



Thyroid hormone-regulated cardiac microRNAs are predicted to suppress pathological hypertrophic signaling

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Cardiomyocyte size in the healthy heart is in part determined by the level of circulating thyroid hormone (TH). Higher levels of TH induce ventricular hypertrophy, primarily in response to an increase in hemodynamic load. Normal cardiac function is maintained in this form of hypertrophy, whereas progressive contractile dysfunction is a hallmark of pathological hypertrophy. MicroRNAs (miRNAs) are important modulators of signal-transduction pathways driving adverse remodeling. Because little is known about the involvement of miRNAs in cardiac TH action and hypertrophy, we examined the miRNA expression profile of the hypertrophied left ventricle (LV) using a mouse model of TH-induced cardiac hypertrophy. C57Bl/6J mice were rendered hypothyroid by treatment with propylthiouracil and were subsequently treated for 3 days with TH (T3) or saline. T3 treatment increased LV weight by 38% ($p < 0.05$). RNA was isolated from the LV and expression of 641 mouse miRNAs was determined using Taqman Megaplex arrays. Data were analyzed using RQ-manager and DataAssist. A total of 52 T3-regulated miRNAs showing a >2 -fold change ($p < 0.05$) were included in Ingenuity Pathway Analysis to predict target mRNAs involved in cardiac hypertrophy. The analysis was further restricted to proteins that have been validated as key factors in hypertrophic signal transduction in mouse models of ventricular remodeling. A total of 27 mRNAs were identified as *bona fide* targets. The predicted regulation of 19% of these targets indicates enhancement of physiological hypertrophy, while 56% indicates suppression of pathological remodeling. Our data suggest that cardiac TH action includes a novel level of regulation in which a unique set of TH-dependent miRNAs primarily suppresses pathological hypertrophic signaling. This may be relevant for our understanding of the progression of adverse remodeling, since cardiac TH levels are known to decrease substantially in various forms of pathological hypertrophy.

Keywords: thyroid hormone, hyperthyroidism, microRNA, pathway analysis, physiological hypertrophy, pathological hypertrophy

INTRODUCTION

An increase in the hemodynamic load placed on the heart induces cardiomyocyte hypertrophy. The resulting increase in ventricular mass is aimed at normalizing wall stress and maintaining cardiac output. In the context of chronic pressure and/or volume overload, such as in hypertension, aortic stenosis, valvular disease, or following myocardial infarction, the initially adaptive response is associated with progressive contractile dysfunction and heart failure (1). This pathological remodeling is characterized by distinct changes in cardiac gene expression, increased cardiomyocyte apoptosis, and fibrosis. Typical phenotypic changes include a shift in expression from the fast contractile protein myosin heavy chain α (*Myh6*) to the slow β isoform (*Myh7*) (2), reduced expression of sarcoplasmic reticulum Ca^{2+} -ATPase (*Serca2a*) (2, 3), and increased expression of fetal genes [e.g., α -skeletal actin, atrial natriuretic peptide (*Anp*), B-type natriuretic peptide (*Bnp*), deiodinase type III (*Dio3*) (4–6)].

In contrast, cardiac hypertrophy in response to increased levels of circulating thyroid hormone (TH) (hyperthyroidism) is not associated with adverse remodeling or impairment of contractility in both animals and human beings (7–9), in spite of the chronically

increased hemodynamic load, which also in this case is the principal trigger of cardiomyocyte growth (10, 11). TH-induced remodeling is, therefore, considered physiological and is similar to physiological ventricular hypertrophy induced by pregnancy or chronic exercise training, and is equally reversible (12–14).

The distinction between stimuli and the related signal-transduction pathways that cause either pathological or physiological hypertrophy has not been without discussion. It has been presumed that the duration of the stimulus, i.e., chronic or intermittent, is the key difference determining the two forms of hypertrophy. Nevertheless, studies using intermittent pressure overload showed that although the resulting hypertrophy appeared to be adaptive, the cellular characteristics already showed all features of pathological remodeling (15). This suggests that certain aspects of the different stimuli and down-stream processes determine the phenotype of hypertrophy, rather than the duration of the exposure to the stimuli (16).

Gain- and loss-of-function studies have delineated numerous signal-transduction pathways which relay the various neurohumoral and mechanical signals that drive cardiac hypertrophy [see Ref. (16) for an extensive review]. There is considerable overlap

and cross-talk between these pathways and although some pathways appear to mediate physiological, and others, pathological hypertrophy, the mechanisms underlying development of either one of these distinct phenotypes in response to chronic overload are not clear (16). In the case of TH, some effects of pathological signaling will be countered by direct transcriptional regulation of genes such as *Myh6*, *Myh7*, and *Serca2a* (7, 17), mediated by the TH receptors $\alpha 1$ or $\beta 1$ (18). Both receptors are capable of mediating the transcriptional T3 effect, and they are also implicated in the non-genomic T3 effect involving stimulation of the phosphoinositide 3-kinase [PI3K (p110 α)]-Akt pathway (19–21). However, additional mechanisms are expected to account for the full suppression of adverse remodeling.

Recent studies have shown an important role for microRNAs (miRNAs) in the regulation of cardiac gene expression by virtue of their ability to induce degradation of specific target mRNAs or reduce the efficiency of translation (22, 23). Although miRNAs are generally considered to fine-tune gene expression, critical roles of individual miRNAs have been established in various aspects of pathological cardiac hypertrophy (22–25). For example, miR-208a is required for overload-induced pathological remodeling, including cardiomyocyte hypertrophy, *Myh* isoform switching, and fibrosis (26, 27). Furthermore, forced expression of miR-208a induces pathological remodeling (27), whereas blocking of miR-208a attenuates hypertension-induced cardiac dysfunction (28). This miRNA is encoded in an intron of the *Myh6* gene, which is transcriptionally stimulated by TH. Consequently, and perhaps counter intuitively, expression of this “pathological” miRNA is increased in hyperthyroidism (27, 29). Little else is known about the TH-dependency of miRNA expression in the heart, despite the obvious potential of miRNA-mediated effects. However, studies in liver and skeletal muscle (30, 31) have identified a number of TH-responsive miRNAs, which are also known to play a role in remodeling adult cardiomyocytes and differentiating cardiomyocyte progenitor cells (32–35).

As a first step in elucidating a role of miRNAs in cardiac TH action, we set out to identify TH-responsive cardiac miRNAs using a mouse model of physiological hypertrophy induced by TH. To increase the sensitivity and to limit long-term secondary effects, TH-deficient mice were treated for 3 days with T3, the active form of TH. Target analysis of the 52 differentially regulated miRNAs indicated limited potentiation of pathways involved in physiological hypertrophy, but a marked suppression of pathways associated with pathological hypertrophy. Our data suggest that cardiac TH action includes a novel level of regulation in which a unique set of TH-dependent miRNAs suppresses pathological hypertrophic signaling.

MATERIALS AND METHODS

ANIMALS

Two groups of six male C57BL/6 mice (10–12 weeks, Charles Rivers) were used. Both groups were allowed *ad libitum* access to food containing PTU (Teklad + 0.15% propylthiouracil) for 44 days to induce hypothyroidism. Six mice were injected (IP) with a supra-physiological dose of 5 μ g T3 in 20 μ l saline at day 42, 43, and 44 (corresponding to 0.21–0.24 μ g T3/g BW); the remaining

six hypothyroid mice were injected with 20 μ l saline. Animals were sacrificed at day 45. The heart was excised and left and right ventricle (LV, RV) and septum were dissected, weighed, immediately frozen in liquid nitrogen, and stored at -80°C . Tibia length was determined for normalization purposes. Plasma T3 levels were determined by AccuBind ELISA kits (Monobind Inc., Lake Forest, CA) according to the manufacturer’s instructions. Animals were housed individually and all experiments complied with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication no. 86-23, revised 1996) and were approved by the Institutional Animal Care and use Committees of VU University Medical Center Amsterdam.

RNA ISOLATION

Total RNA was isolated from approximately 20 mg LV using the mirVana Paris isolation kit (Ambion, Foster City, CA). To improve cell lysis, frozen samples were sliced into 10 μ m sections using a cryostat. The tissue sections were dissolved in 2 ml cell disruption buffer. Three aliquots of 500 μ l lysate were transferred to Eppendorf tubes and 500 μ l of 2 \times denaturation solution, containing guanidinium thiocyanate, was added to each tube to prevent RNA degradation by cellular ribonucleases. Subsequently, an equal volume of acid-phenol:chloroform was added to separate RNA from DNA and protein (36). The aqueous phase was transferred to a fresh tube. To make sure there was no DNA or protein left, we repeated the previous step. Next, 1.25 volumes of 100% ethanol was added before each extract was transferred onto an individual filter cartridge, centrifuged, and washed. The bound RNA fraction was pretreated with DNaseI (Qiagen, Venlo, the Netherlands) before 105 μ l elution buffer was added to each filter cartridge to collect the total RNA fraction. It should be noted that the capacity of the filter is limited, requiring the use of three cartridges per sample. RNA concentration and quality were measured either with the Nanodrop1000 (Thermo Scientific, Wilmington, DE) or the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples were stored at -80°C , at which temperature miRNAs are stable (37).

QUANTITATIVE REAL-TIME PCR

To analyze mRNA levels of the TH-regulated myosin heavy chains (*Myh6*, *Myh7*), 10 μ l DNaseI-treated total RNA was transcribed to cDNA using Cloned AMV first strand cDNA synthesis (Invitrogen, Carlsbad, CA, USA). RNA concentration was adjusted to 12.5 ng/ μ l. Quantitative real-time polymerase chain reaction (qPCR) was performed using MESA GREEN qPCR MasterMix Plus for SYBR assay (Eurogentec, Seraing, Belgium) and specific primers (38) for *Myh6* (sense: 5'-GACCAGGCCAATGAGTACCG-3', antisense: 5'-GCCTAGCCAACCTCCCGTTC-3') and *Myh7* (sense: 5'-CGCTCCACGCACCCTCACTT-3', antisense: 5'-GTCCATCACCCT GGAGAC-3') in an Applied Biosystems model 7700 (Applied Biosystems, Foster City, CA). Expression levels of *Hprt* (sense: 5'-TCCCTGGTTAAGCAGTACAGCC-3', antisense: 5'-CGAGAGGTCTTTTACCAGC-3') were used for normalization. For comparison of the relative gene expression [$2^{-(\text{Ct HPRT} - \text{Ct target})}$] of the two groups, a two-tailed Student *t*-test was performed to calculate *p* values.

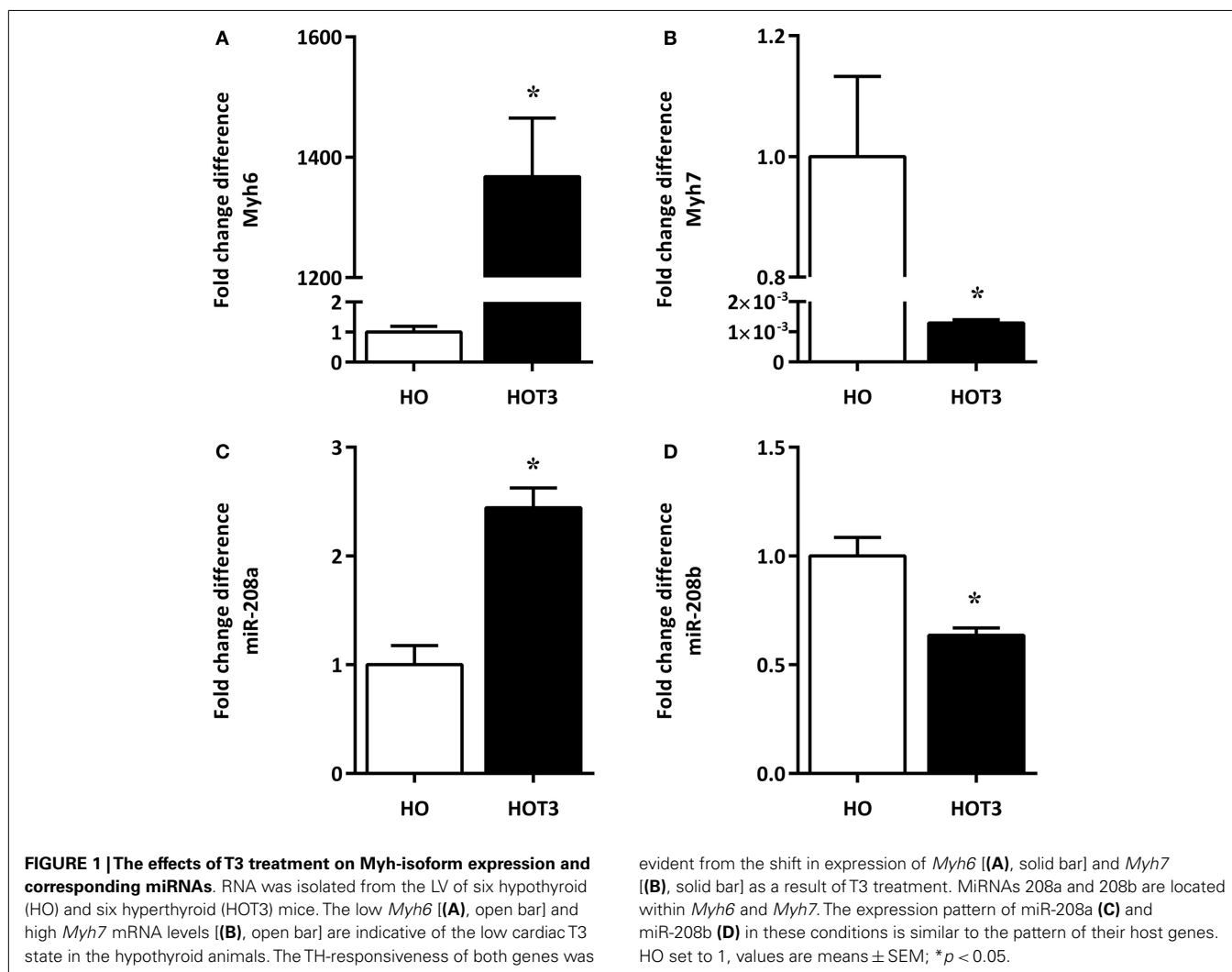
Table 1 | Effects of short-term T3 treatment on BW, HW, and plasma T3 levels.

	HO	HOT3
Anatomic data		
Body weight day 0 (g)	22.1 ± 0.5	22.1 ± 0.6
Body weight day 44 (g)	22.7 ± 0.4	22.6 ± 0.5
Total heart weight/TL (mg/cm)	41.3 ± 1.1	58.1 ± 0.6*
LV weight/TL (mg/cm)	24.0 ± 1.4	33.1 ± 1.2*
Thyroid hormone status		
T3 levels (nM)	0.1 ± 0.1	47.5 ± 4.2*

Twelve mice were allowed ad libitum access to food containing PTU for 44 days. Six randomly chosen mice were injected with T3 at day 42, 43, and 44. Controls received vehicle. Mice were sacrificed at day 45. Body weight remained unchanged during the experiment. Total heart weight increased by 41%, and LV weight increased by 38%. T3 treatment resulted in 475 times higher T3 levels in the hyperthyroid mice. Heart weight was corrected for TL, tibia length; HO, hypothyroid; HOT3, hyperthyroid. Values are means ± SEM; * $p < 0.05$.

MicroRNA EXPRESSION ANALYSIS

TaqMan miRNA Megaplex array (Applied Biosystems, Foster City, CA) was used to analyze miRNAs expression profiles. To synthesize cDNA, 3 μ l RNA was added to 4.5 μ l of RT-PCR reaction mix consisting of specially designed Megaplex RT primers (Rodent pool A or B, v3), dNTPs, MultiScribe reverse transcriptase, 10 \times reaction buffer, MgCl₂, and RNase inhibitor. Subsequently, the mixture was subjected to 40 cycles for 2 min at 16°C, for 1 min at 42°C, and for 1 s at 50°C, followed by 5 min at 85°C and cooling to 4°C. A pre-amplification was performed to increase the cDNA quantity, thereby improving the sensitivity of miRNA detection (39). The pre-amplification mixture consisted of 2.5 μ l RT product, 12.5 μ l TaqMan PreAmp master mix, and Megaplex PreAmp primers. The mixture was incubated for 10 min at 95°C, 2 min at 55°C, 2 min at 72°C followed by 12 cycles of 15 s 95°C and 4 min at 60°C, inactivated for 10 min at 99.9°C, and cooled down to 4°C. Each product was diluted by adding 75 μ l 0.1 \times TE (pH 8.0). After 9 μ l diluted pre-amplified target cDNA was added to 450 μ l 2 \times TaqMan Universal master mix (w/o amperase) and 441 μ l nuclease free water, 100 μ l mixture



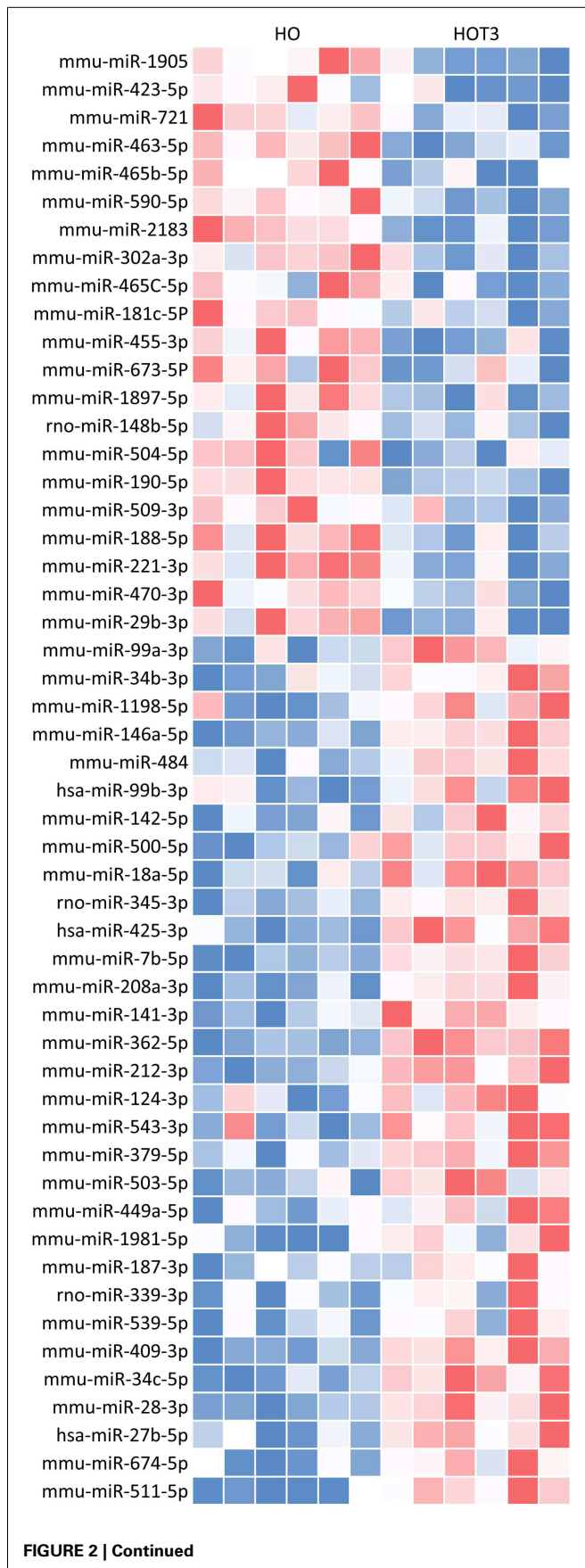


FIGURE 2 | Continued

T3 treatment for 3 days revealed a unique miRNA profile in the hypertrophied LV.

Expression data of 641 known mouse miRNAs were obtained with TaqMan Megaplex arrays (v3, A and B, rodent) and analyzed using RQ-manager (v1.2) and DataAssist (v3.0). The depicted heat map represents a unique profile of 52 differentially expressed miRNAs after 3 days of T3 treatment with a FC > ± 2 and a *p*-value < 0.05. Blue, low; red, high.

per lane was added to the TaqMan array card. The qPCR was performed on a 7900HT system with TLDA arrays. MammU6 and snoRNA202 were the most stable miRNAs and, therefore, chosen as endogenous controls. Comparative threshold (Ct) values were analyzed by SDS software v2.3 before being exported to RQ-manager v1.2 (Applied Biosystems). Data were next exported to DataAssist v3.0 (Applied Biosystems) for statistical analyses. MiRNAs with Ct's above 38 were designated "not-expressed" and excluded from the analyses. Data were only considered when expression of a particular miRNA was observed in at least five mice of each group. A two-tailed Student *t*-test was used for two-group comparisons.

PREDICTION OF miRNA TARGETS

To obtain an overview of the predicted miRNA targets and their role in biological processes, we analyzed significantly differentially regulated miRNAs with Ingenuity Systems Pathway Analysis software (IPA, Ingenuity Systems, www.ingenuity.com). Briefly, after analyzing the expression of all the detected miRNAs using DataAssist, a list of significantly differentially regulated miRNAs was uploaded to IPA. The uploaded miRNAs were analyzed using the microRNA Target Filter to find experimentally validated miRNA targets from Tarbase and miRecords, as well as highly predicted miRNA-mRNA pairing from Targetscan. Additionally, IPA includes peer-reviewed biological literature describing miRNA-target interactions. We focused only on miRNAs that were up- or downregulated by at least a fold change (FC) of > ± 2 and *p*-value of < 0.05. Targets expressed in heart tissue were used for further *in silico* analysis.

RESULTS

LEFT VENTRICULAR HYPERTROPHY INDUCED BY T3 TREATMENT

Hypothyroidism was induced using the established 40-day protocol of *ad libitum* access to food containing PTU (40). Indicative of the low plasma T3 levels and in agreement with earlier studies, growth was arrested in these animals (Table 1) (40). T3 treatment for three consecutive days resulted in a ~475-fold increase of plasma T3. Cardiac hypertrophy was evident from a 41% increase in total heart weight, and a 38% increase in weight of the LV free wall, which was used in all further analyses (Table 1).

A total RNA fraction was isolated from approximately 20 mg LV tissue. Expression analysis of the TH-regulated *Myh6* and *Myh7* genes indicated the low cardiac T3 state in the hypothyroid animals, showing low *Myh6* and high *Myh7* mRNA levels (2). The TH-responsiveness of both genes was evident from the shift in expression of *Myh6* and *Myh7* as a result of T3 treatment (Figures 1A,B).

ALTERED miRNA EXPRESSION PROFILE INDUCED BY T3 TREATMENT

Using a platform based on stem-loop primers, we observed 45 significantly upregulated and 104 significantly downregulated miRNAs as a result of T3 treatment (Table S1 in Supplementary Material). Among these miRNAs, 208a and 208b are examples of miRNAs known to be up- and downregulated, respectively, in response to increasing TH levels (27, 29). Both miR-208a and 208b followed the expression of their corresponding host genes *Myh6* and *Myh7*, respectively (Figures 1C,D).

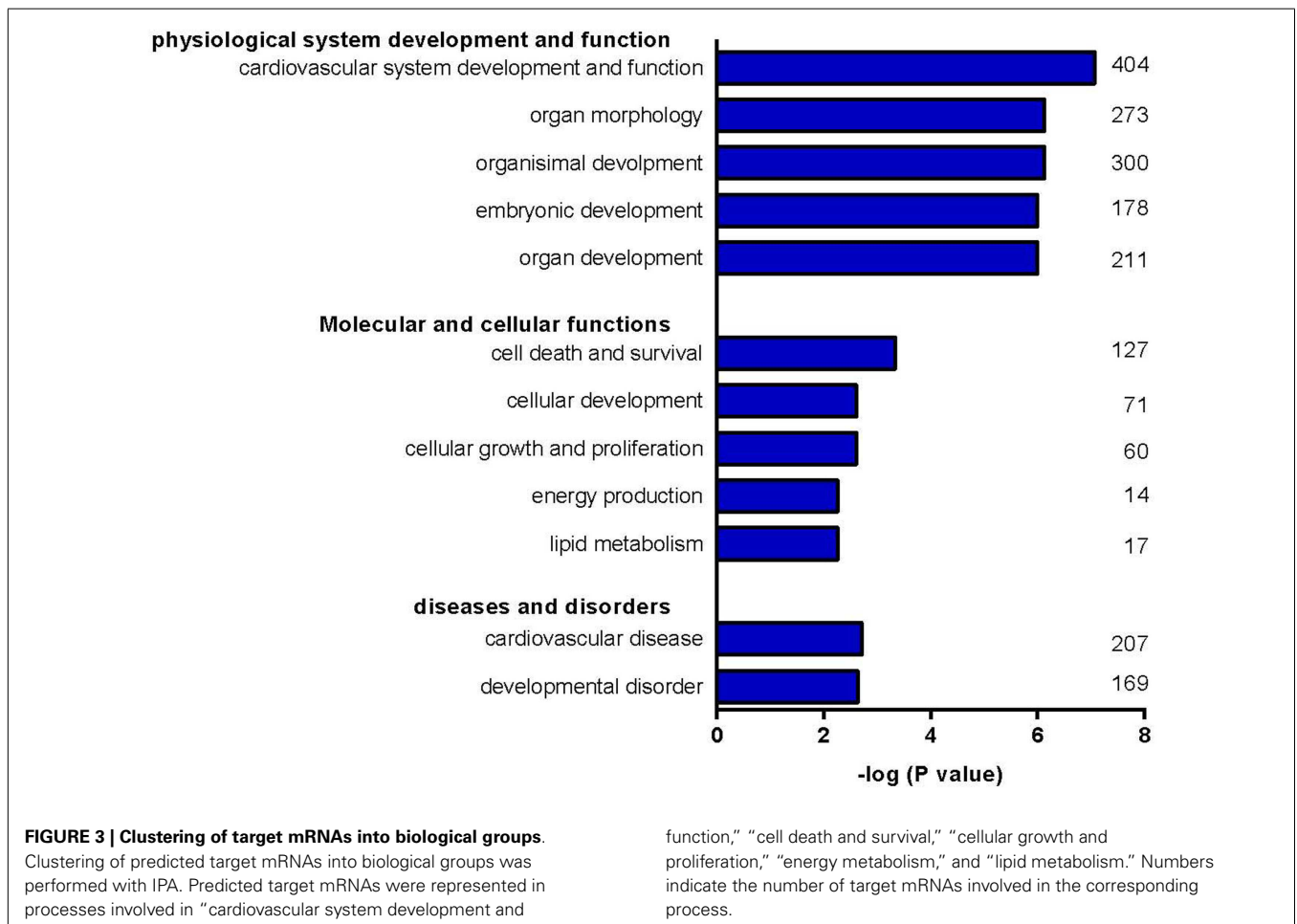
ANALYSIS OF POTENTIAL TARGET mRNAs OF T3-REGULATED miRNAs

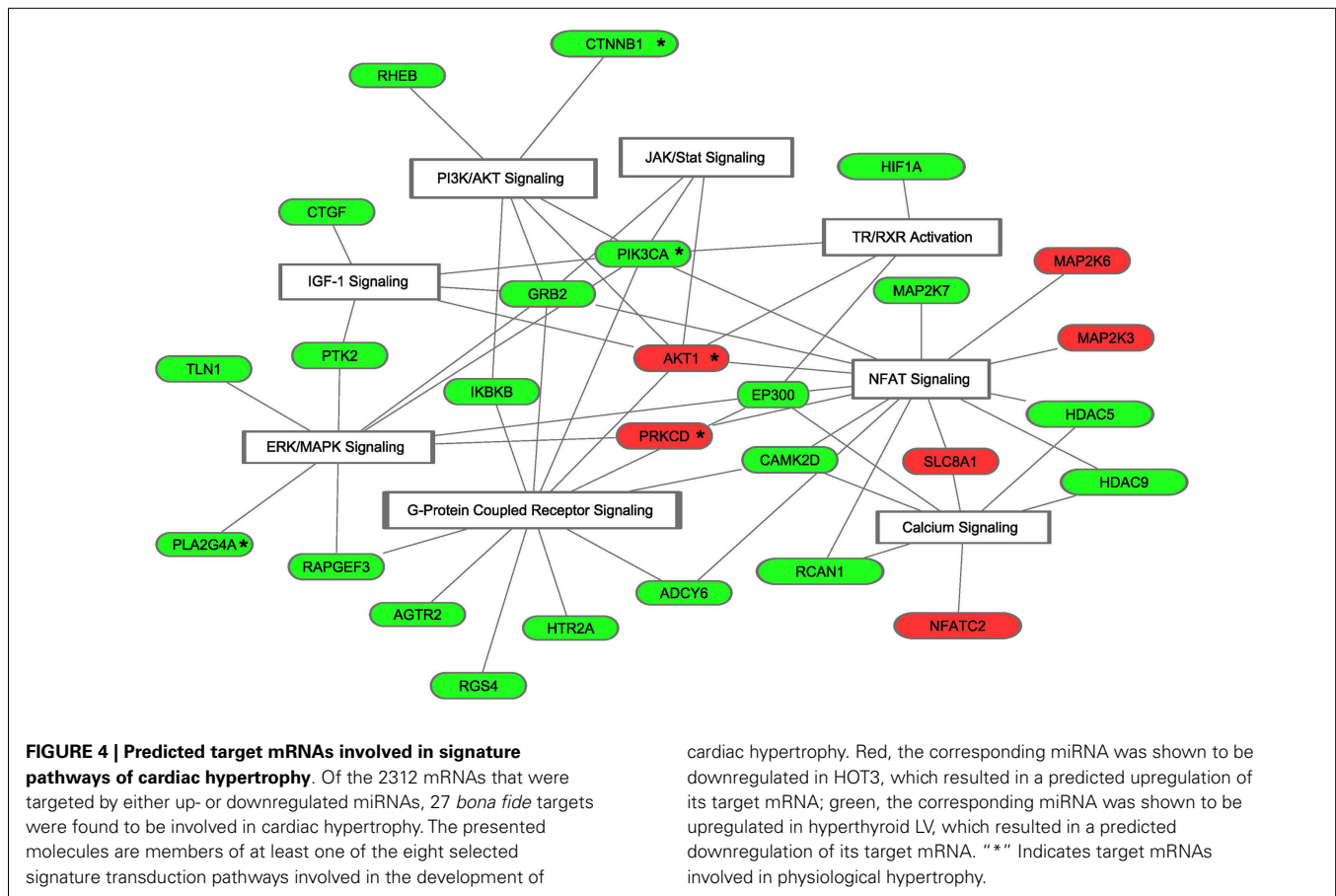
Of the total of 149 differentially regulated miRNAs, 52 met the criteria of at least a twofold increase or a 50% decrease and having a *p*-value < 0.05. This group of 52 forms a unique signature of miRNAs differentially regulated after 3 days of T3 treatment (Figure 2). To test for potential target mRNAs, *in silico* target prediction was performed using a web-based entry tool, IPA. Thirty-one out of the 52 miRNAs had targeting information available in the Ingenuity database and could be used in the IPA analysis (Table S2 in Supplementary Material). The predicted interaction between miRNA and its target mRNA was based on sequence homology of the miRNA seed region and the target sequence present in the 3' UTR of the mRNA. The analysis was furthermore restricted to mRNAs known to be expressed in heart tissue. In this way, a total of 3274 mRNAs were identified as potential targets of the group of

31 miRNAs. An IPA "core" analysis was subsequently performed to relate the predicted target mRNAs to known biological functions and processes. This revealed that the predicted target mRNAs were significantly represented in "cardiovascular system development and function," "cell death and survival," "cellular growth and proliferation," "energy metabolism," and "lipid metabolism" (Figure 3).

PATHWAY ANALYSIS OF POTENTIAL TARGET mRNAs INVOLVED IN T3-INDUCED CARDIAC HYPERTROPHY

We next focused our analyses on target mRNAs encoding proteins which have been shown to be critical components of hypertrophic signaling pathways, i.e., the IGF-1-, NFAT-, Ca²⁺-, GPCR-, JAK/STAT-, PI3K/Akt-, ERK/MAPK-, and TH-signaling pathways (16) and which have been validated in mouse models of pathological and/or physiological hypertrophy. Analysis was furthermore limited to mRNAs that are solely targeted by either up- or down-regulated miRNAs, so that the effect of miRNA regulation on the expression of these signaling components is unequivocal. This approach resulted in 27 candidates (Figure 4). In depth analyses using IPA and additional literature searches revealed that the predicted regulation of 5 of these targets (19%) results in enhancement of physiological hypertrophy, whereas regulation of 15 of these targets (56%) results in suppression of pathological remodeling (Table 2).





DISCUSSION

In this paper, we describe an unbiased *in silico* approach to analyze differentially expressed cardiac miRNAs in the physiologically hypertrophied mouse heart as a result of T3 treatment. Our data show that next to the known extra-cardiac effects of T3 on the heart due to an increase in hemodynamic load (10, 11), and modulation of TH-regulated genes (2, 3), a T3-dependent miRNA profile is expressed which is predicted to primarily suppress pathways involved in pathological hypertrophy, with some enhancement of pathways involved in physiological hypertrophy.

We chose to use hypothyroid mice treated for only 3 days with T3 to optimize the identification of T3-responsive miRNAs. The reciprocal regulation of the TH-regulated myosin genes, *Myh6* and *Myh7*, and the 41% increase in heart weight are in line with previous findings (14, 66, 67). Earlier studies established that the T3-induced increase in heart weight is caused by an increase in cell size and is not accompanied by fibrotic deposition (68, 69). Some of the observed changes in the miRNA expression profile of the hypertrophied LV, following T3 treatment, also confirmed earlier results. The observed reciprocal expression of the miRNAs 208a and 208b, which originate from introns of *Myh6* and *Myh7*, respectively, has been reported before (27, 29). As already mentioned in the Section "Introduction," expression of miR-208a has been implicated in the development of pathological hypertrophy. Mir-208a targets the Thyroid Hormone Receptor Associated

Protein 1 (*Thrap1*) (26, 27, 70), which is a component of the TH-receptor/co-factor complex that mediates transcriptional regulation of T3-dependent genes (26, 27, 71, 72). Increased miR-208a expression is, therefore, expected to decrease T3 action, because *Thrap1* expression will be decreased. However, our data suggest that in the presence of high levels of T3, the effect of miR-208a on the efficiency of T3-regulated gene expression and pathological signaling is minimal, since it does not prevent T3-induced gene expression nor does it result in a pathological phenotype under this condition. Also, in line with earlier results, the cardiac miRNAs, miR-206 and miR-1, which were shown to be downregulated by TH in liver and skeletal muscle (30, 31), were found to be suppressed by T3 treatment in the present study.

Furthermore, our results are consistent with data obtained using a model of exercise-induced cardiac hypertrophy (73), showing a significant downregulation of miR-26b, 27a, and 143 after 7 days of exercise. Moreover, a trend toward downregulation of miR-195 and 499 was observed in that study, where we found significant downregulation of both miRNAs after 3 days of T3 treatment. Exercise-induced physiological cardiac growth has been considered similar to TH-induced physiological hypertrophy (16), and our data suggest that this similarity may involve regulation by miRNAs.

In-depth analysis of our set of 149 differentially expressed miRNAs revealed 52 with a FC $> \pm 2$ and a *p*-value < 0.05 , which

Table 2 | Predicted interaction of T3-induced miRNA expression and hypertrophic signaling pathways.

mRNA	miR		Induction	Reference
UPREGULATED TARGET MRNAS INVOLVED IN PATHOLOGICAL HYPERTROPHY				
<i>Map2k3</i>	590	Dominant negative <i>Map2k3</i> increases hypertrophy	TAC, AngII, Iso	(41)
<i>Map2k6</i>	29b	Dominant negative <i>Map2k6</i> increases hypertrophy	TAC, AngII, Iso	(41)
<i>Slc8a1</i>	721	Reduced levels of <i>Slc8a1</i> caused hypertrophy	TAC	(42)
DOWNREGULATED TARGET MRNAS INVOLVED IN PATHOLOGICAL HYPERTROPHY				
<i>Agtr2</i>	539	Chronic loss of <i>Agtr2</i> attenuates left ventricular hypertrophy	AngII	(43)
<i>Camk2d</i>	539, 7b	Inhibition prevents maladaptive remodeling	TAC, Iso	(44, 45)
<i>Ctgf</i>	124, 212, 18a	Inhibition attenuates LV remodeling in pressure overload-induced heart failure	TAC, AngII	(46, 47)
<i>Ep300</i>	212	Specific reduction of <i>Ep300</i> content or activity diminishes stress-induced hypertrophy	TAC	(48, 49)
<i>Grb2</i>	124, 141	<i>Grb2</i> ^{+/-} mice showed a reduced hypertrophic response in response to overload	TAC	(50)
<i>Hif-1α</i>	18a	Deletion of <i>Hif-1α</i> prevents hypertrophy	TAC	(51)
<i>Htr2a</i>	34c	Blockade of <i>Htr2a</i> has a beneficial effect on the development of hypertrophy	TAC	(52)
<i>Ikbkb</i>	503	<i>Ikbkb</i> negatively regulates the anti-hypertrophic action of Irf7/Nf-kappa B in pathological hypertrophy	TAC, Pe, AngII	(53, 54)
<i>Ptk2/Fak</i>	379	Reduced levels of <i>Ptk2</i> were accompanied by prevention, as well as reversal, of load-induced LV hypertrophy	TAC	(55)
<i>Rapgef3</i>	539, 721	KO reduces β-adrenergic stimulation-induced hypertrophy	TAC	(56)
<i>Rheb</i>	141	<i>Rheb</i> activates mTORC1 signaling-dependent hypertrophy	TAC, MI	(57)
<i>Tln1</i>	124, 503	Reduction of <i>Tln1</i> expression improves cardiac remodeling	TAC	(58)
UPREGULATED TARGET MRNA INVOLVED IN PHYSIOLOGICAL HYPERTROPHY				
<i>Akt</i>	302	Increased <i>Akt</i> is involved in physiological hypertrophy	MI, Ex	(16, 59, 60)
<i>Prkcd</i>	181c	<i>Prkcd</i> activates non-pathological hypertrophy	MI	(61)
DOWNREGULATED TARGET MRNA INVOLVED IN PHYSIOLOGICAL HYPERTROPHY				
<i>Ctnnb1</i>	141	Down regulation is required for adaptive remodeling	AngII	(62)
<i>Pik3ca</i>	124	Constitutively active <i>Pik3ca/p110α</i> increases the hypertrophic response in TAC; is activated in physiological hypertrophy	TAC, Ex	(63, 64)
<i>Pla2g4a</i>	543	Hypertrophic growth is increased in <i>Pla2g4a</i> ^{-/-} in TAC; increasing <i>Igf-1</i>	TAC	(65)

IPA and additional literature were used to evaluate the role of 27 bona fide target mRNAs in the development of cardiac hypertrophy (see **Figure 4**). Twenty of the initial 27 target mRNAs were shown to be validated in mouse models for cardiac hypertrophy, as indicated in the table, together with the relevant literature and description of the principal findings. These models included transverse (ascending) aortic constriction (TAC), LV remodeling following myocardial infarction (MI), and Angiotensin II (AngII), isoproterenol (Iso), and phenylephrine (Pe) induced pathological hypertrophy, and exercise (Ex) induced physiological hypertrophy. The identified T3-regulated miRNAs are depicted in green (reduced expression) or red (increased expression). The direction of change of the predicted target mRNAs is similarly color coded. Fifteen target mRNAs are predicted to be regulated by the indicated miRNAs in such a way that pathological signaling is suppressed. For example, *Map2k3* has been shown in a TAC model to suppress pathological remodeling. Consequently, its upregulation by decreased miR-590 expression is considered to limit pathological signaling. Likewise, *Camk2d* is required for TAC-induced pathological remodeling and its suppression by the upregulated miRNAs 539 and 7b is again considered to limit pathological signaling. The remaining five target mRNAs are regulated in such a way that physiological signaling is enhanced.

formed a miRNA signature unique for short-term T3 treatment in the LV. To test for potential target mRNAs, *in silico* target prediction was performed using IPA. Thirty-one of the 52 had targeting information available in the database, resulting in 3274 confirmed or highly predicted targets. Core analysis included in IPA was used to interpret the target mRNA data in the context of biological functions and processes. Besides “cardiovascular system development and function,” processes in which target mRNAs are

known to be involved included “cell death and survival,” “cellular growth and proliferation,” “energy metabolism,” and “lipid metabolism.” These findings are in accordance with the concept that TH plays a determining role in cell proliferation, differentiation, and metabolism (74, 75).

Next, the analysis was limited to target mRNAs that were solely targeted by either up- or downregulated miRNAs, so that the effect of miRNA regulation on the expression of these signaling

components was unequivocal, resulting in 2312 target mRNAs. Subsequently, we searched for target mRNAs involved in cardiac hypertrophy pathways. Signature cardiac hypertrophy pathways, such as IGF-1-, PI3K/Akt-, JAK/STAT-, and TH-signaling pathways, are mostly associated with physiological hypertrophy, whereas GPCR-, Ca²⁺-, ERK/MAPK-, and NFAT signaling pathways are associated with pathological hypertrophy (16). Analysis revealed 27 target mRNAs that were indicated by IPA to be part of at least one of the signature pathways mentioned above and have been validated in models of pathological and/or physiological hypertrophy. In the setting of T3-induced hypertrophy, the predicted regulation of 19% of these targets indicates enhancement of physiological hypertrophy, while 56% indicates suppression of pathological remodeling.

For instance, *Hif1 α* and *Camk2d* are upregulated in both human and mouse pathological hypertrophy (51, 76). Inactivation of *Hif1 α* in TAC mouse models resulted in a beneficial effect preventing cardiac growth (51, 77). Transgenic mouse models overexpressing *Camk2d*, the predominant isoform expressed in the heart, established the involvement of *Camk2d* in pathological hypertrophy (78). Specific inhibition of *Camk2d* attenuated cardiac growth in response to several hypertrophic stimuli (44, 45, 79). It was consequently suggested that target-specific inhibition of *Camk2d* may be a useful treatment of cardiac hypertrophy (80). Another example is connective tissue growth factor, *Ctgf*, which in the present study is targeted by three upregulated miRNAs, miR-212, 124, and 18a, suggesting a strong downregulation. It has been reported that while the initial overexpression of *Ctgf* activates *Akt*, triggering adaptive hypertrophy, the prolonged *Ctgf* overexpression eventually leads to heart failure (46, 81). These, and other target mRNAs listed in **Table 2** are predicted by IPA to be downregulated by T3-dependent miRNAs, suggesting that fine-tuning by these miRNAs attenuates the development of pathological hypertrophy.

Likewise, it has been described that increased expression of the serine/threonine kinase *Akt* results in cell growth (82), both in pathological and in physiological hypertrophy (83). Recent studies using *Akt* knockout mouse models suggest that *Akt* is more likely to be required for physiological hypertrophy than for pathological hypertrophy (59). It has been shown that short-term activation of *Akt* is associated with cardiac protection (84), utilization of glucose, instead of free fatty acids, and improvement of cardiac contractility (85). For *Ctnnb1*/Beta-catenin, a key molecule in pathways associated with cardiac hypertrophy, it has been shown that decreased expression levels are important for the process of adaptive left ventricular remodeling (62), suggesting that the predicted fine-tuning of these target mRNAs enhances physiological hypertrophy.

Although we limited our analyses to miRNAs that were strongly responsive to T3 treatment, and to target mRNAs that have been validated in mouse models, future studies should be aimed at confirming the predicted regulation of the target mRNAs and their proteins. At present, our data suggest that cardiac T3-action includes a novel level of regulation in which a unique set of T3-dependent miRNAs enhances some aspects of physiological hypertrophy, but primarily suppresses pathological hypertrophic signaling. This suggests a mechanism that may in part explain why cardiac hypertrophy due to high circulating T3 levels does not

progress to dysfunction, in spite of the chronic hemodynamic overload, which in the context of cardiovascular disease is the principal trigger of pathological hypertrophy (10). This proposed mechanism may be relevant for our understanding of the progression of adverse remodeling. As in other severe illnesses, heart failure is often associated with reduced plasma T3 levels, the so-called non-thyroidal illness syndrome (86). Clinical studies have shown that the extent of the reduction is an independent predictor of mortality in patients with chronic heart failure (87, 88). In addition, we and others have shown that increased cardiac activity of the TH inactivating enzyme, *Dio3*, results in a local hypothyroid condition in the pathologically remodeled heart due to pressure overload or following myocardial infarction (38, 89, 90). Indeed, alterations in gene expression in the failing heart are to some extent similar to the genetic profile of a hypothyroid heart (4). Taken together, this suggests that reduced T3-signaling in the hemodynamically overloaded heart will aggravate adverse remodeling by releasing the T3-dependent miRNA brake on these pathological signaling pathways. Manipulation of these miRNAs may be an additional option in the ongoing development of miRNA-based therapeutics for the treatment of heart failure (91, 92).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fendo.2014.00171/abstract>

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