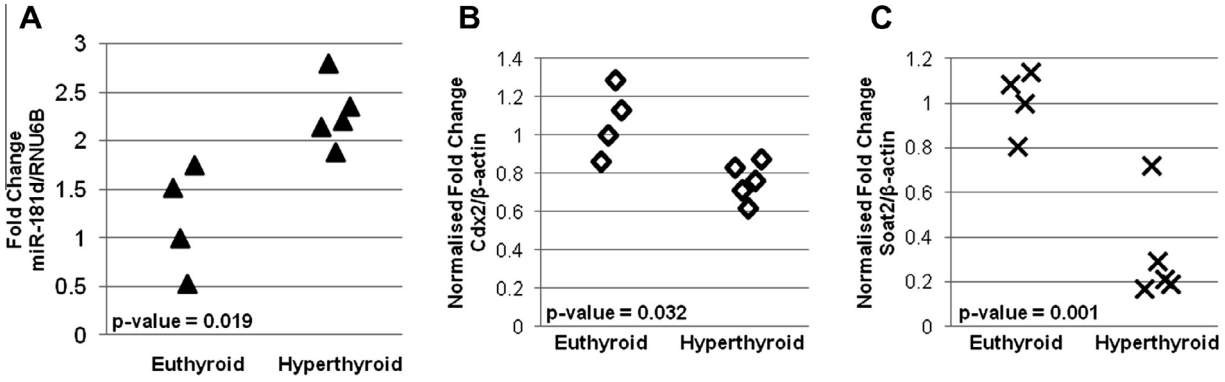

Fig. 3. T_3 downregulated Soat2 in mouse liver cells by upregulating a miRNA, miR-181d. C57/bl6 mice were either injected with 10 μg/100 g body weight of T_3 (hyperthyroid) or with saline (euthyroid). After 3 consecutive days of injections, the animals were sacrificed and total RNA purified from their liver cells.(A) MiR-181d expression was induced by T_3 *in vivo*. (B) *Cdx2*, which has a much lower expression in mouse liver cells compared to the HepG2 cell line, was similarly downregulated by T_3 treatment. (C) *Soat2* was also downregulated by T_3 in mouse livers, potentially via the downregulation of *Cdx2* by miR-181d.

CDX2 mRNA expression [8]. This binding site is conserved between man and mouse. Additionally, CDX2 directly binds to the promoter of *SOAT2*, and induces *SOAT2* gene expression [9]. Our data showed that stimulation of miR-181d expression by T_3 downregulated *SOAT2* expression by decreasing *CDX2* in human hepatic cells and mouse liver. This novel signaling cascade is an example of negative regulation by TH that does not require direct TR binding to the promoters of either the *CDX2* or *SOAT2* gene; hence, it represents a novel mechanism that requires TR but utilizes non-TR effectors (*i.e.,* miRNAs and transcription factors) to mediate downstream regulation of transcription by TH.

TH also regulates other miRNAs in the liver and the heart [3,12,13]. In separate screens for liver miRNAs, Huang et al. reported that TH induced miR-21 [13] whereas Dong et al. found that TH induced miR-1, miR-206, miR-133a and 133b [12]. The difference between these results is most likely due to different cell lines

and techniques used for their screens. However, when they are taken together with our results, it suggests that TH regulates a significant subset of hepatic miRNAs.

MiR-181d was initially identified in a screen for regulators of lipid droplet levels in hepatic cells [6]. It reduced cellular triglycerides and cholesteryl esters by about 60%. Of note, the expression of miR-181b, a close family member, was decreased in nonalcoholic fatty liver disease (NAFLD) [14]. Recently, it was reported that livers of SOAT2 knockout mice and knockdown mice had decreased mass of hepatic lipid droplets containing cholesteryl esters and triglycerides [10]. In addition, treatment with SOAT2 antisense oligonucleotides reversed pre-existing NAFLD in mice [14]. VLDL produced by the SOAT2 knockdown or knockout mice had triglyceride cores without cholesteryl esters but the number of VLDLs produced remained normal [15]. Since more than 70% of the cholesterol in VLDL is esterified, we measured the total cholesterol output of HepG2-TRβ cells and found that miR-181d decreased cholesterol secretion (Fig. 2D). Patients with hyperthyroidism also have decreased serum total cholesterol levels [16]; thus, the miR181d/CDX2/ SOAT2 cascade likely contributes to this TH-dependent change in cholesterol trafficking. The components of this cascade also are potential drug targets; indeed, SOAT2 inhibitors have been developed to treat hypercholesterolemia [17]. In conclusion, our studies on this novel signaling cascade demonstrate that TH regulation of miRNAs can have profound effects on hepatic and systemic lipid metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.116.

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