Thyroid Hormone Dependent Regulation of Target Genes and Their Physiological Significance

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Thyroid hormone (T_3) regulates growth, development and differentiation. These activities are mediated by nuclear thyroid hormone receptors (TRs), which belong to the steroid/thyroid hormone receptor superfamily of ligand-dependent transcription factors. In an effort to study the mechanism of target genes regulation and their physiological significance after T_3 treatment in a TRα-overexpressing hepatoma cell line (HepG2-TRα), c-DNA microarrays were performed. The data demonstrated that approximately 149 genes represented were positively regulated by T_3, including fibrinogen, transferrin, fibronectin (FN), androgen receptor (AR)-associated protein (ARA70), and dehydroepiandrosterone sulfotransferase family 1A member 2 (SULT2A1). To further confirm the microarray results, a quantitative-reverse transcription polymerase chain reaction (Q-RT-PCR) was applied. The protein synthesis inhibitor, cycloheximide was used to determine whether the regulation was direct or indirect. A promoter assay further showed that T_3 regulation was largely at the level of transcription. Although those genes were isolated from a human tumor cell line, they are regulated similarly in rats and humans. These results indicate that T_3 might play an important role in the process of blood coagulation, inflammation, metabolism and cell proliferation. This may help to explain the association between thyroid diseases and the mis-regulation of the inflammatory and clotting profiles evident in the circulatory systems of these patients. *(Chang Gung Med J 2008;31:325-34)*

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Thyroid hormone, 3, 3', 5-triiodo-L-thyronine (T_3) has an important role in eukaryotic cell development, differentiation and metabolism.[1-14] These activities are mediated by nuclear thyroid hormone receptors (TRs), of which two principal genes have been identified and mapped to human chromosomes 17 and 3.[4,8,10-14] Each gene encodes at least two TR isoforms (TRα1 and 2, and TRβ1 and 2) that are generated as a result of alternative splicing and/or promoter choice.[4,8,10-14] Most TRs are expressed in virtually all vertebrate tissues, except for TRβ2, with expression primarily restricted to the adult pituitary and hypothalamus.[4,14,15] The nature of the transcriptional response is dictated by cell type, promoter

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context, and hormone status.[15] In most cases, TRs are transcriptional repressors without their cognate hormone (T_3 or T_4) and are turned into activators by ligand binding (Fig. 1).[13,16]

Similar to other nuclear hormone receptors, TRs are ligand-dependent transcription factors which are comprised of modular functional domains mediating the binding of hormones (ligands), DNA binding, receptor homo- and hetero-dimerization, and interaction with other transcription factors and co-factors.[1,3,13,15,17,18] The gene regulating activity of TRs is mediated by binding to specific DNA sequences, known as thyroid hormone response elements (TREs), located at the promoter regions of thyroid hormone target genes (Fig. 1).[4] TR can bind DNA as a monomer or homodimer, or function as a heterodimer with retinoic X receptor (RXR), the receptor of 9-*cis*-retinoic acid (Fig. 1).[19-22] The partnerships are mainly dependent on the promoter context. In the absence of a cognate ligand, corepressors, such as silence mediator of RXR and TR (SMRT), histone deacetylase (HDAC), nuclear receptor corepressor (N-CoR), and mSin3 are recruited to the promoter by TRs to facilitate gene repression.[23-27] Alternatively, coactivators, such as cAMP response element-binding (CREB) binding protein (CBP/p300), steroid receptor coactivator (SRC), and p300 and CBP associated factor (pCAF) are tethered to transcriptional complexes to coactivate target gene expression.[18,28] The transcriptional regulation activities of TRs are also affected by extracellular stimuli. Phosphorylation of TRs at specific serine/threonine sites has been shown to alter their transcriptional activities and tissue-specific stability.[21,29-31]

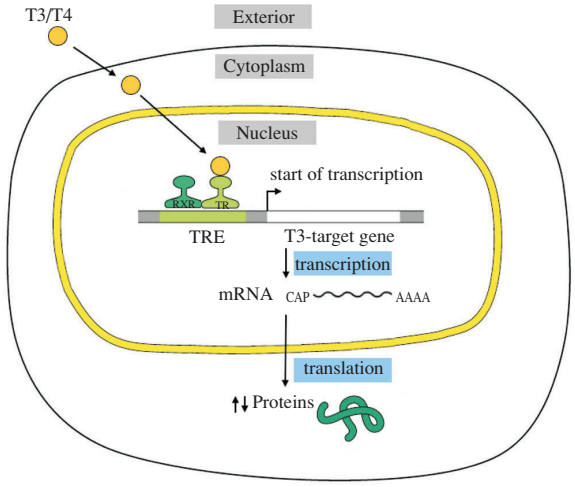

Fig. 1 Model of thyroid hormone receptor action in the nucleus. TR: thyroid hormone receptor; TRE: thyroid hormone response element; RXR: retinoid X receptor. T_3/T_4 binds to its nuclear receptor (TR) to induce or repress T_3-target gene expression.

The liver is clearly recognized as a target organ for thyroid hormones. In fact, Chamba et al. reported roughly equal quantities of TRα1 and TRβ1 protein in human hepatocytes.[32] HepG2 is a well-differentiated hepatocellular carcinoma cell-line without detectable TR protein expression. However, it secretes all 15 plasma proteins and preserves many liver-specific functions, and thus can serve as an *in vitro* model.[33] Therefore, HepG2 cell lines could allow for a model system to study the cell type-specific and TR isoform-specific regulation of the T_3-target genes in the liver.

The aim of this review is to investigate and identify T_3 target genes and their physiological significance in isogenic hepatocellular carcinoma (HCC) cell lines when treated with T_3. The HCC cells over-expressing TR proteins can provide for investigating the effect of various receptor levels on the regulation of target gene expression. Microarray assay, a powerful tool to quest for mechanisms or to study the cellular functions of TR in normal and aberrant situations has been proven.[34-38] Therefore, this technique was utilized to identify T_3 target genes in HepG2 cells over-expressing TRα1 (HepG2-TRα1). The results showed that 149 genes were up-regulated by treatment with 100 nM T_3 for 48 h.[39] Among these are genes involved in metabolism, detoxification, signal transduction, cell adhesion, cell migration, transcription factors, oncogenes, and cell cycle (Fig. 2). Surprisingly, a very high proportion of these genes are involved in the systemic/cellular inflammatory response, which is not traditionally associated with thyroid hormone function. Accordingly, in this review we focused on several genes from microarray analysis, including fibrinogen, fibronectin (FN), transferrin, dehydroepiandrosterone (DHEA)-sulfotransferase family 1A member 2 (SULT2A1) and androgen receptor (AR)-associated protein (ARA70). Their physiological significance in those regulations is also discussed.

Human fibrinogen is up-regulated by T_3

Human fibrinogen is a circulating 340 kDa gly-

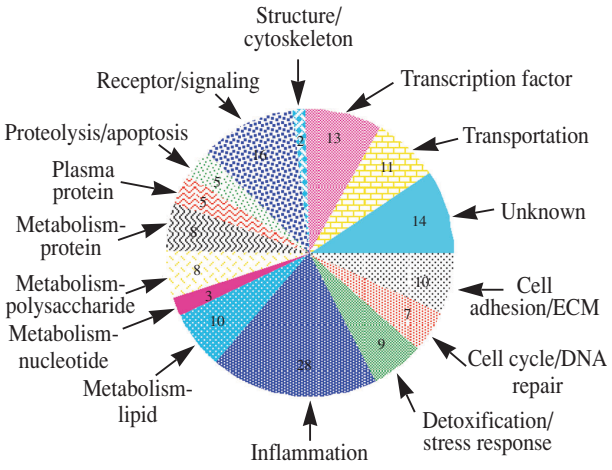

Fig. 2 Pie chart diagram summary of expression profiling data of 149 up-regulated genes from microarray experiments carried out from HepG2-TRα1 cells treated with 100 nM T_3 for 48 h.

coprotein, primarily synthesized by hepatocytes.[40-42] It is comprised of two symmetric half molecules, each consisting of one set of three different polypeptide chains termed Aα, Bβ and γ. The molecule is highly heterogenous due to alternative splicing, extensive post-translational modification and proteolytic degradation. Fibrinogen is cleaved by thrombin to form fibrin, the most important component in the blood clotting reaction.[43] Simpson-Haidaris et al. reported that fibrinogen alters the migratory ability of breast cancer cells.[44] Additionally, the role of TR/T_3 in the process of blood clotting and cell migration is currently unknown. Our previous data indicated that several plasma proteins, including prothrombin, angiotensinogen, haptoglobin, complement, lipoproteins and fibrinogen, are up-regulated by T_3 in a hepatoma cell line which highly expressed TRα1, as well as in the liver of thyroidectomized rats.[39] Burggraaf et al. reported that excess T_3 was associated with elevated levels of plasma fibronectin and fibrinogen, while plasminogen was decreased.[45] Previous studies from this laboratory indicated that T_3, acting through TRs, inhibits expression of Nm23-H1 and promotes tumor metastasis.[46] The function of Nm23-H1 is associated with anti-metastasis.[47,48] Overall, the experimental data indicate that T_3 plays an important role during blood coagulation as well as cell migration.

Transferrin and apolipoprotein are T_3-target genes

Transferrin is a plasma glycoprotein for iron delivery which expresses in all mammals and is synthesized in the liver.[49-54] A study from this laboratory revealed that T_3 induced an abundance of transferrin mRNA and protein expression in a time- and dose-dependent manner in HepG2-TRα1 but barely in HepG2-Neo cells that do not express detectable TRα1. The T_3-regulated transferrin is at the transcriptional level as determined by nuclear run-on experiments. Also, the results imply that the induction of transferrin by T_3 is direct and may in fact be mediated by a TRE in the promoter region.[55]

T_3 plays an important role in the homeostasis of cholesterol in the vascular endothelium.[56-58] Functional positive and negative TREs coexist within the rat apolipoprotein AI promoter and both elements contribute to the control of apolipoprotein AI gene expression,[59] a component of high density lipoprotein (HDL) particles. Previous study indicated that T_3 also positively regulates apolipoprotein CI, CII, and CIII.[39] T_3 induces several acute phase inflammatory proteins as well, including orosomucoid, complement component, amyloid A, and bikunin. Thus, T_3 may play an important role in the regulation of several serum proteins during the inflammatory response.

Fibronectin (FN) is indirectly regulated by T_3

FN mediates a wide variety of key interactions between cells and the extracellular matrix (ECM).[60,61] It also plays a significant role in cell adhesion, migration, growth, and differentiation.[62] FN was found to be up-regulated by T_3 in TRα1 and β1 over-expressing HepG2 cells in a dose-dependent manner at the mRNA and protein levels. Blockade of protein synthesis by cycloheximide almost completely inhibited the concomitant induction of *FN* mRNA by T_3, indicating that T_3 indirectly regulates FN. Furthermore, nuclear-run on and *FN* promoter assay clearly demonstrated that the presence of T_3 can specifically increase the number of *FN* transcriptional initiations. In addition, the up-regulation of FN by T_3 was mediated, at least in part, by transforming growth factor-β (TGF-β), because the induction of FN was blocked by the addition of TGF-β neutralizing antibody. The involvement of the mitogen activated protein kinase/c-Jun N-terminal

kinase/p38 MAPK (MAPK/JNK/p38) pathway in T_3 and TGF-β mediated induction of FN expression was determined. The results demonstrated that T_3 regulates *FN* gene expression indirectly, mediated by an as yet unidentified transcription factor with the participation of the MAPK/JNK/p38 pathway and the TGF-β signaling pathway.[63]

Dehydroepiandrosterone sulfotransferase family 1A member 2 (SULT2A1) is mediated by steroidogenic factor 1 and indirectly regulated by T_3

Mammalian sulfotransferase (SULT) has been classified into at minimum two groups (SULT1 and SULT2 families) based on similarities in their amino acid sequences and enzymatic properties.[64-71] Among them, SULT2A1 is a cytosolic enzyme that mediates the sulfate conjugation of many hydroxysteroid substrates including estrogens, pregnenolone, androgen precursor dehydroepiandrosterone, androgens, benzylic alcohol procarcinogens and other hormonal and xenobiotic substrates.[68,72-74] Human SULT2A1 expression occurs predominantly in the liver, intestine and adrenal glands.[65,75]

SULT2A1 was indirectly up-regulated both at the protein and mRNA level after T_3 treatment in HepG2-TRα1 cells. Moreover, SULT2A1 has been reported to be regulated by two transcription factors, GATA and steroidogenic factor 1 (SF1), in the human adrenal gland.[76] Interestingly, T_3 also induced SF1 at the protein level and RNA level in HepG2-TRα1 cells.[77] Approximately seven SF1 binding sites exist on the *SULT2A1* gene. A series of deletion mutants of *SULT2A1* promoter fragments in pGL2 plasmid were constructed to identify and localize the critical SF1 binding site. The promoter activity of the *SULT2A1* gene was enhanced by T_3 in HepG2-TR cells. The sequence of -228/+1 (based on the transcription start site) SF1 binding site was identified as the most critical site, as deleting this region reduced T_3-induced expression. Actually, transcription factor SF1 application enhanced only the -228/+1 but not other reporter plasmid activities. SULT2A1 and SF1 up-regulation at the protein and RNA levels in thyroidectomized rats occurred after T_3 application. In summary, the *SULT2A1* gene was mediated by SF1 and indirectly regulated by T_3.[77]

Tagawa et al. showed[78] that serum DHEA-S levels were decreased in patients with hypothyroidism and increased in patients with hyperthyroidism. T_3 may enhance the synthesis of this steroid, therefore, DHEA sulfotransferase levels could be increased in hyperthyroidism. These findings strongly support the findings that T_3 up-regulates SULT2A1. Patients with hyperthyroidism usually have increased serum SULT2A1 levels, indicating that modulation of T_3 levels is critical to controlling SULT2A1 *in vivo*.

ARA70 is regulated by T_3

ARA70 is one gene found to be up-regulated by T_3. ARA70 is a ligand-dependent coactivator for the AR and is significantly increased by T_3 treatment at the protein and mRNA level in HepG2-TRα1 cells.[79-87] Similar findings were obtained in thyroidectomized rats after T_3 application. This regulation is direct because cycloheximide treatment did not suppress induction of *ARA70* transcription by T_3. A series of deletion mutants of *ARA70* promoter fragments in the pGL2 plasmid were generated to localize the TRE. The results indicated DNA fragments (-234/-190 or +56/+119) enhanced promoter activity by T_3. Thus, two TRE sites exist in the upstream-regulatory region of *ARA70*. The interaction between TR and TRE found on the *ARA70* gene was further confirmed with electrophoretic mobility shift assays. Additionally, ARA70 can interfere with TR/TRE complex formation. The data indicated that ARA70 suppresses T_3 signaling in a TRE-dependent manner. These experimental results suggest that T_3 directly up-regulates *ARA70* gene expression. Subsequently, ARA70 negatively regulates T_3 signaling.[88]

The ARA70 protein has been isolated from human brain[89] and prostate cDNA libraries.[80] The protein ARA70 increases the transcriptional activity of AR co-transfection assays. Notably, a study using laboratory rats indicated ARA70 likely plays some role in Sertoli cells in testis development.[89] Postnatal Sertoli cell maturation is characterized by steadily increasing AR expression.[90] T_3 stimulates production of *AR* mRNA *in vitro*,[90] another process considered important for normal maturation of Sertoli cells.[90] Experimental results suggest that T_3 stimulates ARA70 expression in coordination with AR to regulate Sertoli cell maturation. AR plays a key reproductive role in males.[91] Hyperthyroidism appears to alter spermatogenesis and fertility.[92] Our

previous study provided molecular evidence that T_3/TR could be mediated by ARA70 and influence the male reproductive system.[88]

The function of ARA70 can also inhibit TR signaling. Moreover, T_3 increased AR levels in a prostatic carcinoma cell line,[93] and in rat Sertoli and peritubular cells.[90] Consequently, ARA70 may be a functional link between modulation of TR/AR crosstalk in T_3-signaling models.

TR stability is increased by phosphorylation

Binding of T_3 to TR also induces the degradation of TR, resulting in the desensitisation of the cells to further T_3 treatment. It has been shown that phosphorylation of TR plays a critical role in its activity and stability following T_3 binding.[31] However, the kinases in control of phosphorylating TR in the nucleus have not been identified previously. In this study, the results indicate that MAPKs are possible candidates responsible for the nuclear phosphorylation of TR.[31] Suppression of MAPKs with specific inhibitors repressed TR transcriptional activity. Over-expression of the MAPK activator, MKK6, and its constitutively active mutant, MKK6EE, significantly increased TR activity and protected TR from degradation. Additionally, MAPKs enhanced the DNA-binding affinity of TR.[21,29,30] The results suggest that MAPKs are the major kinases responsible for the nuclear phosphorylation of TR and are critical factors modulating the transcriptional activity and protein stability of TR subsequent to ligand-binding.[31]

T_3 inhibits cell proliferation in a hepatoma cell line

What is the physiological significance of T_3 regulation? One of the phenotypic changes is cellular proliferation. Growth of a HepG2-TR stable line was inhibited more than 50% following treatment with T_3. TGF-β neutralizing antibody could reverse the cell growth inhibition effect of T_3, but the control antibody could not. Flow cytometric analysis indicated that the growth inhibition was apparent at the transition point between the G1 and S phases of the cell cycle. The expression of major cell cycle regulators, cdk2 and cyclin E,[94-99] was down-regulated in HepG2-TR cells. Moreover, p21 was up-regulated by T_3 treatment both at the protein and mRNA levels. Furthermore, phospho-retinoblastoma (ppRb) protein was down-regulated by T_3. The expression of TGF-β was studied to delineate the repression mechanism. TGF-β was stimulated by T_3 and its promoter activity was enhanced by T_3 also. Both T_3 and TGF-β repressed the expression of cdk2, cyclin E, and ppRb. On the other hand, TGF-β neutralizing antibody but not control antibody blocked the repression of cdk2, cyclin E, and ppRb by T_3.[100] These results demonstrated that T_3 represses the proliferation of hepatoma cells rather promotes it. Actually, T_3 significantly suppresses the growth of only HepG2 over-expressing TR but not HepG2-Neo cells that did not express detectable TR. Examining the expression of a number of factors that are known to be significantly involved in the cell cycle, for example cdk2, Rb, p21, and cyclin E, confirmed that the suppressive effect of T_3 treatments on hepatoma cell proliferation did not result simply from the toxic effects of this hormone.[100] Additionally, our previous work[100] used the GC cell line, which is known to promote proliferation when stimulated by T_3.[101] We found that T_3 repressed hepatoma cell growth by lengthening the G1 phase of the cell cycle, concomitantly decreased in the expression of cdk2 and cyclin E.

Conclusions

To study the target genes regulated by T_3 in TRα1–overexpressing hepatoma cell lines, we performed oligo-microarrays. Among the remaining T_3 induced genes are several components of coagulation-related and inflammation-related or transcriptional factors. Several plasma proteins are up-regulated by T_3 in a hepatoma cell line that highly expresses TRα1, as well as in thyroidectomized rats. Microarray data indicates that the other plasma proteins such as plasminogen, and α-fetoprotein are down-regulated by T_3. Thus, further investigation of the hepatic plasma proteins regulated by T_3 is required to continue elucidating this importance, but so far relatively unappreciated, mechanism.

The microarray technology allowed us to determine the TRα1-dependent, T_3 regulated expression of downstream target genes. The consequences presented here gives greater insight into the action of TRα1 in hepatoma cell lines. Although those genes were isolated from a human tumor cell line, they are regulated similarly in rats and humans. This may help to explain the association with thyroid diseases

(hyper- and hypo-thyroidism). Further study is required to investigate the regulation of tumor-specific T_3 target genes.

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甲狀腺素調控目標基因的機制及其生理意義

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甲狀腺素 (T_3) 主要透過與甲狀腺受體 (thyroid hormone receptors, TRs) 的結合，而具有調控細胞生長、發育及分化的能力。TRs 屬於固醇類／甲狀腺受體家族成員之一，在細胞內功能爲轉錄因子。爲探討肝癌細胞中，T_3 調控目標基因的機制，過量表現 TRα 的肝癌細胞株 (HepG2-TRα) 做爲實驗材料。將此細胞株經 T_3 處理後，利用 microarray 廣泛鑑定出可能受 T_3 調控之基因。初步經 microarray 分析顯示，約 3560 個基因受到 T_3 的正向調控，這些基因包括 fibrinogen、數種參與凝血過程的分子、fibronectin、ARA70 及 SULT2A1 等。這些基因利用 Q-RT-PCR 進一步確認受 T_3 調控的情形，結果顯示與 oligo-microarray 分析的數據非常類似。而蛋白合成抑制劑“cycloheximide”則被用來決定標的基因是受 T_3 的直接或間接調控。另外，promoter assay 可用來瞭解 T_3 影響基因的表現是否發生在轉錄時期。雖然這些基因乃由人類腫瘤細胞株鑑定出，但與大鼠及人體實驗結果相類似。然而甲狀腺疾病的病人是否有發炎及凝血調控異常的問題，藉由這些研究結果，或可解釋這兩者之間的關聯性。(長庚醫誌 2008;31:325-34)

關鍵詞：凝血，甲狀腺素，受體，轉錄，調控

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