Thyroid Hormone May Regulate mRNA Abundance in Liver by Acting on MicroRNAs

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Abstract

MicroRNAs (miRNAs) are extensively involved in diverse biological processes. However, very little is known about the role of miRNAs in mediating the action of thyroid hormones (TH). Appropriate TH levels are known to be critically important for development, differentiation and maintenance of metabolic balance in mammals. We induced transient hypothyroidism in juvenile mice by short-term exposure to methimazole and perchlorate from post natal day (PND) 12 to 15. The expression of miRNAs in the liver was analyzed using Taqman Low Density Arrays (containing up to 600 rodent miRNAs). We found the expression of 40 miRNAs was significantly altered in the livers of hypothyroid mice compared to euthyroid controls. Among the miRNAs, miR-1, 206, 133a and 133b exhibited a massive increase in expression (50- to 500-fold). The regulation of TH on the expression of miR-1, 206, 133a and 133b was confirmed in various mouse models including: chronic hypothyroid, short-term hyperthyroid and short-term hypothyroid followed by TH supplementation. TH regulation of these miRNAs was also confirmed in mouse hepatocyte AML 12 cells. The expression of precursors of miR-1, 206, 133a and 133b were examined in AML 12 cells and shown to decrease after TH treatment, only pre-mir-206 and pre-mir-133b reached statistical significance. To identify the targets of these miRNAs, DNA microarrays were used to examine hepatic mRNA levels in the short-term hypothyroid mouse model relative to controls. We found transcripts from 92 known genes were significantly altered in these hypothyroid mice. Web-based target prediction software (TargetScan and Microcosm) identified 14 of these transcripts as targets of miRs-1, 206, 133a and 133b. The vast majority of these mRNA targets were significantly down-regulated in hypothyroid mice, corresponding with the up-regulation of miRs-1, 206, 133a and 133b in hypothyroid mouse liver. To further investigate target genes, miR-206 was over-expressed in AML 12 cells. TH treatment of cells over-expressing miR-206 resulted in decreased miR-206 expression, and a significant increase in two predicted target genes, Mup1 and Gpd2. The results suggest that TH regulation of these genes may occur secondarily via miR-206. These studies provide new insight into the role of miRNAs in mediating TH regulation of gene expression.

Introduction

Thyroid hormones (TH) are critically important for development, tissue differentiation, and maintenance of metabolic balance in mammals through direct and indirect regulation of expression in target genes [1]. Severe disruption of TH action during fetal and early neonatal development leads to a suite of permanent deficits in experimental animals and humans [1]. The liver plays a critical role in metabolism, serum glucose and lipid regulation and is a major target organ of TH. Previous studies using comprehensive transcriptional arrays have shown that TH regulates the expression of genes involved in these important physiological processes [2,3]. However, the mechanism by which TH regulates the expression of these genes, whether by direct actions on transcriptional activity or by indirect actions on mechanisms that control cellular levels of miRNAs, is not well understood.

MicroRNAs (miRNAs) are small non-coding RNAs of 19–24 nucleotides in length that are important regulators of crucial biological processes, such as metabolism, cell growth, apoptosis and carcinogenesis [4,5]. The number of known miRNAs has rapidly increased over the past years. Recently, the Sanger Institute released the latest version of their database of known miRNAs (miRBase 14.0; Sep 2009, http://microrna.sanger.ac.uk); 786 mature mouse miRNA sequences are currently reported. Long primary miRNAs are transcribed by RNA polymerase II in the nucleus, and then modified by an enzyme complex containing DROSHA and DGCR8 to form pre-miRNA. Subsequent cleavage of pre-miRNA by an RNase III, DICER 1, results in mature miRNA, which suppresses translation and enhances degradation of target gene transcripts by binding to complementary regions within the target transcripts [4,5].

Considering the importance of TH in regulating fundamental processes governed by hepatic function, and the potential importance of miRNAs in regulating genes coding for proteins important in these function, we sought to test the hypothesis that TH regulates specific miRNAs. Therefore, we employed DNA microarrays and TaqMan low density arrays (TLDA) to analyze gene and miRNA expression profiles in a juvenile hypothyroid mouse model induced by short-term exposure of dams to goitrogen before weaning. TLDA offers a high throughput and
sensitive approach for detection of miRNAs [6]. Selected miRNAs were examined in more detail in other animal models with altered TH levels and in an in vitro system to confirm TH regulation. The target genes of one miRNA (miR-206) were investigated with cell lines stably transfected with the corresponding miRNA. Analysis of miRNA expression changes in combination with global mRNA levels provides a powerful approach to determine the effect of TH perturbation on miRNA expression and function. The results of this study provide insight into the role of miRNAs in mediating the actions of TH on liver function.

Materials and Methods

Ethics Statement

All animal care and handling was in accordance with Canadian Council for Animal Care Guidelines and was approved by the Health Canada Animal Care Committee. Permit number is 2007006.

Animal models with altered TH level

C57BL/6 mice were purchased from Charles River (St. Constant, QC, Canada) and were housed in hanging polycarbonate cages under a 12:12 hour light-dark cycle at 23°C with food (Purina rodent chow 5010; Ralston-Purina, St. Louis, MO, USA) and sugar water (1%) available ad libitum. All cages contained shelters and nesting material. After a 10 day acclimation period breeding was initiated by transferring two sexually mature female mice (8 weeks post natal) into the home cage of a sexually mature (10 weeks) male. After 4 nights of co-housing each female was transferred to a separate cage. Dams were allowed to litter naturally and litter numbers were not adjusted. On post natal day (PND) 12, half of the dams with litters were provided with sugar water containing methimazole (MMI, 0.05%, Sigma Chemical, St. Louis, MO) and perchlorate (1%, Sigma Chemical), while other half were provided sugar water. Four hours before sacrifice on PND 15, pups with sugar water were injected with PBS (control group) or T4/T3 (hypo group) and perchlorate treated pups were injected with PBS (hypo group) or T4/T3 (hypo+ group, 20/2ug/100g bw).

For PTU induced hypothyroidism, C57BL/6 time-pregnant mice (13 day gestation, GD 13) were purchased from Charles River (St. Constant) and supplied ad libitum with 0.3% diet cherry Kool-aid in water containing 0%(control) or 0.04% PTU (6-propylthiouracil; Sigma Chemical) from GD 13 to PND 15.

On PND 15, a male pup from each litter was sacrificed by exsanguination under isoflurane anaesthesia. Serum, prepared using Serum Separator Tubes (BD Biosciences, Mississauga, ON, Canada) was retained for T4 analysis with RIA kits (MP Biomedicals, Medicorp, Mississauga, ON, Canada) was labelled with Cyanine 3-CTP (Cy3) using Agilent Quick Amp Labelling kits (Agilent Tech. Inc.). Labelled sample and common reference cRNA were hybridized to Agilent mouse oligo microarrays (product number G4122F; 4x44K arrays) according to the manufacturer’s instructions. Slides were washed and scanned on an Agilent G2505B microarray scanner and data were acquired with Agilent Feature Extraction software version 10.1.1.1.

Microarray hybridization

Relative transcript levels were determined using a 2 colour reference hybridization design where each RNA sample was labelled with Cyanine 3-CTP (Cy3) and universal mouse reference total RNA (Stratagene, Cedar Creek, TX, USA) was labelled with Cyanine 3-CTP (Cy5) using Agilent Quick Amp Labelling kits (Agilent Tech. Inc.). Labelled sample and common reference cRNA were hybridized to Agilent mouse oligo microarrays (product number G4122F; 4x44K arrays) according to the manufacturer’s instructions. Slides were washed and scanned on an Agilent G2505B microarray scanner and data were acquired with Agilent Feature Extraction software version 10.1.1.1.

Microarray normalization and analysis of differential gene expression

Background fluorescence was measured using the (-)3xSLv1 probes. Probes exhibiting median signal intensities less than the trimmed mean (trim = 5%) plus three trimmed standard deviations of the (-)3xSLv1 probe were flagged as absent (within the background signal). Data were normalized using the transform. madata function in the MAANOVA library in R using a global lowess with a span of 0.2 [7,8]. Genes that were differentially expressed as a result of treatment were determined using the MAANOVA library in R. The F statistic [9] was used to test for treatment effects. P-values were estimated by the permutation method using residual shuffling, followed by adjustment for multiple comparisons using the false discovery rate (FDR) approach [10]. The fold change calculations were based on the least-square means. Significant genes were identified as those where the F statistic had a Benjamini-Hochberg corrected p<0.05. All data are MIAME compliant and available through the Gene Expression Omnibus (GEO, accession number GSE21277).

miRNA expression analysis with TLD

Four male mice from each of the control and hypo groups respectively were used to comprehensively analyze miRNA expression. Samples containing 750 ng RNA were used to perform reverse transcription with the Taqman miRNA Reverse Transcription kit and Megaplex RT Primers Rodent Pool A and B (Applied Biosystem). RT-PCR reactions were performed with TaqMan Rodent miRNA Array A and B (containing up to 600 rodent miRNAs) by the Institut de Recherche en Immunologie et en Cancérologie (IRIC), University de Montreal, with the 7900 HT system. Using the log2 of the delta Ct values, differentially
expressed miRNAs were identified using an F-test with U6 as a housekeeping miRNA. The critical value of the F-test statistic was determined by bootstrapping the residuals from the one way ANOVA model [11] using the R [7] software. Residuals were re-sampled within each treatment condition to avoid making the common variance assumption [12]. Multiple comparison adjustment was applied to the final results using the FDR approach [10]. The dataset is available through GEO (accession number GSE21277).

miRs-1, 206, 133a and 133b expression in animal models or cultured cell lines

Taqman miRNA Reverse Transcription kits (Applied Biosystem) were used for reverse transcription reactions with 10 ng total RNA as template and specific primers from the Taqman miRNA Assay Kits. PCRs were performed with Taqman universal PCR Master Mix according to the manufacturer’s instructions. Three animals from each group or 3 batches of cultured cells were used. Relative miRNA expression was analyzed using the ΔΔCt method with U6 as a housekeeping miRNA and one of the control samples as the calibrator. Significant differences in expression were determined using a Student’s t-test and called significant if \( p < 0.05 \).

MiRNA target predictions

TargetScan mouse 5.1 (http://www.targetscan.org/mmu_50/) and MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl) were used to predict the targets of miRs-1, 206, 133a and 133b. Genes predicted by either of algorithm were considered to be the targets. These softwares apply different algorithms to identify the highly complementary sites and are widely used for miRNA target prediction [13–15].

qRT-PCR analysis of target genes and precursors of miRNAs

Reverse transcription was carried out with SuperScript III (Invitrogen) using SYBR-Green and a GFX system (BioRad, Mississauga, ON, Canada). Primers were designed using Beacon design 2.0 (Premier BioSoft International, Palo Alto, CA, USA). PCR reactions were performed in duplicate, and the values of the threshold cycles were averaged. Gene expression levels were normalized to Hprt. PCR efficiency was examined using the standard curve for each gene. The primer specificity was assured by the melting curve for each gene. A Student’s t-test was used for statistical evaluation. The sequences of primers are available upon request.

Results

Validation of the short-term MMI/perchlorate-induced hypothyroid juvenile mouse model

Serum T4 levels in PND 15 male pups of dams treated from PND 12 to PND 15 with drinking water containing 0.05% MMI/1% perchlorate were significantly reduced (\( p < 0.001 \), Fig. 1A). Functional hypothyroidism was further confirmed by the observation of a 50% reduction in malic enzyme (a known TH regulated gene in liver [3]) expression in hypothyroid mouse liver (Fig. 1B).

MiRNA expression in hypothyroid mice

The TLDA analysis revealed that 40 miRNAs were significantly altered (\( p < 0.1 \)) in the liver of hypothyroid mice compared with controls. Among them, 11 miRNAs exhibited a fold change greater than 4 (Table 1); 8 of these 11 (70%) were up-regulated in hypothyroid mice. Three miRNA families (miRs-1 and 206, miRs-133a and 133b as well as miRs-135a and 135b) exhibited very large increases in expression (ranging from 50- to 500-fold). Data for these findings are available through GEO, accession number GSE21277.

The expression of miRs-1, 206, 133a, 133b in other animal models with altered TH levels

To further investigate the effect of TH on hepatic miRNA expression, we examined the expression of the most differentially regulated miRNAs (miRs-1, 206, 133a, 133b) in the livers of (a) hypothyroid mice induced by PTU treatment (PTU hypothyroid); (b) hyperthyroid mice created by injecting T3/T4 four hours before sacrifice (hyperthyroid) and (c) hypothyroid mice induced by MMI/Perchlorate treatment but receiving T4/T3 injection four hours before sacrifice (corrected hypothyroid). Three mice were chosen from each group and their serum T4 levels were shown in Table 2. As shown in Fig. 2A, all of four selected miRNAs were significantly increased in the livers of PTU induced...
hypothyroid mice, while significantly decreased in the livers of hyperthyroid mice. Corrected hypothyroid animals had serum T4 levels intermediate between control and hyperthyroid animals although these were only significantly different from the hyperthyroid T4 levels (p = 0.046 vs hyperthyroid and 0.067 vs control; Table 2). Similarly, hepatic expression of all 4 miRNAs was also intermediate between control and hyperthyroid mice with only miR206 being significantly reduced relative to control animals (Fig 2B).

The expression of miRs-1, 206, 133a and 133b in AML 12 cells treated with TH

To further explore the effects of TH on miRNA regulation in liver, we treated AML 12 cells (derived from mouse hepatocytes) with 10 nM T3 for 1 hour or 24 hours. The expression of miRs-1, 206, 133a and 133b was examined with the Taqman miRNA Assay. TH treatment caused down-regulation of all miRNAs examined at both 1 hour and 24 hours, although this effect was only statistically significant for miRs-1, 206 and 133b at 24 hours (Fig. 3A).

Since mature miRNAs are derived from the cleavage of precursors by the RNase-III enzyme DICER, we investigated the effects of TH on the levels of the precursors of the selected miRNAs in AML 12 cells. As significant decreases of mature miRNAs were only found at 24 hours, we examined the precursor miRNAs at 24 hours as well. Precursors of all 4 miRNA species were reduced by at least 50% and this reduction was statistically significant (p < 0.05) for mir-206 and mir-133b (Fig. 3B) even with the small sample size used.

Target Genes of miRs-1, 206, 133a and 133b in hypothyroid mouse liver

The livers of five male pups from the transient hypothyroid model were analysed using Agilent gene expression microarrays alongside controls. Significantly altered genes (p < 0.05) were found for 103 transcripts between euthyroid and hypothyroid pups. Among them, 92 genes have known functions and include genes known to be regulated by TH, such as Dio1, Spot 14 and Vldlr [3]. The expression of malic enzyme was also decreased in these hypothyroid mice (1.4-fold, unadjusted p-value = 0.005), consistent with the result of qRT-PCR (Fig. 1B). Detailed analysis of the expression profiles of these mice will be presented as part of another publication (Paquette et al., in preparation). The dataset is available through GEO (accession number GSE21277). Because miRs-1 and 206 as well as miRs-133a and 133b families exhibited such large fold changes, the targets of these miRNAs were predicted using TargetScan and MicroCosm. The overlap between predicted target genes and significantly changed genes with fold change >1.5 and p<0.05 in hypothyroid mice is shown in Table 3. Of the 14 predicted targets, 11 were significantly down-regulation in hypothyroid pups, corresponding with the up-regulation of miRs-1, 206, 133a and 133b. None of the targets of miRs-1, 206, 133a and 133b that were curated in TarBase (http://diana.cslab.ece.ntua.gr/tarbase/) were altered in the hypothyroid mouse livers.

To provide direct evidence of the regulation of the expression of target genes by miRNAs in response to TH, we established AML 12 cells that over-expressed mir-206 by stable transfection of

| Table 1. Differentially expressed miRNAs in hypothyroid mouse liver (p<0.1, Fold change >4). |

| Gene Name | Fold Change | p-value | Clustered miRNA* | Function |
|-----------|-------------|---------|-----------------|----------|
| miR-133 family | mmu-miR-133b | 501.39 | 0.05 | mmu-miR-206 | Apoptosis [24], muscle development [25,26] |
| | mmu-miR-133a | 100.36 | 0.02 | mmu-miR-1 | Apoptosis [24], muscle development [25,26] |
| miR-1/206 family | mmu-miR-1 | 92.11 | 0.01 | mmu-miR-133a | Apoptosis [24], muscle development [25,26] |
| | mmu-miR-206 | 58.90 | 0.07 | mmu-miR-133b | Apoptosis [24], muscle development [25,26] |
| miR-135 family | mmu-miR-135b | 85.11 | 0.09 | | Colorectal cancer [27] |
| | mmu-miR-135a | 14.62 | 0.09 | | Colorectal cancer [27] |
| Others | mmu-miR-138* | 27.77 | 0.08 | | Squamous cell carcinoma [28] |
| | mmu-miR-199b* | 4.96 | 0.05 | | Choriocarcinoma [29] |
| | mmu-miR-148a* | -4.20 | 0.04 | | DNA methyltransferase [30] |
| | mmu-miR-582-5p | -5.14 | 0.07 | | Malignant mesothelioma [31] |
| | mmu-miR-200a* | -5.49 | 0.10 | | Cervical cancer [32] |

*indicates they are in the same chromosome and apart less than 10kb (based on database of TargetScan).

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| Table 2. Serum T4 levels of male pups in the various animal models (n = 3). |

| T4 (μg/dl) | p |
|-----------|---|
| Chronical Hypothyroid | |
| Control | 9.93 |
| PTU (0.04%) | 0.57 | 0.000 |
| Transient Models | |
| Control | 9.6 |
| Hyper | 39.4 | 0.000 |
| Hypo+ | 16.6 | 0.007 (to Control) |
| | | 0.046 (to Hyper) |

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vector containing mir-206 precursor. The expression of miR-206 was approximately 1500 times higher in miR-206 over-expressing cells than in mir null control vector transfected cells (Fig. 4A). We selected 4 down-regulated genes from the predicted miR-206 targets in Table 3. The expression of these genes was examined in these cells and the results are shown in Figure 4B. Expression of three out of the four putative target genes were significantly decreased in miR-206 over expressed cells suggesting that these three genes are true targets of miR-206.

To investigate whether these 3 true target genes are regulated by TH, we examined the expression of these 3 genes and that of miR-206 in miR-206 transfected AML 12 cells treated with T3 for 24 hours. T3 treatment decreased the expression of miR-206 by roughly 50% (p<0.05) in miR-206 transfected AML 12 cells (Fig. 4C), while the expression of target genes Gpd2 and Mup1, but not Nrp1, significantly increased (Fig. 4D).

**Discussion**

TH are essential for normal development and for normal adult physiology. In both development and in adulthood, an important role of TH is to control metabolism and, perhaps, body weight. Recent studies on the effects of a TRβ-selective drug that targets the liver indicate that activation of the TRβ receptor can lower serum lipids, can decrease global fat stores, and can improve insulin tolerance in ob/ob mice [16]. Thus, liver is an important target of TH in regulating energetic metabolism and physiology [2,3]. TH primarily exert their effects through interaction with TR, which, upon heterodimerization with the retinoic X receptor, acts as ligand-activated transcription factors to initiate or block target gene expression by binding to TH response elements (TREs) in the gene promoter regions. Indeed, much of the focus of work in our laboratory has been on the identification of TREs associated with promoters [17]. However, a direct TR-TRE mechanism may not explain all TH actions. For example, non-genomic action of TH, which is often related to activation of signalling pathways, is another well characterized TH response [18]. MiRNAs represent an additional mechanism by which TRs may regulate or coordinate TH response genes. MiRNAs are involved in many biological processes and functions in controlling protein expression through targeting degradation of mRNA, translational suppression of protein production, and directing chromatin structure (reviewed in [19]). As such, in the current work we explored the role of miRNAs in governing TH response.

To examine the possibility that TH action may influence miRNA expression which could, in turn, alter mRNA levels, we generated hypothyroid mice by short-term treatment with MMI and perchlorate. Global expression of miRNAs and mRNAs were studied with TLDA and DNA microarray technologies. TLDA (a modified qRT-PCR method) was applied to generate miRNA expressions...
profiles. This high throughput method has increased sensitivity and accuracy relative to microarrays, and demonstrates a 100% miRNA expression validation rate against standard qRT-PCR [6]. We detected 40 significantly altered miRNAs (p, 0.1) in the livers of hypothyroid mice. Among these, 11 miRNAs exhibited fold change >4 (Table 1) and included 8 up-regulated and 3 down-regulated miRNAs. Remarkably, miRs-1, 206, 133a and 133b exhibited fold changes in excess of 50-fold. MiR-1 and miR-133a cluster on chromosome 2 within 10 kb, while miR-206 and miR-133b cluster on chromosome 1 within 4 kb. The results demonstrate a highly robust induction of miRNAs in response to hypothyroidism for specific genomic sites.

To demonstrate that the increased miRNA expression was induced by TH deficiency, not the toxicity of MMI and perchlorate, we examined the expression of miRs-1, 206, 133a and 133b in chronic hypothyroid, short-term hyperthyroid and a TH-supplemented transient hypothyroid animal models using RT-PCR. All of the miRNAs significantly increased in the chronically hypothyroid mouse liver, although the fold change was smaller than in the transient hypothyroid model (Fig. 2A). This may be attributed to the different animal models or different detection systems used. Alternatively, there may be some adaptation following chronic hypothyroidism, or the miRNAs may exhibit a large initial response to the TH perturbation. This increased miRNA expression was not seen in TH corrected transient hypothyroidism, while miR-206 was significantly decreased (Fig. 2B). In addition, all selected miRNAs were significantly decreased in hyperthyroid mouse livers. That both serum T4 (Table 2) and hepatic expression of these miRNAs in the corrected hypothyroid animals are intermediate between control and hyperthyroid animal suggests an inverted dose dependent regulation by TH of these miRNA levels. These observations were confirmed in an in vitro model of mouse hepatocytes (AML 12 cells), where treatment with T3 caused a rapid (1 hour) reduction in the levels of miRs-1, 206, 133a and 133b. Further reduction of miRs-1 and 133a was found 24 hours post-treatment (Fig. 3A). The expression of miRNA precursors decreased with T3 treatment at 24 hrs (Fig. 3B). Although some of these changes were not significant at the p<0.05 level (only three biological replicates were used in these analyses), the data in vitro provide more evidence that TH reduce the cellular levels of miRs-1, 206, 133a and 133b at the transcript level.

TH regulation of these miRNAs is also supported by recent studies on muscle miRNA expression in hypothyroid humans [20]. In this work Visser et al. collected skeletal muscle biopsies from clinically hypothyroid patients who were being treated with TH or not. The authors found that TH induced a large down-regulation of primary miRs-206 and 133b. Levels of the primary transcript associated with miRs-1 and 133a were also reduced but to a lesser extent. Together these findings provide strong evidence that TH...
Table 3. Targets of miRs-1, 206, 133a and 133b that were significantly altered in hypothyroid mouse liver (p<0.05, Fold change >1.5).

| Accession Number | Gene Symbol | p-value | Fold Change | Description |
|------------------|-------------|---------|-------------|-------------|
| Targets of miR-1/206 | | | | |
| NM_013703a | Vldlr* | 0.001 | 1.669 | very low density lipoprotein receptor |
| NM_001013785a | Akr1c19 | 0.000 | 1.612 | aldo-keto reductase family 1, member C19 |
| NM_029692a | Upp2 | 0.008 | -1.562 | uridine phosphorylase 2 |
| NM_010274b | Gpd2 | 0.000 | -1.695 | glycerol phosphate dehydrogenase 2, mitochondrial |
| NM_031188a | Mup1 | 0.003 | -1.815 | major urinary protein 1 |
| NM_008737b | Nrp1* | 0.000 | -2.000 | neuropilin 1 |
| NM_026460a | Serpini2 | 0.001 | -3.080 | serine (or cysteine) peptidase inhibitor, clade I, member 2 |
| Targets of miR-133a/b | | | | |
| NM_016919ab | Col5a3 | 0.000 | 1.752 | procollagen, type V, alpha 3 |
| NM_182959a | Slc17a8 | 0.000 | -1.637 | solute carrier family 17 |
| NM_025989a | Gp2 | 0.002 | -1.727 | zymogen granule membrane glycoprotein 2 |
| NM_009344a | Phlda1* | 0.000 | -1.815 | pleckstrin homology-like domain, family A, member 1 |
| NM_008693a | Klk1b3 | 0.002 | -2.103 | kallikrein 1-related peptidase b3 |
| NM_007769a | Dmbt1 | 0.004 | -4.236 | deleted in malignant brain tumors 1 |

*indicates the genes that were found in chronic hypothyroidism in our previous study [3]. a indicates the genes were predicted with the database of MicroCosm Targets and b indicates with TargetScan Mouse.

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Figure 4. Identification of the target genes of miR-206 that are regulated by TH. A. Levels of miR-206 in AML 12 cells stably transfected to ectopically express miR-206. AML12 cells were transfected with pEGP-mmu-mir-206 expression vector or pEGP-mir null control vector and selected with puromycin and green florescence protein by microscopy. The expression of miR-206 was examined with the Taqman miRNA Assay (n = 5). B. The expression of genes that are putative targets of miR-206 in the two cell types shown in figure 4A was examined with RT-PCR (n = 3). C. The effects of TH on the expression of miR-206 in the transfected cells. The miR-206 transfected cells were treated with 10 nm T3 for 24 hours. MiR-206 expression was analyzed with Taqman miRNA Assay (n = 3). D. The effect of TH on the expression of the miR-206 target genes in the transfected cells. The miR-206 transfected cells were treated with 10 nm T3 for 24 hours. The expression of target genes was examined with RT-PCR (n = 3). Data are presented as mean ± SE. A two-tailed Student’s t-test was used to calculate significance. * indicates p<0.05.
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down-regulate the expression of miR-1, 206, 133a and 133b in liver and skeletal muscle. However, the targets of these miRNAs and the potential biological implications are not known.

In order to explore the correlation between miRNA and mRNA levels, gene expression microarrays were used to quantify transcript abundance in the livers of the same mice (hypothyroid and euthyroid). Significant alterations in mRNA levels (p<0.05) were found for 92 transcripts with known functions between euthyroid and hypothyroid pups. Changes in gene expression may be regulated by TH through TR-TRE, miRNAs, non-genomic signalling or other mechanisms. Targets of miRs-1, 206, 133a and 133b were predicted using TargetScan and MicroCosm. The analysis predicts that these miRNAs target 14 genes that also exhibited changes in mRNA levels in hypothyroid mice relative to controls (Table 3). The vast majority of these mRNA targets (11/14) were down-regulated under hypothyroid conditions, corresponding with the increased expression of miRs-1, 206, 133a and 133b in these mice. Three targets (Vldr, Nrp1 and Phlda1) have previously been found to be differentially expressed following alterations in TH levels in mouse livers [3]. We further validated the targets of miR-206 by establishing over-expressing miR-206 in AML 12 cells by stable transfection. The expression of miR-206 was approximately 1500 times higher in the transfected cells relative to control cells (Fig. 4A). The expression of four predicted targets of miR-206 that were down-regulated in hypothyroid livers was examined; 3 of them were decreased significantly in miR-206 transfected cells (Fig. 4B). These findings strongly suggest that these 3 genes are putative targets of miR-206. To further demonstrate that miR-206 targets are regulated by TH, we treated the miR-206 transfected cells with TH for 24 hours and found that the expression of miR-206 decreased significantly (Fig. 4C), and was accompanied by increases in the expression of 2 of the miR-206 target genes (Mup1 and Gpd2, Fig. 4D). Mup1 mediates chemical signalling by acting as the pheromone ligand and regulates systemic glucose and lipid metabolism [21,22], while Gpd2 plays a role in thermogenesis [23]. TH are involved in the regulation of the activities of Mup1 and Gpd2. Our current findings provide possible mechanisms by which TH regulates these activities via post-transcriptional control by miR-206.

These findings suggest that TH may regulate the cellular levels of several species of miRNA which, in turn, regulate the mRNA levels of some genes. To our knowledge, this represents the first observation of miRNA mediating the physiological action of a hormone in the development of the liver. At present, it is not clear if TH reduces the levels of these specific miRNAs by blocking transcription of primary genes or through some other mechanisms. Further confirmation of the target genes and the physiological significance of miRNAs in regulating TH function are underway in our laboratory.

In conclusion, the present study applied global miRNA and mRNA expression profiling to reveal a potential regulatory role for miRNAs in response to TH in the developing mouse liver. Two target genes of miR-206 affected by TH were confirmed in vitro. The work provides insight into the mechanisms leading to abnormal metabolism and development in the liver following TH imbalance.

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Author Contributions

Conceived and designed the experiments: HD RTZ MGW CY. Performed the experiments: HD MP. Analyzed the data: HD AW MGW CY. Wrote the paper: HD RTZ MGW CY.

References

1. Boelaert K, Franklin JA (2005) Thyroid hormone in health and disease. J Endocrinol 187: 1–15.
2. Stahlberg N, Merino R, Hernandez LH, Fernandez-Perez I, Sandelin A, et al. (2005) Exploring hepatic hormone actions using a compilation of gene expression profiles. BMC Physiol 5: 8.
3. Dong H, Yauk CL, Williams A, Lee A, Douglas GR, et al. (2007) Hepatic gene expression changes in hypothyroid juvenile mice: characterization of a novel negative thyroid-responsive element. Endocrinology 148: 3932–3940.
4. Chen XM (2009) MicroRNA signatures in liver diseases. World J Gastroenterol 15: 1665–1672.
5. Lynn FC (2009) Meta-regulation: microRNA regulation of glucose and lipid metabolism. Trends Endocrinol Metab 20: 452–459.
6. Mees ST, Schliechter C, Marlin WA, Colombo-Benkmann M, et al. (2009) Analyzing miRNAs in Ductal Adenocarcinomas of the Pancreas. J Surg Res.
7. Team R (2008) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.
8. Wu HH, SW, Cui X, Churchill GA (2003) MAANOVA: a software package for the analysis of spotted cDNA microarray experiments.
9. Nieto T, Inoue T, Node K (2008) Alternative spliced variants in the pantetheinase family of genes expressed in human neutrophils. Gene 426: 57–64.
10. Benjamin YHY (1995) Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society 57: 289–300.
11. Kerr MKCG (2001) Bootstrapping cluster analysis: assessing the reliability of conclusions from microarray experiments. Proc Nail Acad Sci USA 98: 8961–8965.
12. Higgins J (2003) An introduction to modern nonparametric statistics. Brooks/Cole, Pacific Grove, CA.
13. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian miRNAs are conserved targets of microRNAs. Genome Res 19: 92–105.
14. Grimson A, Farh KK, Johnston WK, Garrett-Engle P, Lim LP, et al. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27: 91–103.
15. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15–20.
16. Grigorova G, Effendic S, Klahn A, Rehmke M, Korner P, et al. (2008) Anti-obesity, anti-diabetic, and lipid lowering effects of the thyroid receptor beta subtype selective agonist KB-141. J Steroid Biochem Mol Biol 111: 262–267.
17. Dong H, Yauk CL, Rowan-Carroll A, You SH, Zoller RT, et al. (2009) Identification of thyroid hormone receptor binding sites and target genes using ChIP-on-chip in developing mouse cerebellum. PLoS One 4: e6150.
18. Jordanov A, Hadzopoulou-Cladaras M, Lazou A. Non-genomic effects of thyroid hormone in adult cardiac myocytes: relevance to gene expression and cell growth. Mol Cell Biochem.
19. Valenzuela-Sanchez MA, Liu J, Hannon GJ, Parker R (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 20: 515–524.
20. Visser WE, Heemstra KA, Swaggers MK, Ozgur Z, Corsumit EP, et al. (2009) Physiological thyroid hormone levels regulate numerous skeletal muscle transcripts. J Clin Endocrinol Metab 94: 3487–3496.
21. Logan DW, Marton TF, Stowers L (2008) Species specificity in major urinary proteins by parallel evolution. PLoS One 3: e3290.
22. Zhou Y, Jiang L, Liu L (2009) Identification of MUP1 as a regulator for glucose and lipid metabolism in mice. J Biol Chem 284: 11152–11159.
23. Dossantos RA, Allafda A, Eto K, Kadowaki T, Silva JE (2003) Evidence for a compensated thermogenic defect in transgenic mice lacking the mitochondrial glycerol-3-phosphate dehydrogenase gene. Endocrinology 144: 5469–5479.
24. Xu C, Lu Y, Pan Z, Zhu W, Luo X, et al. (2007) The muscle-specific microRNA mir-1 and miR-133 produce ongoing effects on apoptosis by targeting HSPO, HSPO7 and caspase-9 in cardiomyocytes. J Cell Sci 120: 3045–3052.
25. Swertman D, Godjarak E, Rathjen T, Oztunay S, Braun T, et al. (2008) Specific requirements of MRFs for the expression of muscle specific microRNAs, miR-1, miR-206 and miR-133. Dev Biol 321: 491–499.
26. Kato Y, Miyaki S, Yokoyama S, Omori S, Inoue A, et al. (2009) Real-time functional imaging for monitoring miR-133 during myogenic differentiation. Int J Biochem Cell Biol 41: 2225–2231.
27. Nagel R, le Sage C, Diosdado B, van der Waal M, Oude Vrielink JA, et al. (2008) Regulation of the adenomatous polyposis coli gene by the miR-155 family in colorectal cancer. Cancer Res 68: 5795–5802.
28. Jiang L, Liu X, Kolokythas A, Yu J, Wang A, et al. Down-regulation of the Rho GTPase signaling pathway is involved in the microRNA-138 mediated inhibition of cell migration and invasion in tongue squamous cell carcinoma. Int J Cancer.
29. Chao A, Tsai CL, Wei PC, Hsueh S, Chao AS, et al. (2009) Decreased expression of microRNA-199b increases protein levels of SET (protein phosphatase 2A inhibitor) in human choriocarcinoma. Cancer Lett.
30. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R (2008) miR-148 targets human DNMT3b protein coding region. Rna 14: 872–877.
31. Guled M, Lahti L, Lindholm PM, Salmenkivi K, Bagwan I, et al. (2009) CDKN2A, NF2, and JUN are dysregulated among other genes by miRNAs in malignant mesothelioma -A miRNA microarray analysis. Genes Chromosomes Cancer 48: 615–623.
32. Hu X, Schwarz JK, Lewis JS, Jr., Haettner PC, Rader JS, et al. A microRNA expression signature for cervical cancer prognosis. Cancer Res 70: 1441–1448.