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Identification of Novel Retinal Target Genes of Thyroid Hormone in the Human WERI Cells by Expression Microarray Analysis

Yan Liu[a], **Li Fu**[a], **Ding-Geng Chen**[b,c], and **Samir S. Deeb**[a]

[a]Departments of Medicine and Genome Sciences, University of Washington, Seattle, WA 98195

[b]Department of Mathematics and Statistics, South Dakota State University Brookings, SD 57007

[c]Department of Surgery, Sanford School of Medicine, University of South Dakota, Sioux Falls, SD 57105

Abstract

Using the human WERI-Rb1 cell line as a model system, we performed a genome-wide search for retinal target genes of thyroid hormone (TH) via expression microarray analysis followed by quantitative real-time RT-PCR verification. We identified 12 novel retinal targets of TH, including 10 up-regulated genes (*OPN1MW*, *OPN1LW*, *TIMP3*, *RP1L1*, *GNGT2*, *CRX*, *ARR3*, *GCAP1*, *IMPDH1*, and *PDE6C*) and 2 down-regulated genes (*GNGT1* and *GNB3*). In addition, we found a number of novel TH targets that are not currently known to be retinal genes. This is the first report of human retinal targets regulated by thyroid hormone.

Keywords

thyroid hormone; target gene; microarray; retina; photoreceptor

INTRODUCTION

Thyroid hormone (TH) action is known to direct the development and function of many tissues, especially the brain, liver and heart (reviewed in (Yen 2001;Viguerie and Langin 2003)). Recent studies in mice have revealed that TH and its β2 receptor (TRβ2) also play a pivotal role in retinal development. A dorsal-ventral gradient of TH is established in developing mouse retina around postnatal day 10, correlating with the spatial and temporal distribution of the middle-wave-sensitive (M) cone opsin expression (Roberts et al. 2006). In addition, TRβ2 expression is restricted to cone photoreceptors in mouse retina (Roberts et al. 2005) and more importantly, deletion of the gene encoding for TRβ2 in mice results in complete loss of M cones (Ng et al. 2001). These results indicate that TH action is essential for M cone development and differentiation, possibly via regulation of M cone opsin expression, as well as other cone-specific genes.

Different from rodents, which are dichromatic with only M and S (short-wave-sensitive) cones, humans are trichromatic with L (long-wave-sensitive), M, and S (short-wave-sensitive) cones

Address correspondence to: Yan Liu, Division of Medical Genetics, Department of Medicine, BOX 357720, University of Washington, Seattle, WA 98195-7720, ywliu@u.washington.edu.

in the retina. The human L and M cone opsin genes (*OPN1LW* and *OPN1MW*, respectively) are located on the X chromosome and are arranged in a head-to-tail tandem array (reviewed in (Deeb 2004)). These two opsins are highly homologous with only 15 out of 364 amino acids being different. In fact, the L-opsin gene is derived from the M-opsin gene via a duplication event that occurred during evolution and includes both the coding and promoter regions. Furthermore, a regulatory sequence upstream of the L-opsin gene, called the locus control region (LCR), can control the expression of both L- and M-opsin genes (Wang et al. 1992). Interestingly, while these two genes have almost identical promoter sequence, their expression in human retina is mutually exclusive. Despite all that is known about these genes, the mechanism of regulation of L- and M-opsin gene expression remains largely unknown.

Several lines of evidence suggest that the role of TH and its receptor in mouse retina is similar in the human system. In 1977, a case report from Germany showed that a deletion in the human TRβ gene in a patient was associated with S cone monochromacy, indicating that both the L and M cones were missing (Newell, 1977 #170). In addition, transgenic mice bearing a reporter construct driven by the upstream regulatory sequence of the human L/M locus showed a similar expression pattern to that seen with the endogenous mouse M-opsin (Wang et al. 1992; Fei and Hughes 2001). These data strongly support the hypothesis that expression of both human L and M cone opsins is regulated by TH and its receptors during retinal development.

To test whether thyroid hormone regulates the transcription of human L- and M-opsin genes, we took advantage of a human retinoblastoma cell line WERI-Rb1 (hereafter referred as WERI). WERI is an early stage cone lineage cell line, expressing low levels of L- and M-opsin (Shaaban and Deeb 1998). Previously, we had found that these cells express L- and M- opsin in a mutually exclusive pattern, similar to the human retina and the choice of expressing L- or M-opsin can be reset during cell division (Deeb 2006). In this paper, we showed that WERI cells are also ideal for investigation of TH action on human retinal genes. Using WERI as a model system we determined by expression microarray and quantitative real-time RT-PCR (qRT-PCR) analyses that the L- and M-opsin genes are transcriptional targets of thyroid hormone. In addition, a number of other novel TH-targets were also identified.

METHODS

Cell Culture

The human retinoblastoma cell line WERI-Rb1 (WERI) was obtained from the American Type Culture Collection and maintained as a suspension culture in RPMI 1640 supplemented with L-glutamine and 10% fetal bovine serum (Hyclone). For experiments with thyroid hormone treatment, WERI cells were shifted 2 or 3 days before the treatment to medium with 2% B27, a defined serum-free substitute (Invitrogen) instead of fetal bovine serum. The triiodothyronine (T3) stock solution was prepared in 1N NaOH at a concentration of 100 uM. All-trans retinoic acid (ATRA) and 9-cis retinoic acid stock solutions were prepared in DMSO at a 10 mM concentration. All chemicals were obtained from Sigma.

RNA Extraction, RT- PCR and qRT-PCR

Total RNA was extracted from WERI cells using either the Versagene RNA extraction kit (Gentra Systems) or Trizol reagent (Invitrogen) and reverse transcribed (RT) using iScript cDNA synthesis kit (Biorad) in a 10 or 20 ul reaction. cDNA samples from the RT reactions were used as templates for PCR amplification using LA-taq polymerase (TAKARA). The sequence of the isoform-specific primers for human TRα1, TRα2, TRβ1and TRβ2 are given in Malo et al (Malo et al. 2004). Primer sequences for PCR gel analysis and SYBR Green-based quantitative PCR (qPCR) are listed in Table 1. Taqman-based qPCR assays were purchased from Applied Biosystems (ABI) and are listed as follows: APOE

(Hs00171168_m1), ARR3 (Hs00182888_m1), CRX (Hs00230899_m1), CRYM (Hs00157121_m1), CST11 (Hs00370023_m1), DELGEF (Hs00183730_m1), DPP4 (Hs00175210_m1), GAPDH (Hs99999905_m1), GNB3 (Hs00157740_m1), GNGT1 (Hs00184207_m1), GNGT2 (Hs00258864_m1), GCAP1 (Hs00181172_m1), HEG1 (Hs00419997_m1), HR (Hs00218222_m1), IMPDH1 (Hs00265302_m1), LIPG (Hs00195812_m1), LMOD1 (Hs00201704_m1), OPN1LW/OPN1MW (Hs00241039_m1), PDE6C (Hs00196421_m1), PDE6H (Hs00196432_m1), PYY (Hs00373890_g1), RP1L1 (Hs00698865_m1), RRAD (Hs00188163_m1), SAG (Hs00167021_m1), SALL1 (Hs00231307_m1), TIMP3 (Hs00165949_m1).

All qPCR analyses were performed using the Applied Biosystems 7500 apparatus and analyzed by the Relative Quantification ddCt method. SYBR Green-based qPCR reactions were performed using Power SYBR Green Master Mix (ABI). Taqman probe-based qPCR reactions were performed using Taqman Universal Master Mix (ABI). The cycling parameters for SYBR-qPCR analysis are listed in Table 1 with an additional dissociation step to monitor the specificities of amplified products. For Taqman-qPCR, we use the default cycling conditions (40 cycles of 95°C 15 seconds and 60°C 1 minute).

Microarray Analysis

WERI cells were treated in triplicates for 48 hours with either 100 nM T3 or solvent (0.001N NaOH) and harvested for RNA extraction. The array experiment was performed by the Center of Expression Arrays at the University of Washington (http://expression.washington.edu/). Briefly, each sample with 5 ug RNA was reverse transcribed, biotin-labeled by *in vitro* transcription, and then hybridized to human whole genome U133 plus 2.0 oligonucleotide chips (Affymetrix) following the Affymetrix recommended procedures. The chips were stained with streptavidin-phycoerythrin and scanned using the GeneChip Scanner (Affymetrix). The data files were then analyzed by ArrayAssist software (Stratagene) and lists of differentially expressed genes (fold-change ≥ 2, p-value ≤ 0.05 or fold-change ≥ 4, p-value ≤ 0.01) were generated using the PLIER probe level analysis algorithm (Affymatrix) and two-sample Student's *t*-test. Selected target genes identified by microarray were verified by Taqman-based qRT-PCR assays as described above.

RESULTS

WERI cell line as a model system to examine T3 action on human retinal genes

To date, no studies of human retinal genes regulated by T3 have been reported, most likely due to the limitation on available human retinal tissues. An alternative approach is to use cell lines for such a study. Human WERI retinoblastoma cells have been shown to express the thyroid hormone receptors (TR) and many retinal-specific genes (Li et al. 2003). Since TRs include different isoforms and form heterodimers with members of the retinoid X receptor (RXR) family to mediate T3 action, we first examined which isoforms of TR and RXR are present in WERI cells. RT-PCR analysis was performed using isoform-specific primers (Malo et al. 2004). As shown in Figure 1, WERI cells express all four TR isoforms: TRα1, TRα2, TRβ1 and TRβ2, with TRβ2 being at the highest concentration. Interestingly, we found that the TRβ1 PCR product was smaller (about 350bp) than previously reported (484bp) (Malo et al. 2004). Sequence analysis of this smaller TRβ1 product indicated that it matches the TRβ1 sequence, but is missing exons 2 and 3 for a total loss of 136 nucleotides. In addition, WERI cells express all three RXR isoforms. RXRγ is expressed at the highest level among the RXR isoforms (Figure 1). These results suggest that TRβ2/RXRγ heterodimers are likely the predominant form of thyroid hormone receptor complexes in WERI cells.

Expression of the L/M opsin mRNA has been shown to be up-regulated by retinoic acid in WERI cells (Li et al. 2003). We tested whether its expression is also regulated by T3. RNA extracted from WERI cells treated with either T3 or retinoic acid (ATRA or 9-cis RA) were used to perform RT-PCR analysis to compare the expression of L/M opsin mRNA with that of COUPTF1, a known RA-responsive gene (Li et al. 2003). We need to point out here that due to the high homology between the L- and M-opsin genes, many studies do not distinguish between these two genes. In this paper, we consider the L- and M- opsin genes as one unit when the assay amplifies the mRNA of both genes (shown as L/M, or *OPN1LW/OPN1MW*), but as two units when the assay specifically amplifies the mRNA of either L or M opsin gene. As shown in Figure 2, the L/M opsin mRNA was dramatically induced by T3 (10 nM) in WERI cells, but only weakly activated by ATRA or 9-cis RA (Figure 2, top panel). In contrast, the expression level of COUPTF1 was highly induced by ATRA or 9-cis RA, but not by T3 (Figure 2, middle panel). On the other hand, the house keeping gene, GAPDH, was expressed at similar levels among all the samples (Figure 2, bottom panel). These results indicate that WERI cells are T3-responsive and suitable for identification of retinal targets of thyroid hormone.

Identification of TH-targets in WERI cells by expression microarray analysis

To systematically identify target genes of TH in WERI cells, we performed a genome-wide search using expression microarray analysis. To ensure robust signals, we chose to treat WERI cells with 100 nM T3. Although this dose is much higher than physiological concentration (around 1 nM), no toxic effects on WERI cells were observed. The Affymetrix U133+2.0 chip used for the microarray analysis contains approximately 54K probe sets representing about 38,000 genes, including all known retinal-specific genes. Three biological replicates were used for statistical analysis. After 48 hours of T3 treatment, WERI cells were harvested for RNA extraction. The quality of the RNA samples was tested by an Agilent Bioanalyzer. Induction of L/M opsin in T3 treated samples was examined by RT-PCR analysis to assure that the T3 treatment worked well (data not shown). Six samples of RNA (3 mock treated, 3 treated with 100 nM T3) were then labeled and hybridized to U133+2.0 chips. The raw data generated were analyzed by the ArrayAssist software (Stratagene) using the PLIER algorithm for probe level analysis and a two-sample Student's *t*-test for the determination of statistically significant changes in mRNA concentrations. As shown in Figure 3A and 3C, T3 treatment induces a global gene expression profile change in WERI cells with minimal variation between the three biological replicates. From a total of 54,675 probe sets (some genes contain multiple probe sets on the chip) tested, we found that 534 probe sets showed up-regulation of 2 fold or more (p-value $\leq$0.05) and 479 probe sets showed down-regulation of 2 fold or more (p-value $\leq$0.05). A total of 97 probe sets, representing 69 genes, were induced 4 fold or more with a p-value of $\leq$ 0.01 (Figure 3B, Table 2), and 61 probe sets, representing 50 genes, were down-regulated 4 fold or more with a p-value of $\leq$ 0.01 (Figure 3B, Table 3). The complete list of differentially expressed genes with $\geq$2 fold, p-value $\leq$0.05 can be found in the Supplementary Material.

Not surprisingly, we found that the induction level of the L/M opsin gene (*OPN1LW/OPN1MW*) was the highest (44.68 fold) on the list of up-regulated genes (Table 2). In addition to the L/M opsin gene, the following four retinal genes were induced more than 4 fold ($p \leq 0.01$): *CRX* (cone rod homeobox), a homeodomain-containing transcription factor that is essential for the development of cone and rod photoreceptors; *TIMP3* (tissue inhibitor of metalloproteinases), the gene that is mutated in Sorsby's fundus dystrophy, a retinal degenerative disease; *GNGT2* (the γ subunit of cone-specific transducin), and RP1L1 (RP1-like protein 1), a gene with sequence similarity to *RP1* (retinitis pigmentosa 1). The term retinal gene here is defined as a gene found on the lists of retinal disease genes in the RetNet database (www.sph.uth.tmc.edu/Retnet) and retina enriched genes derived from EST, SAGE and microarray data (Qian et al. 2005).

Thyroid hormone receptors can also function as transcriptional repressors in a ligand-dependent or independent manner (reviewed in (Yen 2001)). There are a number of retinal candidates that could potentially be regulated by thyroid hormone negatively: the S-opsin, which was found to be inhibited by thyroid hormone in mouse models and *in vitro* reporter analysis (Yanagi et al. 2002;Roberts et al. 2006); *NRL* and *NR2E3*, two rod-specific transcription factors which positively regulate rod cell differentiation and rod-specific gene expression but have negative impact on the cone pathway (Chen et al. 2005;Cheng et al. 2006;Oh et al. 2007). However, although the U133+2.0 chip contains probes for these genes, no significant change in mRNA levels in response to T3 were observed by microarray analysis. Instead, *GNGT1* (the γ subunit of rod-specific transducin) was the only retinal gene found to be down-regulated by more than 4 fold (-19.90 fold) We suspect that some negative targets of thyroid hormone could be missed using the WERI model system since the cells were most likely derived from a precursor cell that was committed to the L/M cone lineage.

Unexpectedly, we found that most differentially expressed genes identified (Table 2, 3) have not previously been reported to be expressed in the retina and to be TH-responsive. Only one gene on our 4 fold induced list, the hairless gene (*HR*), has been previously reported to be responsive to TH (Thompson 1996).

Verification of microarray data by qRT-PCR

The success of detecting induction of the L/M opsin gene by microarray analysis indicates that the array hybridization experiments were well executed. However, to get a better sense of the performance of the microarray data, we decided to verify additional genes on our list. Since we only found 6 retinal genes (*OPN1LW* and *OPN1MW* are counted as one unit here) on the 4 fold (p-value ≤0.01) list, we decide to expand our search to retinal genes found on the 2-fold list (p-value ≤0.05). We identified a total of 13 retinal genes from the 2-fold (p-value ≤0.05) list, including 6 previously identified from the 4 fold list (Table 4). Quantitative real-time RT-PCR confirmed the microarray results in 12 out of 13 retinal genes tested (Table 4 and Figure 4). Change in expression of SAG, the rod arrestin gene, was the only false positive retinal gene found. This gene was identified as a weakly induced gene by microarray analysis (2.24 fold), but showed no significant change by qRT-PCR analysis. The fold inductions measured by qRT-PCR were generally higher than those obtained from microarray analysis. This is a common phenomenon for microarray experiments due to the saturation of array hybridization signals.

We also examined by qRT-PCR analysis a subset of the non-retinal genes identified by expression microarray analysis. Similar to what was found with the retinal genes, 23 out of 24 non-retinal genes were confirmed by qRT-PCR (Table 4). The T cell receptor α (*TRA@*) was found to be the only false positive. Although *TRA@* was found to be highly induced (10.81 fold) by microarray analysis, it was not detectable in the presence or absence of T3 by qRT-PCR analysis.

In summary, we verified by qRT-PCR a total of 35 out of 37 T3-responsive genes identified by microarray analysis, which is 94.6%. These results indicate that our expression microarray experiment was well performed and the data are reliable.

The identified retinal targets are regulated by TH at physiological concentration

Since the microarray experiment was performed using 100 nM T3, which is much higher than the physiological concentration (about 1 nM), we wanted to confirm that the target genes identified would also be regulated at physiological concentrations. To address this question, we performed a dose-response experiment on all the retinal target genes identified. WERI cells were treated for 48 hours with different doses of T3 (1nM, 10 nM and 100 nM), as indicated

in Figure 5, and the expression of each gene was measured by qRT-PCR. As expected, the induction of L/M opsin mRNA was substantial. Even at 1 nM T3 concentration, L/M opsin mRNA level increased more than 200 fold compared to the untreated samples. To determine the effect of T3 on L-opsin versus M-opsin expression, we developed a qRT-PCR assay using L-specific or M-specific primers. The expression of both genes was shown to be induced by T3, although at 1 nM T3 the induction of M-opsin mRNA level (350 fold) was more dramatic than that of the L-opsin mRNA level (40 fold). *TIMP3*, another gene dramatically induced by T3, was up-regulated more than 15 fold by 1 nM T3 (Figure 5B). The induction of the other 8 retinal targets was below 10 fold (Figure 5C), but nevertheless showed a good dose response. Most importantly, except for *PDE6H*, all these genes showed a significant induction (p-value ≤ 0.05) at 1 nM T3 compared with the untreated group (No T3). On the other hand, the two down-regulated genes showed no dose-dependency of the T3 repression (Figure 5D). Treatment with 1 nM T3 resulted in similar levels of reduction as that seen with 100 nM T3 treatment. This indicates that T3-dependent repression is very sensitive and saturated at doses higher than 1 nM. We conclude from this dose-response experiment that *PDE6H* is not statistically significantly affected by T3 treatment at physiological concentration but the other 12 retinal TH-targets (*OPN1LW* and *OPN1MW* are counted as two genes here) identified by microarray are physiologically relevant.

Next, we decided to measure the kinetics of the T3-response of above retinal genes. In general, the induction of a target gene that is directly activated by thyroid hormone receptor should be faster than a gene that is indirectly activated via an intermediate transcription factor. Therefore, the kinetics may give us some clues about the mechanism of induction. A time course experiment was performed using 5 nM T3 at various time points (0, 6H, 24H, and 48H) as indicated in Figure 6. A 5 nM T3 dose was chosen because it can generate robust signals and is close to the physiological concentration. Based on the kinetics, we categorized the up-regulated genes into 3 groups, fast (F, ≥ 3 fold induction at 6H time point), medium (M, ≥ 3 fold induction at 24H time point) and slow (S, < 3 fold at both 6H and 24H time points). As shown in Figure 6A, the activation of the L- and M-opsin genes by T3 was not only potent, but also very fast. After just 6 hours, the induction reached its maximal level and remained at similar levels at both 24 and 48 hour treatments. *TIMP3*, *CRX*, *GNGT2* and *IMPDH1* also fell into the F group that was induced more than three fold only after 6 hour T3 treatment. *ARR3*, *GCAP1*, *PDE6C* and *RP1L1* fell into the M group which reached 3 fold inductions at 24 hour time point. Only one gene (*PDE6H*) fell into the S group. Significant induction of PDE6H was not observed until the 48H time point. Combining this result with the insignificant induction by 1 nM T3, we concluded that the effect of T3 on *PDE6H* is most likely an *in vitro* artifact due to the high T3 dose and probably not important *in vivo*. Therefore, we removed *PDE6H* from our final list of retinal targets of thyroid hormone.

While most induced target genes reached their maximal expression level by the 6H or 24H time points, maximal repression of *GNGT1* and *GNB3* by T3 was not observed until the 48H time point (Figure 6D). This is probably due to the time it takes to degrade the mRNA of these genes in the cells. Nevertheless, we believe that the inhibition took effect immediately after T3 treatment, which is supported by the quick decrease of expression at 6H. Such an inhibition could be mediated by two different mechanisms, binding of TR to a negative responsive element in these genes and recruitment of transcriptional co-repressor(s) or titrating away transcriptional co-factor(s) that are required for activation of these genes. Further work will be required to elucidate the actual mechanism of the T3-dependent inhibition of *GNGT1* and *GNB3*.

DISCUSSION

Transcriptional regulation of *OPN1LW* and *OPN1MW* opsin genes by thyroid hormone

Using expression microarray and quantitative RT-PCR analysis, we showed in this paper that the expression of both human L and M opsin genes (*OPN1LW* and *OPN1MW*) was rapidly and highly induced by T3 in WERI cells. This result is consistent with the *in vivo* data showing thyroid hormone and its receptor TRβ2 are required for M-cone development in mice (Ng et al. 2001; Roberts et al. 2006), and strongly suggests that the L and M opsin genes are direct transcriptional targets of T3. However, the cis-regulatory element responsible for the T3 response of the L/M locus has not been identified yet. Yanagi et al reported that the locus control region (LCR) contributes to the T3 response of the L/M opsin gene (Yanagi et al. 2002). They found that T3 (at 1 uM) induced luciferase activity about 2-3 fold in 293 cells co-transfected with a reporter construct containing the LCR region of the human L/M locus (fused with a thymidine kinase promoter) and a TRβ2 expression vector. This weak induction is inconsistent with the substantial induction (≥ 500 fold by 100nM T3 treatment) of the endogenous L/M opsin genes observed after T3 treatment. Furthermore, the LCR does not contain a consensus thyroid hormone responsive element (TRE). We analyzed a 10 kb segment upstream of the L/M locus using Alibaba software (http://www.gene-regulation.com/pub/programs/alibaba2) and found a number of potential TREs. Two sites match the consensus DR4 structure of a TRE (direct repeat of two half-site AGGTCA separated by 4 nucleotides), while others contain only a single half-site, which may also function in transcriptional activation by tethering a second TRβ2 or RXRγ molecule. In the future, we will perform chromatin immunoprecipitation (ChIP) and reporter analysis to identify the regulatory sequences that are responsible for induction of the L and M opsin genes by T3.

Other retinal genes regulated by thyroid hormone

We identified a total of 12 retinal genes whose expression is regulated by thyroid hormone. Among them, 8 genes are involved in phototransduction signaling. Seven are in the cone pathway (*OPN1MW*, *OPN1LW*, *ARR3*, *GCAP1*, *GNGT2*, *PDE6C*, and *GNB3*) and one is in the rod pathway (*GNGT1*). Interestingly, genes involved in the cone pathway are mostly induced except for *GNB3*, which is weakly down-regulated. On the other hand, the only gene involved in the rod pathway, *GNGT1*, the rod-specific gamma subunit of transducin, is the most highly repressed gene. What is worth pointing out is that *GNGT2*, the counterpart of *GNGT1* in the cone, is significantly up-regulated by thyroid hormone. These results suggest that thyroid hormone may direct cone photoreceptor development and differentiation through both positive regulation of cone-specific genes and negative regulation of rod-specific genes.

CRX, the cone-rod homeobox gene, is the only transcription factor identified among the retinal targets. CRX is known to be essential for the development and function of both cone and rod photoreceptors (Freund et al. 1997; Furukawa et al. 1997; Furukawa et al. 1999). Mutations of the *CRX* gene are linked to a number of retinal disorders, such as cone-rod dystrophy (Swain et al. 1997) and Leber congenital amaurosis (Swaroop et al. 1999). A number of cone and rod specific genes have been found to be regulated by CRX, including several of the TH targets we identified (L/M opsin gene, ARR3, GCAP1 and IMPDH1) (Chen et al. 1997; Peng et al. 2005; Qian et al. 2005). Chromatin IP and reporter analysis have shown that CRX can bind to the promoter and locus control region (LCR) of the L/M gene locus and activate its transcription (Peng et al. 2005). However, based on the potency and kinetics of induction of L/M opsin versus CRX, we do not think CRX is an intermediate factor required for T3 action on L/M opsin gene. Instead, it is likely that both CRX and TRβ2 directly bind and activate the L/M opsin gene. On the other hand, CRX may mediate the activation of ARR3 and GCAP1 by T3 based on the relatively slower kinetics of these genes. The fact that TR and CRX share a number

of common targets and that CRX is also a T3-target provides new insight into the mechanism of cone photoreceptor differentiation.

TIMP3 is an inhibitor of matrix metalloproteinases (MMP) and is mutated in Sorsby's fundus dystrophy (SFD) (Weber et al. 1994), an autosomal dominant retinal degenerative disease. Interestingly, mutant TIMP3 retains its MMP inhibitor activity and the expression of the TIMP3 gene is increased, rather than decreased in the eyes from patients with SFD (Fariss et al. 1998; Langton et al. 1998). Increased expression of TIMP3 has also been found in other retinal diseases, such as simplex retinitis pigmentosa (Jones et al. 1994; Jomary et al. 1995). Our finding that the expression of TIMP3 can be significantly up-regulated by T3 suggests that TIMP3 could be involved in thyroid-associated ophthalmopathy. It will be interesting to examine the levels of TIMP3 in patients with hyperthyroidism, such as Grave's diseases, to see if such a connection can be substantiated.

We need to point out that the mechanism of T3 action on these retinal genes could occur at both transcriptional and/or post-transcriptional levels as well as directly or indirectly. At this point, we are not able to distinguish between these possibilities. *In silico* analysis of the 3kb promoter regions of CRX and TIMP3 revealed a few potential TREs for each gene. However, future experiments are required to determine whether these potential TREs indeed contribute to the T3-response of these target genes *in vivo*.

Non-retinal genes regulated by thyroid hormone

Thyroid hormone has profound roles in growth, differentiation and metabolic homeostasis. However, after an extensive search for TH target genes (reviewed in (Viguerie and Langin 2003; Kahaly and Dillmann 2005)), the number of TH targets identified is still much lower than expected. Surprisingly, using the WERI retinoblastoma cell line as a model system, we identified a large number of novel targets of TH. We also verified, using qRT-PCR, the TH-regulation of 23 genes that are not currently known to be retinal genes (defined as non-retinal genes here). Apparently, even though the retina may not be the primary tissue for expression of these genes, their activation by thyroid hormone is still permissive in WERI cells. Many of the differentially expressed genes we identified have been previously implicated in thyroid hormone action. For example, *LMOD1*, the 2nd most highly induced gene, was found to be overexpressed in patients with Grave's disease, a hyperthyroid disorder (Kromminga et al. 1998); *DPP4*, a dipeptidyl peptidase involved in metabolic inactivation of incretin hormones, has been found to be highly expressed in thyroid tumors by several gene expression studies (Huang et al. 2001; Jarzab et al. 2005; Taniguchi et al. 2005); *PYY1*(peptide YY), *APOE* (apolipoprotein E) and *LIPG* (endothelial lipase) are all involved in lipid metabolism, providing new mechanisms by which TH could modulate lipid metabolism. We also found three induced genes, *SALL1*(sal-like 1 protein, +6.06 fold), *CRYM* (mu crystalline, +2.59 fold), and *DELGEF* (deafness locus associated putative guanine nucleotide exchange factor, +2.61 fold) that are linked to deafness (Uhlmann et al. 1999; Abe et al. 2003; Botzenhart et al. 2005). It is possible that loss of expression of these genes could be responsible for the deafness phenotype observed in TRβ knock out mice.

In summary, we have identified a number of novel TH target genes using expression microarray analysis followed by qRT-PCR verification. Our findings may contribute significantly to the understanding of the mechanism of thyroid hormone action during photoreceptor development, as well as its involvement in human diseases associated with alterations of thyroid status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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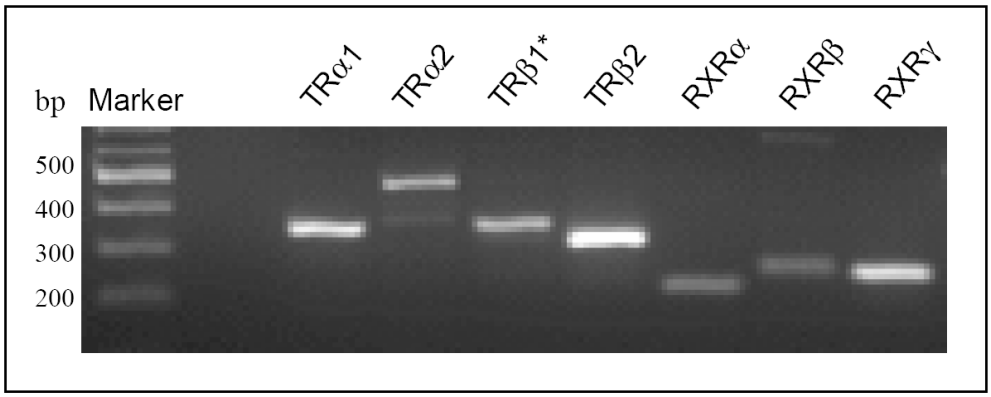

Figure 1. WERI cells express high levels of TRβ2 and RXRγ

RNA extracted from WERI cells was used to perform RT-PCR analysis using isoform-specific primers as described in Table 1. The predicted product size for TRα1 = 346 bp, TRα2 = 467 bp, TRβ1= 484 bp, TRβ2 = 307 bp, RXRα = 201 bp, RXRβ = 239 bp, RXRγ = 228 bp. * = TRβ1 PCR product size is smaller than predicted (484 bp). See text for explanation.

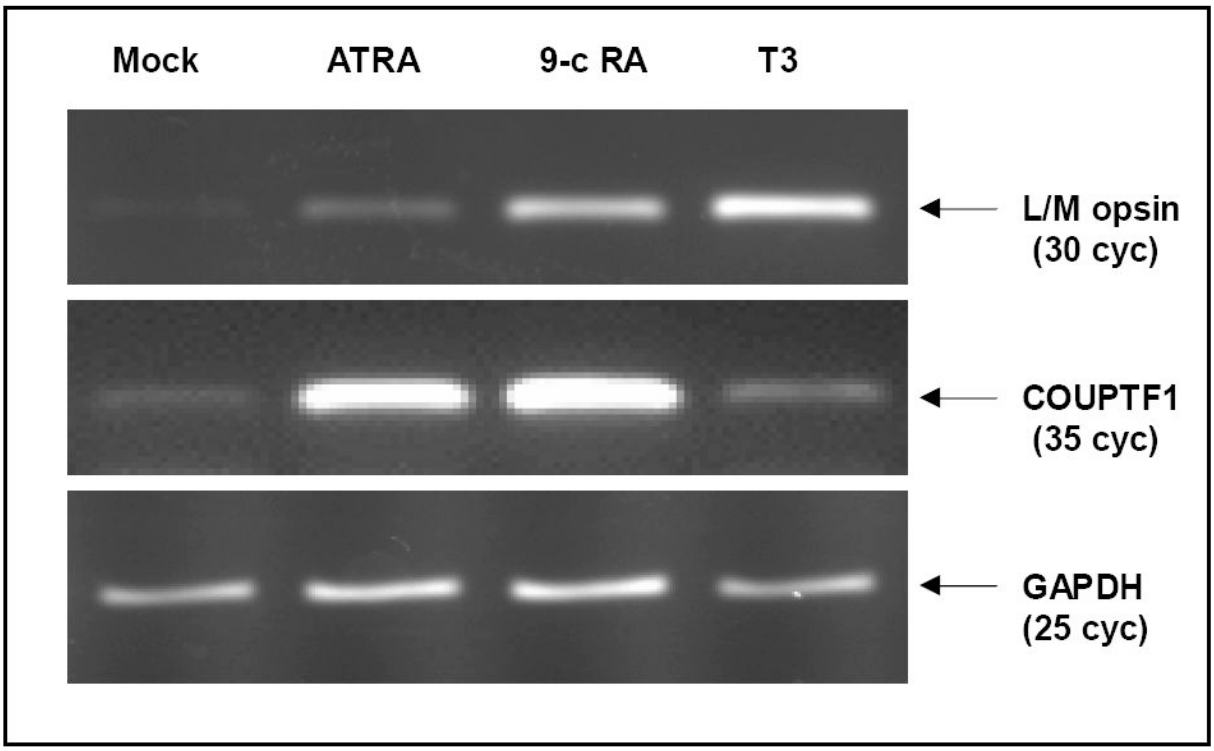

Figure 2. T3 induces the transcription of the L/M opsin gene in WERI cells

WERI cells were either treated with vehicle (mock), 10 uM all-trans retinoic acid (ATRA), 10 uM 9-cis retinoic acid (9c-RA), or 10 nM T3 (T3) for 48 hours. RNA extracted from these samples was used to perform RT-PCR analyses using primer pairs that amplify the L/M opsin gene (top panel), COUPTF1 (middle panel), or the housekeeping gene GAPDH (bottom panel).

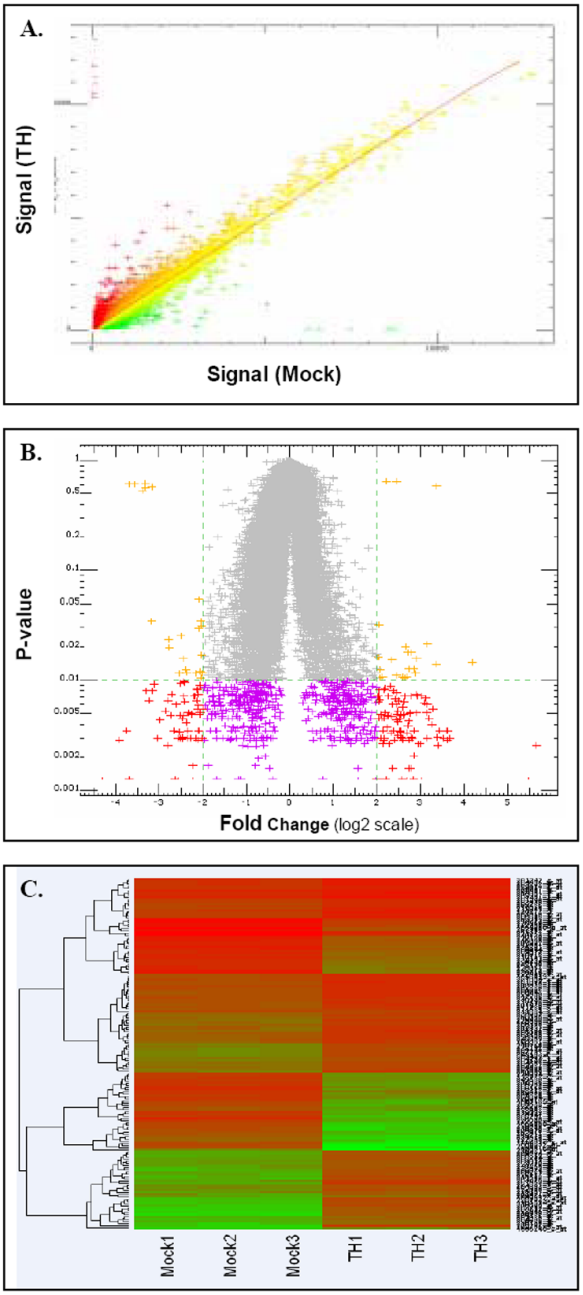

Figure 3. Microarray Expression profile of WERI cells treated with T3

(A). Scatter plot of hybridization signals of RNA from WERI cells treated with 100 nM T3 (TH, Y-axis) versus signals from a mock treated sample (Mock). Up-regulated transcripts are indicated by red + sign; down-regulated transcripts are indicated by green + sign; yellow + sign indicate transcripts with similar expression levels in the TH and Mock samples. **(B).** Scatter plot of p values (Y-axis) versus fold change (X-axis, log2 scale). The red + sign on the bottom left are transcripts down regulated by 4 fold or more with p value of ≤0.01 ; red + signs on the bottom right represent transcripts that are up-regulated by 4 fold or more with p values of ≤0.01. **(C).** Heatmap of probe sets on ±4 fold, p≤0.01 list generated by cluster analysis. Note the three biological replicates show similar levels of expression.

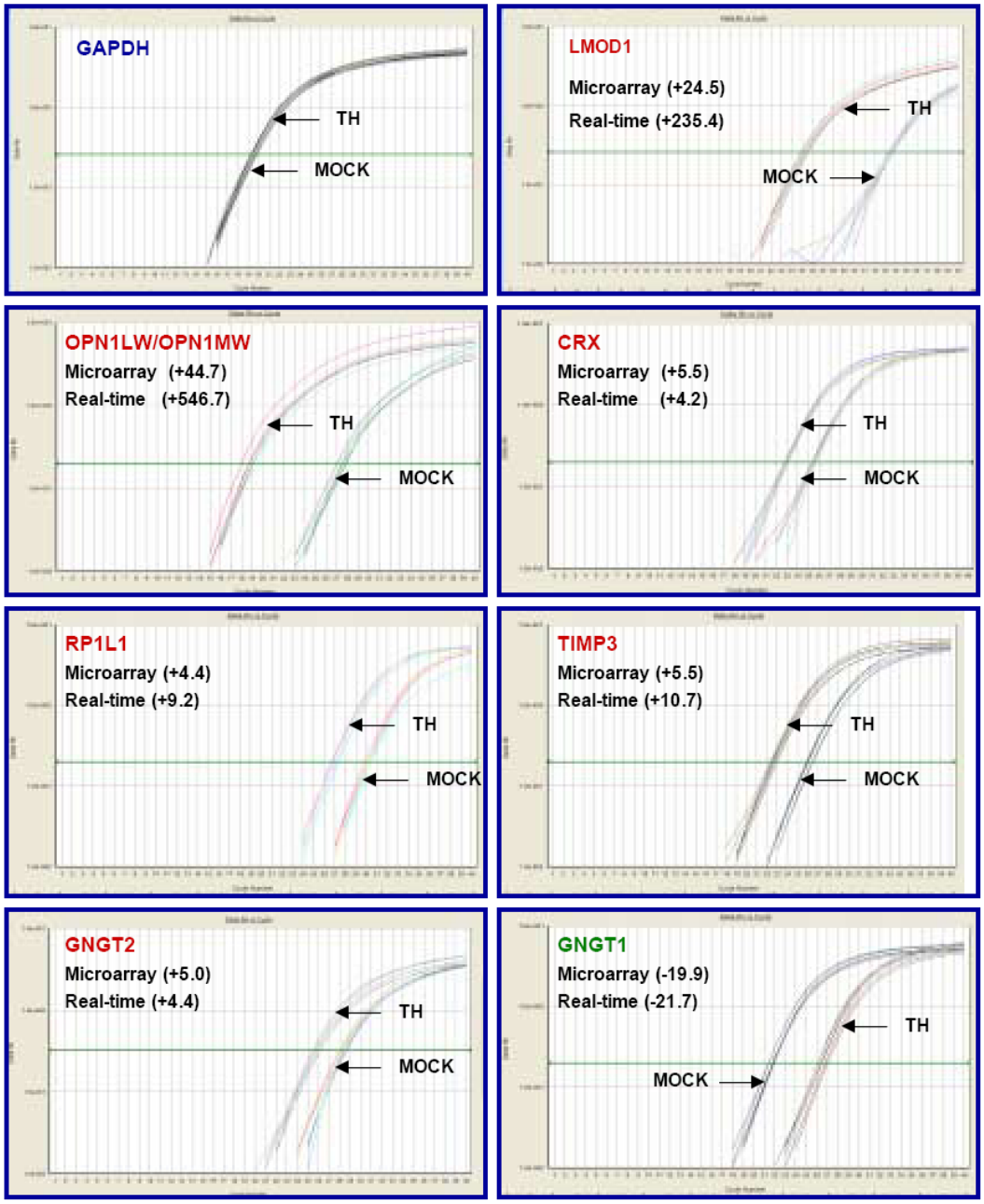

Figure 4. Verification of microarray data by qRT-PCR analysis

RNA samples used for microarray analysis mock (Mock) and 100 nM T3 (TH), were subjected to qRT-PCR to measure the expression levels of the 37 target genes identified by microarray analysis. The data for all 37 genes can be found in Table 4. This figure shows the amplification curves of 8 genes: GAPDH (used for normalization), LMOD1 (an up-regulated non-retinal gene), 5 up-regulated retinal genes (OPN1LW/MW, CRX, RP1L1, TIMP3 and GNGT2), and 1 down-regulated retinal gene (GNGT1). Each sample contains three biological replicates and was assayed in duplicate.

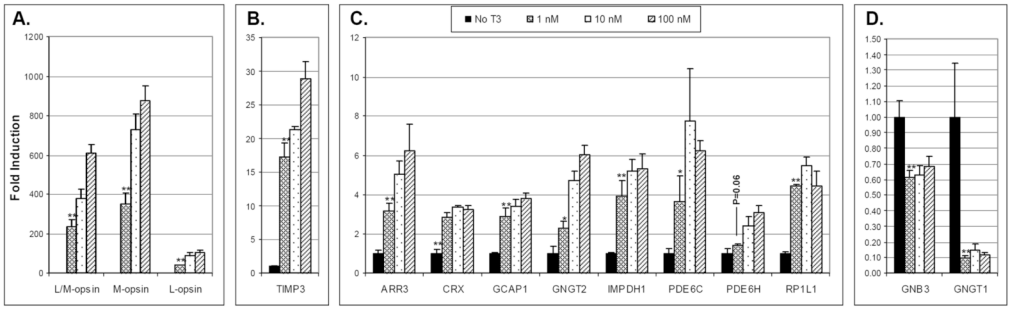

Figure 5. Dose-response analyses on identified retinal TH-targets

RNA samples from WERI cells treated for 48 hours with either vehicle control (No T3) or different concentrations of T3 (1nM, 10 nM, 100 nM) were used to perform qRT-PCR to measure the expression level of each gene as indicated. Fold inductions was calculated using the ddCt method which show the relative amount of each sample compared to the control (No T3). The data shown are normalized mean values ± SD of three biological replicates done in duplicate. Statistical analyses were performed using the Student's *t*-test and compared the 1 nM group to the control group (No T3). The * sign indicates $p \leq 0.05$, ** sign indicates $p \leq 0.01$.

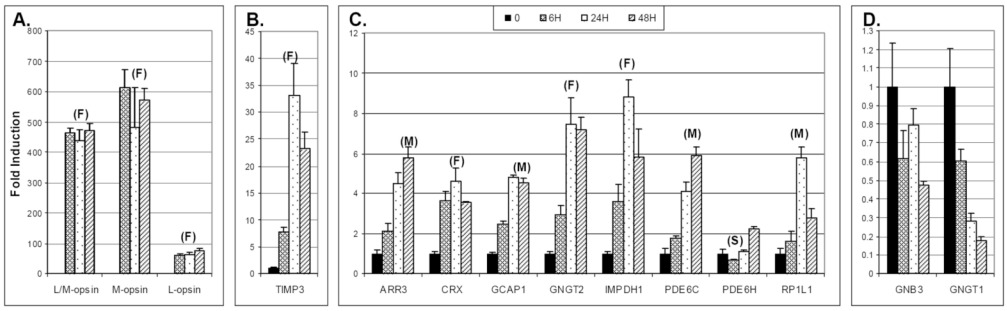

Figure 6. Time course analyses on identified retinal TH-targets

RNA samples from WERI cells treated with 5 nM T3 at different time points were used to perform qRT-PCR to measure the expression levels of each gene as indicated. GAPDH was also measured for normalization. Fold induction was calculated using the ddCt method which shows the relative amount of samples from each time point compared to the 0 time point. The data shown are normalized mean values ± SD of three biological replicates done in duplicate. F=fast, M=medium, S=slow.

Table 1

Primers used for RT-PCR and SYBR-based qRT-PCR analyses

Methods	Gene Symbol	Primer	Sequence (5' to 3')	Cycling Condition
Gel analysis	GAPDH	GAPDH1F	CGCTGAGTACGTCGTGGAGTC	95°C 15 s and 64°C 50s(25 cycles)
		GAPDH1R	CACAGTCTTCTGGGTGGCAGT	
	RXRA	hRXRα-F	CAT CTT TGA CAG GGT GCT GAC	95°C 15 s, 62°C 30s, 72°C 40s (35 cycles)
		hRXRα-R	TGC TCT GGG TAC TTG TGC TTG	
	RXRB	hRXRβ-F	GAG TAG GAG CCA TCT TTG ATC G	
		hRXRβ-R	TAG CAG CAG CTT GGC AAA CCG	
	RXRG	hRXRγ-F	GGT CGG CTC CAT CTT TGA CAG	
		hRXRγ-R	TTG GCA AAC CTG CCT GGC TG	
	L/M opsin	CB196	TACCCCGGGGTGCAGTCTTAC	98°C 10 s and 66°C 30 s (30 cycles)
		CB78	TTGGCAGCAGCAAAGCATGCG	
SYBR-qPCR	M-opsin (OPN1MW)	CB7G	ACCCCACTCAGCATCATCGT	95°C 15 s and 62°C 40 s (40 cycles)
		CB79G	CCAGCAGAAGCAGAATGCCAGGAC	
	L-opsin (OPN1LW)	CB7R	ATCCCACTCGCTATCATCAT	
		CB79R	CCAGCAGACGCAGTACGCAAAGATC	
	GMPR	GMPR-F1	CGTGTTCAGCTAACCCTGGGGAC	95°C 15 s and 65°C 40s (40 cycles)
		GMPR-R1	ACCATTCAGGAGCAGCCAGAAGC	
	ITM2C	ITM2C-F1	AAGCAAGGAGCTAGGACCCCCAG	
		ITM2C-R1	GACTGAGCAGTGACCTTGCCTGC	
	KIAA1755	KIAA1755-F1	TCATTGTGGAAAGACCTGTCGGC	
		KIAA1755-R1	ACCCGAGGGGAGAGCTGTGTATG	
	MARCO	MARCO-F1	GGGACAATTTGCGATGACGAGTG	
		MARCO-R1	CCAGCTCCCACTTTGTACAGGGC	
	PAMLM2-AKAP2	PALM2-AKAP2-F1	TGCATTCTGCCGTGTTTATAGGTG	
		PALM2-AKAP2-R1	TGCCACTGACAGACCCTGTTTCC	
	RARI14	RAI14-F1	ACGCTTGCAACTTCCCTTATGGC	
		RAI14-R1	ACTGAGGCCAAGCAGCCTTGTG	
	SPON2	SPON2-F1	CTGCTCTCAGCCTCCTCCTCCTG	
		SPON2-R1	CCCCTGGACGATGAAGGACAATC	
	TFF1	TFF1-F1	TCGACGTCCCTCCAGAAGAGGAG	
		TFF1-R1	GCAGAAGCGTGTCTGAGGTGTCC	
	THEDC1	THEDC1-F1	GTACATTCAAAGGCCTGGCATCG	
		THEDC1-R1	CTTCAGCAAAATGCTTGGGGGTG	
	TP53I3	TP53I3-F1	AGGCAAGATCGTCCTGGAACTGC	
		TP53I3-R1	TAAACGGCTCTGGAGGAAGCACC	
	TRA@	TRA@-F1	CTCGAACCGAACAGCAGTGCTTC	
		TRA@-R1	TCTCTCAGCTGGTACACGGCAGG	
	TU3A	TU3A-F1	TGGTGTGAGGACCATGCTGTGAG	

Methods	Gene Symbol	Primer	Sequence (5’ to 3’)	Cycling Condition
		TU3A-R1	GTTGCAGAAGTGGGGTGGGAATC	

Table 2

T3-induced genes in WERI cells (fold-change ≥ 4, p-value ≤0.01)

Rank	Gene Symbol	UniGene ID	Gene Name	Fold
1	**OPN1MW**	Hs.247787	cone opsin, medium-wave-sensitive	44.68
	OPN1LW	Hs.592247	cone opsin, long-wave-sensitive	
2	LMOD1***	Hs.519075	leiomodin 1 (smooth muscle)	24.46
3	PALM2-AKAP2	Hs.591908	PALM2-AKAP2 protein	12.75
4	KIAA1755	Hs.472690	KIAA1755 protein	12.33
5	Unknown	Hs.26039	Homo sapiens, clone IMAGE:3869276,	12.00
6	DPP4**	Hs.368912	dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	10.68
7	CST11	Hs.128100	cystatin 11	10.60
8	TRA@**	Hs.74647	T cell receptor alpha locus	9.85
9	TP53I3	Hs.50649	tumor protein p53 inducible protein 3	9.64
10	TU3A	Hs.506357	TU3A protein	9.15
11	RAI14	Hs.431400	retinoic acid induced 14	8.93
12	MARCO	Hs.67726	macrophage receptor with collagenous structure	8.83
13	THEDC1	Hs.24309	thioesterase domain containing 1	8.68
14	ITM2C	Hs.111577	integral membrane protein 2C	8.19
15	PYY	Hs.169249	peptide YY	8.06
16	SPON2	Hs.302963	spondin 2, extracellular matrix protein	7.94
17	TFF1	Hs.162807	trefoil factor 1 (breast cancer, estrogen-inducible sequence expressed in)	7.77
18	GMPR	Hs.484741	guanosine monophosphate reductase	7.69
19	RRAD**	Hs.1027	Ras-related associated with diabetes	7.57
20	TMEM35	Hs.45140	transmembrane protein 35	7.50
21	HR	Hs.272367	hairless homolog (mouse)	7.49
22	FNDC5	Hs.524234	fibronectin type III domain containing 5	7.24
23	MAP2	Hs.368281	Microtubule-associated protein 2	6.90
24	PSCD1	Hs.191215	pleckstrin homology, Sec7 and coiled-coil domains 1(cytohesin 1)	6.84
25	FHOD3	Hs.436636	formin homology 2 domain containing 3	6.77
26	RUTBC2	Hs.474397	RUN and TBC1 domain containing 2	6.75
27	JUP	Hs.514174	junction plakoglobin	6.67
28	HEG***	Hs.477420	HEG homolog 1 (zebrafish)	6.57
29	TXNIP***	Hs.533977	thioredoxin interacting protein	6.45
30	Unknown	Hs.436603	Transcribed locus	6.39
31	APOE***	Hs.110675	Apolipoprotein E	6.06
32	CD52**	Hs.276770	CD52 antigen (CAMPATH-1 antigen)	6.06
33	SALL1**	Hs.135787	sal-like 1 (Drosophila)	6.05
34	MONDOA**	Hs.437153	Mlx interactor	5.99
35	TNC	Hs.143250	tenascin C (hexabrachion)	5.76
36	Unknown	Hs.533721	hypothetical protein KIAA0284	5.73
37	ABCC4	Hs.508423	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	5.64

Rank	Gene Symbol	UniGene ID	Gene Name	Fold
38	RAB37	Hs.351413	RAB37, member RAS oncogene family	5.61
39	Unknown	Hs.7413	Transcribed locus	5.60
40	JARID2***	Hs.269059	Jumonji, AT rich interactive domain 2	5.57
41	**CRX**	Hs.617342	cone-rod homeobox	5.52
42	**TIMP3******	Hs.297324	tissue inhibitor of metalloproteinase 3	5.51
43	DPYSL3**	Hs.519659	dihydropyrimidinase-like 3	5.50
44	GLUL***	Hs.518525	glutamate-ammonia ligase (glutamine synthase)	5.50
45	GAS6***	Hs.369201	growth arrest-specific 6	5.38
46	KIAA1622	Hs.259599	KIAA1622	5.19
47	ATP1B1	Hs.291196	ATPase, Na+/K+ transporting, beta 1 polypeptide	5.17
48	P4HA2	Hs.519568	procollagen-proline, 2-oxoglutarate 4-dioxygenase	5.15
49	Cep164	Hs.504009	KIAA1052 protein	5.05
50	**GNGT2**	Hs.181781	G protein, gamma transducing activity polypeptide 2	5.00
51	EPHX2	Hs.212088	epoxide hydrolase 2, cytoplasmic	4.98
52	LDLRAD3	Hs.205865	Low density lipoprotein receptor	4.86
53	SOX7	Hs.213194	SRY (sex determining region Y)-box 7	4.65
54	FGFR1	Hs.264887	fibroblast growth factor receptor 1	4.58
55	SLC9A3R1	Hs.396783	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1	4.56
56	ANKRD33	Hs.433492	ankyrin repeat domain 33	4.50
57	C19orf30	Hs.326728	chromosome 19 open reading frame 30	4.49
58	Unknown	Hs.446662	Transcribed locus, strongly similar to NP_057175.1	4.46
59	**RP1L1**	Hs.33538	retinitis pigmentosa 1-like 1	4.43
60	PSG9**	Hs.502092	pregnancy specific beta-1-glycoprotein 9	4.40
61	LOXL1	Hs.65436	lysyl oxidase-like 1	4.40
62	ELA3A**	Hs.181289	elastase 3A, pancreatic (protease E)	4.31
63	SCN1B	Hs.436646	sodium channel, voltage-gated, type I, beta	4.25
64	PITPNC1	Hs.591185	phosphatidylinositol transfer protein, cytoplasmic 1	4.22
65	SLC2A3**	Hs.419240	solute carrier family 2, member 3	4.21
	SLC2A14		solute carrier family 2, member 14	
66	LIPG	Hs.465102	lipase, endothelial	4.16
67	CCL26	Hs.131342	chemokine (C-C motif) ligand 26	4.11
68	OLFM1	Hs.522484	olfactomedin 1	4.01
69	HIPK2	Hs.397465	Homeodomain interacting protein kinase 2	4.00

Note: Gene symbols with bold font are retinal genes. Gene symbols with asterisks indicate that the fold induction is the average of multiple probe sets from the array

** = 2 probe sets

*** = 3 probe sets

**** = 4 probe sets

Table 3

T3-repressed genes in WERI cells (fold-change ≤- 4, p-value ≤0.01)

Rank	Gene Symbol	UniGene ID	Gene Title	Fold
1	**GNGT1**	Hs.642688	G protein, gamma transducing activity polypeptide 1	-19.90
2	Unknown	Hs.56400	Similar to Cdc42 guanine nucleotide exchange factor zizimin 1	-13.47
3	LOC387763	Hs.530443	hypothetical LOC387763	-12.82
4	SGPP2**	Hs.210043	Sphingosine-1-phosphate phosphotase 2	-11.00
5	SLIT3	Hs.145601	slit homolog 3 (Drosophila)	-9.83
6	LHX4	Hs.496545	LIM homeobox 4	-9.40
7	NAP5	Hs.516471	Nck-associated protein 5	-8.94
8	LOC440934	Hs.238964	Hypothetical gene supported by BC008048	-8.63
9	PCSK2	Hs.315186	proprotein convertase subtilisin/kexin type 2	-8.25
10	NDRG4	Hs.322430	NDRG family member 4	-7.89
11	LOC440928	Hs.528187	Hypothetical gene supported by AK096649	-7.23
12	SLC27A6	Hs.49765	solute carrier family 27 (fatty acid transporter), member 6	-6.96
13	EBF3	Hs.205433	early B-cell factor 3	-6.66
14	ABCC8	Hs.54470	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	-6.58
15	Unknown	Hs.554010	Transcribed locus	-6.11
16	FLJ34870	Hs.270083	FLJ34870 protein	-5.87
17	FOXP2***	Hs.282787	forkhead box P2	-5.80
18	ADRA2A	Hs.249159	adrenergic, alpha-2A-, receptor	-5.79
19	DDIT4L	Hs.480378	DNA-damage-inducible transcript 4-like	-5.72
20	MFAP4	Hs.296049	microfibrillar-associated protein 4	-5.54
21	CNTN1**	Hs.143434	Contactin 1	-5.53
22	HHIP	Hs.507991	hedgehog interacting protein	-5.39
23	PRDM1***	Hs.436023	PR domain containing 1, with ZNF domain	-5.35
24	PDZK1	Hs.444751	PDZ domain containing 1	-5.32
25	Unknown	Hs.147881	Homo sapiens, clone IMAGE:4826545, mRNA	-5.30
26	ADCY2**	Hs.481545	adenylate cyclase 2 (brain)	-5.27
27	PTPRG	Hs.148340	protein tyrosine phosphatase, receptor type, G	-5.19
28	KIAA1913	Hs.172870	KIAA1913	-5.03
29	LOC643771	Hs.549665	Hypothetical protein LOC643771	-5.03
30	Unknown	Hs.356481	CDNA clone IMAGE:4077090, partial cds	-4.85
31	Unknown	Hs.146050	Transcribed locus	-4.81
32	SV2C***	Hs.482549	Synaptic vesicle glycoprotein 2C	-4.80
33	ISOC1	Hs.483296	isochorismatase domain containing 1	-4.79
34	APCDD1	Hs.293274	adenomatosis polyposis coli down-regulated 1	-4.78
35	GSG1	Hs.558558	germ cell associated 1	-4.70
36	CTNND2**	Hs.314543	catenin (cadherin-associated protein), delta 2	-4.63
37	UNC93A	Hs.145911	unc-93 homolog A (C. elegans)	-4.58
38	RPL10A	Hs.148340	Ribosomal protein L10a	-4.49
39	ERN1	Hs.133982	endoplasmic reticulum to nucleus signalling 1	-4.41

Rank	Gene Symbol	UniGene ID	Gene Title	Fold
40	Unknown	Hs.552087	CDNA clone IMAGE:5922621, partial cds	-4.37
41	SPAG1	Hs.492373	sperm associated antigen 1	-4.36
42	HT017	Hs.558524	HT017 protein	-4.34
43	DOC1	Hs.104672	downregulated in ovarian cancer 1	-4.29
44	NSE2	Hs.124951	breast cancer membrane protein 101	-4.27
45	C10orf13	Hs.466120	chromosome 10 open reading frame 13	-4.26
46	AMPD3	Hs.501890	adenosine monophosphate deaminase (isoform E)	-4.23
47	BAI3	Hs.13261	brain-specific angiogenesis inhibitor 3	-4.18
48	PRG-3	Hs.382683	plasticity related gene 3	-4.17
49	CAP2	Hs.132902	CAP, adenylate cyclase-associated protein, 2 (yeast)	-4.04
50	LGI2	Hs.12488	leucine-rich repeat LGI family, member 2	-4.02

Note: Gene symbols with bold font are retinal genes. Gene symbols with asterisks indicate that the fold induction is the average of multiple probe sets from the array

** = 2 probe sets

*** = 3 probe sets

**** = 4 probe sets

Table 4

Validation of microarray data by qRT-PCR analysis

	Gene Function	Gene Symbol	Unigene Hs. No.	Microarray		Real Time		Validation
				Fold	p-value	Fold	p-value	
Retinal Genes	Cone phototransduction	OPN1MW/ OPN1LW	247787 592247	44.68	≤0.01	546.64	≤0.01	TRUE
		GNGT2	181781	5.00	≤0.01	4.37	≤0.01	TRUE
		ARR3	308	2.93	≤0.01	3.10	≤0.01	TRUE
		GCAP1**	92858	2.70	≤0.01	3.00	≤0.01	TRUE
		PDE6H	54471	2.21	≤0.01	2.22	≤0.01	TRUE
		PDE6C	93173	2.06	≤0.05	2.64	≤0.01	TRUE
		GNB3	534315	-2.41	≤0.05	-2.85	≤0.01	TRUE
	Rod phototransduction	SAG	32721	2.24	≤0.05	1.00	≤0.99	**FALSE**
		GNGT1	642688	-19.90	≤0.01	-21.74	≤0.05	TRUE
	Transcription	CRX	617342	5.52	≤0.01	4.22	≤0.01	TRUE
	Others	TIMP3****	297324	5.51	≤0.01	10.74	≤0.01	TRUE
		RP1L1	33538	4.43	≤0.01	9.17	≤0.01	TRUE
		IMPDH1	534808	2.85	≤0.01	2.90	≤0.01	TRUE
Non-retinal genes	Auditory function	SALL1	135787	6.05	≤0.01	8.08	≤0.01	TRUE
		DELGEF	32470	2.61	≤0.01	3.01	≤0.01	TRUE
		CRYM	924	2.59	≤0.05	3.02	≤0.01	TRUE
	Cardiac function	HEG***	477420	6.57	≤0.01	6.91	≤0.01	TRUE
		APOE***	110675	6.06	≤0.01	7.46	≤0.01	TRUE
	Gene regulation	HR	272367	7.49	≤0.01	90.05	≤0.01	TRUE
	Immune response	TRA@	74647	10.81	≤0.01	Not detectable		**FALSE**
		MARCO	67726	8.83	≤0.01	103.47	≤0.01	TRUE
	Metabolism	LMOD1***	519075	24.46	≤0.01	235.44	≤0.01	TRUE
		DPP4**	368912	10.68	≤0.01	80.05	≤0.01	TRUE
		CST11	128100	10.60	≤0.01	40.28	≤0.01	TRUE
		PYY	169249	8.06	≤0.01	54.93	≤0.01	TRUE

	Gene Function	Gene Symbol	Unigene Hs. No.	Microarray		Real Time		Validation
				Fold	p-value	Fold	p-value	
		GMPR	484741	7.69	≤0.01	33.12	≤0.01	TRUE
		RRAD**	1027	7.57	≤0.01	11.37	≤0.01	TRUE
		LIPG	465102	4.16	≤0.01	7.10	≤0.01	TRUE
	Others	PALM2/ AKAP2	259461	12.75	≤0.01	125.19	≤0.01	TRUE
		KIAA1755	472690	12.33	≤0.01	238.97	≤0.01	TRUE
		THEDC1	24309	10.23	≤0.05	58.24	≤0.01	TRUE
		TP53I3	50649	9.64	≤0.01	14.72	≤0.01	TRUE
		TU3A	8022	9.15	≤0.01	28.85	≤0.01	TRUE
		RARI14	431400	8.93	≤0.01	15.33	≤0.01	TRUE
		ITM2C	111577	8.19	≤0.01	16.37	≤0.05	TRUE
		SPON2	302963	7.94	≤0.01	12.99	≤0.01	TRUE
		TFF1	162807	7.77	≤0.01	24.71	≤0.01	TRUE

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