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An update on human thyroid hormone receptors in health and disease: Chemistry, physiology and pathophysiology

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Summary

Iodothyronines, the tetra- and tri-iodothyronines (T_4 and T_3), commonly known as thyroid hormones (THs), are secreted by thyroid glands. Thyroid hormones influence the growth and differentiation of every organ of the body via specific nuclear receptors (TRs), which belong to the nuclear receptor superfamily. Though thyroid glands secrete predominantly T_4 (which remains bound to its serum binding proteins), T_3 is the biologically active TH. Free T_4 enters the target cells through specific transporters and is converted into T_3 by cell-specific isoforms of cytoplasmic 5' deiodinase, which regulate the circulating T_3 levels and its availability for nuclear TRs in a tissue-specific manner. T_3 is then translocated to the nucleus, with the help of NADPH-dependent cytosolic transporter, where it binds to the monomers of TR subtypes (TRα and TRβ). Prior to the binding of T_3, TR monomer dimerizes with the 9-*cis* retinoic acid or retinoid X receptor (RXR) and the TR-RXR heterodimer, in association with corepressors, binds to specific TR response element (TRE) in the target genes. Upon T_3 binding to the TR monomer of the TR-RXR-TRE complex, corepressors get released paving way for the binding of coactivators, thereby inducing the transcription of T_3-responsive genes. Apart from the canonical nuclear signalling mechanism, membrane-mediated signalling by THs occurs through its interaction with plasma membrane integrin αvβ3. The impact of TH status and TR signalling on a broad range of genes makes studying its effect *in vivo* a difficult task. Studies on knock-in/out/mutant animal models and humans harboring several mutations of TR isoforms have helped explain various disorders of TH action, particularly the hypothyroid condition associated with the resistance to TH action. The aim of this review is to provide the readers with the information on THs biosynthesis along with the recent progress in TR signalling and its physiological impact on human health.

Keywords: Hormone resistance; HPT axis; Nuclear receptors; TRα/β; Thyroglobulin

Introduction

Transduction of hormone signal into the target cell requires the presence of specific high affinity receptor proteins. Receptors are specific signalling biomolecules, which are located on target cell surface (G-protein coupled receptors, cytokine receptors or receptor kinases) or intracellular sites like cytosol (progesterone, cortisol, and androgen receptors) or nucleus (estrogen, thyroid hormone receptors). Any organ or cell which lacks a specific hormone receptor is classified as a non-responsive organ/cell for that particular hormone. The notion that all peptide hormones have cell surface receptors and steroid-thyroid hormones have intracellular receptors has undergone changes with the identification of steroid-thyroid receptors on cell surface and peptide hormone receptors on intracellular sites, respectively. The concept of basic endocrinology and hormone signalling mechanism has undergone tremendous transformation over a period of time. Hence, in the present scenario, hormone receptors are known to occupy a pivotal position in endocrine physiology i.e., the homeostatic events of various physiological functions have become more hormone receptor-centric than hormone-centric. Therefore, a student of endocrinology or a physician needs to have a complete understanding of the hormone receptors and their signalling mechanisms so as to have a proper insight into endocrine physiology and pathology.

The present review attempts to provide an update on thyroid hormone receptors - T_3 receptors (TRs). We have taken utmost care to provide a comprehensive account on the topic, covering all the relevant information available in the literature. If there is omission of information on any publication in the area, it is purely inadvertent and not intentional. Due to space constraint, we have limited our effort to highlight only the aspects directly pertaining to the topic and suggest the readers to refer to the relevant reviews on areas which are not directly related to the topic but to the context. Before

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dealing with TRs, the reader is provided with a brief insight into thyroid heritage and hormone synthesis. There are many wonderful reviews and books on these aspects and interested readers may refer those texts for detailed information (e.g., Nussey and Whitehead, 2001; Braverman and Utiger, 2005; Panicker, 2011).

Thyroid gland

The thyroid gland, first described comprehensively by the anatomist Thomas Wharton in 1656, is located in the neck, just below the larynx. It is a brownish-red organ having two lobes connected by an isthmus and consists of low cuboidal epithelial cells arranged to form small sacs known as follicles. It is a highly vascularized organ which was originally thought to be a vascular shunt that separates the immune system for the brain from other regions of the body. The gland is involved in iodine metabolism. However, the association between thyroid gland and endemic goiter has been known several centuries before the discovery of iodine; the endocrine function of the organ was recognized during the last century. For example, evidence for this association was observed in the inscriptions on stone tablets of 16th century that the Chinese have used seaweed as an empirical therapy for goiter. Following the discovery that amphibian metamorphosis can be induced by feeding thyroid gland extract (Gudernatsch, 1912), it has been recognized that THs play a crucial role in the control of growth, development, differentiation and metabolism of virtually all the tissues of vertebrates.

Thyroid follicles synthesize two iodothyronines, L-3,5,3',5'-tetraiodothyronine or thyroxine (T_4) and L-3,5,3'-triiodothyronine (T_3), collectively known as thyroid hormones (Cheng et al., 2010). In addition, a third hormone calcitonin, a 32-amino acid peptide, is synthesized and secreted by the parafollicular "C" cells, which inhibits bone resorption by regulating the Ca^{2+} levels (Pearse, 1966; Vandernoot et al., 2012). The first iodothyronine was crystallized in 1914 by Kendall who named the hormone as 'thyroxin', because of a wrong notion that the hormone is a derivative of an amine rather than amino acid (Kendall, 1915, 1919). This is the reason why Kendall could not succeed in synthesizing the hormone. Harington and Barger were the ones to synthesize the hormone from the amino acid tyrosine and name it correctly as 'L-thyroxine' by adding the alphabet 'e' with the concurrence of Kendall and described its physiological effects (Harington and Barger, 1927). Kendall left research on thyroid at that time, but won Nobel Prize subsequently for his research on adrenal cortical steroids (see Sawin, 2005). *Therefore, the students of Endocrinology should be aware of the prize of the alphabet 'e' in thyroxine.*

Thyroid hormones

Initially, T_4 was considered as the only active hormone secreted by the gland. *The identification of T_3 in human plasma was a milestone in thyroidology* (Gross and Pitt-Rivers, 1952). T_3 is the biologically active hormone and T_4, the major TH secreted by thyroid gland, acts as precursor or prohormone for T_3 and remains as a buffer stock in circulation (Fisher, 1996; Bianco and Kim, 2006). Conversion of T_4 to T_3 in target tissues is catalyzed by selenoprotein enzymes called deiodinases (type 1 and type 2 deiodinases); type 3 deiodinase catalyzes the conversion of T_3 into inactive metabolites such as reverse T_3 (rT_3) and T_2 (Marsili et al., 2011; Dentice et al., 2013).

The expression and distribution of deiodinases play an important role in TH action *in vivo* by controlling the amount of hormone that is available for binding with the nuclear receptor in specific cell types at different times during development and adulthood (Gereben et al., 2008a, b; Pascual and Aranda, 2013). For instance, in brain the three corresponding deiodinase genes display a complex expression pattern with opposing regulation by TH: hypothyroidism increases type 2 deiodinase gene expression in brain to promote T_3 production, whereas hyperthyroidism increases type 1 deiodinase in liver and type 3 deiodinase in brain to promote rT_3 production (Flamant et al., 2007) (see Fig. 1).

THs are essential for the normal growth and differentiation of most of the organs, especially during fetal development and early childhood. In adults, the primary effects of THs are manifested by alterations in intermediary metabolism, including changes in oxygen consumption, protein, carbohydrate, lipid and vitamin metabolism, and reproduction (Song et al., 2011).

Synthesis and transport of thyroid hormones

Thyroid glandular follicles play a critical role in compartmentalizing the necessary components for TH synthesis. Thyroglobulin (Tg) is the largest glycoprotein known in humans, with a molecular weight of 660 kDa, 10% of which is composed of carbohydrates (Mercken et al., 1985). It is a dimeric protein with identical monomers, each having 2769 amino acids (330 kDa) and comprises of 132 tyrosine residues altogether. It is coded by a single copy gene, 270 kb long that maps on chromosome 8q24 and contains an 8.5 kb coding sequence,

which is divided into 48 exons (Malthiery et al., 1989; Targovnik et al., 2011). It is one of the starting molecules for TH synthesis and fills the follicular lumen through a process of exocytosis (van de Graaf et al., 2001). It acts as an autocrine regulator of thyroid follicular function that counteracts the effects of thyroid stimulating hormone (TSH), which is secreted by the pituitary gland (Suzuki et al., 2011).

Biosynthesis of THs requires iodide uptake into the thyrocytes and efflux into the follicular lumen, where it is then organified on selected tyrosyls of Tg. Uptake of iodide into the thyrocytes is mediated by an intrinsic membrane glycoprotein, the sodium-iodide symporter (NIS), which actively cotransports two sodium cations per each iodide anion. NIS-mediated transport of iodide is driven by the electrochemical sodium gradient generated by the sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase). In humans, the *NIS* gene is located on chromosome 19p12-13.2 and contains 14 introns and 15 exons (Smanik et al., 1997; Bizhanova and Kopp, 2009). NIS consists of 13 transmembrane domains with the amino terminus located extracellularly and the carboxy terminus facing the cytosol (Dohan et al., 2003). After entering the thyroid follicle via NIS on the basolateral side, iodide is shuttled across the apical membrane into the colloid via pendrin, another sodium-independent chloride/iodide transporter protein (encoded by the *SLC26A4* gene in humans). The *SLC26A4* gene is located on chromosome 7q21-31 and contains 21 exons with an open reading frame of 2343 bp (Everett et al., 1997; Lacroix et al., 2004). Pendrin consists of 12 transmembrane domains with both amino and carboxy termini located inside the cytosol (Royaux et al., 2000; Gillam et al., 2004).

Following the iodide transport, TPO, an integral membrane protein anchored in the apical plasma membrane of thyroid epithelial cells, catalyzes the sequential reactions in the formation of THs. In humans, the *TPO* gene is located on chromosome 2 (spanning more than 150 kb) and consists of 17 exons and 16 introns. It encodes a 933-amino acid peptide with a single membrane-spanning region (Kimura et al., 1987; De Vijlder et al., 1988). It first oxidizes iodide to atomic iodine and then oxidizes specific tyrosine residues on Tg. The "organification of iodine", i.e., the incorporation of iodine into oxidized Tg, is non-specific (occurs via reactive iodine species released from TPO) and results in the production of mono-iodotyrosine and di-iodotyrosine. Finally, TPO links two iodotyrosines to produce T_3 and/or T_4, through a process called coupling. All these reactions take place through electron transfer within the lumen. It is to be noted that the chemical reactions catalyzed by TPO occur on the outer apical membrane surface and are mediated by hydrogen peroxide (de Vijlder and den Hartog, 1998; Ruf and Carayon, 2006; Kessler et al., 2008; Mansourian, 2011).

Tg protein serves as the primary internal reservoir of recycling iodine in the body, upon which biosynthesis of THs is based. In rodents and humans, the peptide linkage between iodothyronines and adjacent amino acids in Tg is enzymatically cleaved (Marriq et al., 1989). The TH-containing portion of Tg is internalized by fluid-phase non-specific micropinocytosis at the apical surface of the thyroid epithelial cells (endocytosis). Lysosomes, which contain the hydrolytic enzymes, fuse with the endosomes and release the hormones (Marino and McCluskey, 2000). Free THs then diffuse into the blood, where they reversibly complex with liver-derived binding proteins [thyroxine-binding globulin (TBG), transthyretin (TTR; also called thyroid-binding prealbumin, TBPA), and albumin] (Choksi et al., 2003). These transport proteins protect T_4 and T_3 from degradation and help the hormone to remain as a buffer stock in peripheral circulation for a long time. On demand, the bound hormone is released and the free hormone only enters the target cells.

Metabolism of thyroid hormones

It is generally accepted that deiodination is the major pathway regulating T_3 bioavailability in mammalian tissues. Alternate pathways of TH metabolism also exist such as sulfation and glucuronidation of the phenolic hydroxyl group of iodothyronines, oxidative deamination and decarboxylation of the alanine side chain to form iodothyroacetic acids (Visser, 1996; Kelly, 2000). Sulfation of T_4 and T_3 markedly accelerates deiodination to form inactive metabolites such as rT_3 and T_2, whereby it regulates iodothyronine metabolism. Glucuronidation of TH often precedes biliary-fecal excretion of hormone (Yamanaka et al., 2007). Furthermore, glucuronidates and sulfated iodothyronines can be hydrolyzed to their precursors in gastrointestinal tract in various tissues, making these conjugates a reservoir of biologically active iodothyronines (Wu et al., 2005; van der Heide et al., 2007).

Regulation of thyroid hormone synthesis

Control of circulating concentrations of THs is regulated by negative feedback loops within the hypothalamic-pituitary-thyroid (HPT) axis (Mebis and van

den Berghe, 2009). Thyrotrophin releasing hormone (TRH), secreted by the paraventricular nucleus (PVN) of the hypothalamus into the median eminence, reaches the anterior pituitary through vascular route and binds to G-protein-coupled specific TRH receptors on the plasma membrane of thyroid-sensitive thyrotrophs (Monga et al., 2008). This in turn stimulates the synthesis and secretion of TSH from the thyrotrophs by transducing the signal through protein kinase C pathway and Ca^{2+} ions (Chiamolera and Wondisford, 2009; Costa-e-Sousa and Hollenberg, 2012). TSH, a glycoprotein hormone, plays a pivotal role in TH synthesis. It binds to the G-protein coupled specific TSH receptors located at the basal membrane of thyroid follicle cells and stimulates the expression and post-translational modifications of Tg, TPO and NIS proteins, which are involved in iodothyronine synthesis (Szkudlinski et al., 2002). It also stimulates iodide uptake, H_2O_2 production, oxidation of iodide and tyrosine, iodination, coupling reactions and the expression of TH receptors (Kopp, 2001; Winter and Signorino, 2001; Calebiro, 2011). TSH production is inhibited by the direct effect of T_3 binding to the thyrotrophs (Kleinau et al., 2013). Although hypothalamic TRH is the major stimulator of TSH synthesis and release from the anterior pituitary (Steinfelder et al., 1991), a negative feedback exhibited by THs at the pituitary is the most important physiological regulator of serum TSH levels (Shupnik et al., 1989). T_3 also inhibits the expression of TRH receptors in thyrotrophs and TRH expression in the PVN. Specific response elements of thyroid hormone receptors (TRE) are present in the promoter region of all three genes. Knock-out studies in mice have revealed that TRH neuron is absolutely required for both TSH and TH synthesis and appears to be the locus of the set-point in the HPT axis (Nikrodhanond et al., 2006; Perello et al., 2006). Thus, systemic thyroid status is maintained within a normal reference range by the HPT axis negative feedback control (see Fig. 2), which maintains a physiological inverse relationship between TSH and circulating T_3 and T_4 levels (Zoeller et al., 2007; Bassett and Williams, 2008).

Thyroid hormone action

Entry of TH into target cells

The THs have global action on human and animal systems and control essential functions of growth, development and metabolism in almost all tissues starting from hair to nail. THs, being hydrophobic, were thought to enter into target cell by passive diffusion. However, it has been accepted now that uptake of TH into the peripheral tissues is mediated by several specific monocarboxylate transporters (MCT) such as MCT8 and MCT10, and organic anion transporters (Visser, 2013; Wojcicka et al., 2013). Once inside the cytoplasm of the target cell, T_4 undergoes deiodination by 5' deiodinases to produce the active hormone T_3, which enters the nucleus and brings about the transcriptional response. *In vitro* studies suggest the existence of a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytosolic T_3 binding protein (CTBP), which helps in the transport of the hormone into the nucleus and facilitates its transcriptional activity (Mori et al., 2002). Suzuki et al. (2003) have also attested that CTBP plays a role as a carrier protein for T_3 from cytoplasm to nucleus as evinced by the presence of binding sites for T_3-CTBP (NADP) complex in rat kidney nuclei. These authors have earlier reported two CTBPs in rat kidney and liver namely p58CTBP and p38CTBP (Hashizume et al. 1989, 1991; Kobayashi et al., 1991). Screening of 73 human tissues revealed the maximum expression of p38CTBP in heart and brain (Suzuki et al., 2003).

Thyroid hormone receptors

The physiological actions of T_3 are mediated by nuclear receptors (TRs) that can bind T_3 with high affinity (Lazar, 1993; Aranda et al., 2013). TRs belong to a superfamily of nuclear receptors that shuttles between nucleus and cytoplasm. The discerning of the nucleotide and amino acid sequences of the receptors revealed that TRs are homologous with other nuclear hormone receptors (Harvey and Williams, 2002). The other members of the steroid thyroid superfamily of receptors include the steroid hormone receptors (androgen, estrogen, progesterone and cortisol receptors), and receptors for all-*cis*/*trans* retinoic acid (RAR, RXR), vitamin D (VDR), steroidogenic factor-1 and peroxisome proliferator-activated receptor (PPAR) and a number of orphan receptors for which the ligands are yet to be identified (Wagner et al., 1995; Flamant et al., 2006).

The major difference between cell surface and nuclear receptors is that the latter are ligand-inducible transcription factors, whereas the former have to recruit and activate general transcription factors through intracellular signalling molecules-mediated activation of specific protein kinases. Transcription factors are nuclear proteins, which remain in an inactive state either in the cytosol or nucleus and get activated upon phosphorylation or ligand binding or by interacting with other transcription factors. The activated transcription factor then binds to

specific nucleotide sequences present in the promoter region of target genes, called response elements, with the help of a DNA binding domain (DBD) and finally interact with RNA polymerases to initiate transcription (Darling et al., 1998; Uings and Farrow, 2000; Tata, 2002; Franco et al., 2003). As T_3-inducible transcription factors, TRs bind to specific regulatory DNA sequences (thyroid hormone receptor response elements, TREs) in the target gene promoters as a heterodimer with the retinoid X receptor (RXR) to bring about the transcriptional response in the target cell (Kliewer et al., 1992; Tagami et al., 2009). However, TRs may also form homodimers/heterodimers within their isoforms or heterodimers with other transcription factors such as PPAR and RAR (Glass, 1996; Cheng, 2000).

Structural features of thyroid hormone receptors

Like other members of the nuclear receptor superfamily, TRs have three major functional domains: (i) a transactivation domain at the amino terminus (A/B); (ii) a DNA-binding domain (C) that binds to sequences of hormone response elements in target gene promoter; and (iii) a ligand-binding and dimerization domain (E) at the carboxyl-terminus (Apriletti et al., 1998; Mangelsdorf et al., 1995). A hinge region (D) connects the DNA-binding domain (DBD) and ligand-binding domain (LBD). The N-terminal transactivation region is highly variable and encompasses the activation function-1 (AF-1) domain. It is involved in ligand-independent basal transcription activity and confers isoform specificity. The LBD located in the C-terminal region of the receptor possesses the AF-2, which is involved in ligand-dependent activation of transcription by binding to coactivators and release of corepressors upon ligand binding (Wurtz et al., 1996). This domain also contains the sequences associated with receptor dimerization (homo- and heterodimerization). The hinge region of TR isoforms (TRα1, TRβ1) and the oncoprotein vErbA (Avian erythroblastosis virus - the ancestral gene of the nuclear receptor proteins) has the signal that directs nuclear localization of the receptor i.e., nuclear localization signal (NLS1). Recently, Mavinakere et al. (2012) have identified a novel NLS, designated NLS-2, in the A/B domain of TRα1 that is absent in TRβ1 and remains inactive in the oncoprotein.

While most other members of nuclear receptor family have leucine-rich chromosome region maintenance 1 (CRM1)-dependent and CRM1-independent nuclear export signal (NES) sequences (Kutay and Güttinger, 2005), TRs have three CRM1-independent NES sequences in the LBD. They are specifically located in a highly conserved C-terminal helix 12 region (NES-H12), the helix 3 region (NES-H3), and the helix 6 region (NES-H6). Mutations in these NES markedly reduce both nuclear export and transactivation of TH-mediated gene expression (Mavinakere et al., 2012). The NLS and NES motifs were shown to be sufficient to target a cytosolic protein to the nucleus or a nuclear protein to the cytosol, respectively. In addition, the shuttling of nuclear receptors, including TRs, occurs through the nuclear pore complexes and the process is mediated by a family of soluble proteins called karyopherins, which bind to NLS or NES in the receptors (Umemoto et al., 2012) (see Fig. 3).

Dynamics of ligand binding domain

The structure and dynamics of LBD are essential for transcription regulation. The LBD is composed of 12 amphipathic [peptides/proteins with polar (water- soluble) and non-polar (non- water soluble) portions] helices, some of which specifically interact with coactivators and corepressors (Nagy et al., 1999). Upon ligand binding, TRs modify the conformation of their LBD region - a process that mainly involves H12 and results in the release of corepressors and recruitment of coactivators (Ito and Roeder, 2001). The H12 region is fully conserved between TRα1 and TRβ1, except for the presence of three additional amino acids toward the C terminus in TRα1 (Mavinakere et al., 2012). The residue numbering of amino acids in LBD mentioned in this review is according to TRβ. In TRs, corepressors and coactivators interfaces overlap and are formed by residues V284, K288, I302, and K306 from helices 3, 5, and 6. The corepressors binding surface is further complemented by residues T277, I280, T281, V283, and C309, which also belong to helices 3, 5, and 6 but are spatially closer to H12 in holo-TR, whereas the coactivators require residues L454 and E457 from H12 to interact with TR (Feng et al., 1998; Webb et al., 2000).

The H12 conformation and dynamics are the key factors that modulate ligand-dependent transcription regulation. It is currently accepted that in the absence of ligand, the C-terminal H12 is positioned such that it exposes an interface for corepressor binding. Ligand binding perturbs the dynamic equilibrium of H12, which adopts a novel preferential orientation that favors coactivators, instead of corepressor, recruitment; the detailed view of LBD dynamics can be found in Souza et al. (2011). H12 is docked over residues I280, V283, and C309 in holo-TR structures, so that corepressor binding

requires a conformational shift of H12 from this position. The role of these three residues (I280, V283, and C309) in coactivator and corepressor binding is essential for the comprehension of H12 conformational equilibrium and dynamics. As coactivator, but not corepressor, binding is dependent on direct interactions with H12, deletion of H12 blocks coactivator interactions but increases corepressor association by exposing its interaction surface (Marimuthu et al., 2002). Some mutations in this region are also linked to resistance to thyroid hormone syndrome (RTH), which is usually associated with reduced transcriptional activity and reduced hormone affinity for the receptor (Olateju and Vanderpump, 2006).

Subtypes and isoforms

Thyroid hormone receptor consists of two major subtypes, TRα (Sap et al., 1986) and TRβ (Weinberger et al., 1986). In humans, TRα and TRβ are encoded by two distinct genes located on chromosomes 17q11.2 and 3p24.2, respectively (Sorensen et al., 2008). Both *TRα* and *TRβ* genes consist of 10 exons, and exons 3-10 are the main coding regions (Laudet et al., 1991; Escriva et al., 2000). Both the genes are transcribed as multiple mRNA isoforms/variants as a result of alternative splicing. TRα1 (410 amino acids) and TRα2 (492 amino acids) differ in their C-terminal region, whereas TRβ1 (461 amino acids) and TRβ2 (514 amino acids) differ in their N-terminal region (Williams and Brent, 1995). TRα1 gene encodes one T_3-binding TRα1 and two splice variants (TRα2 and TRα3). Truncated TRs, transcribed from an internal promoter located in intron 7, give rise to the variants TRΔα1 and TRΔα2 that lack amino-terminal A/B and DBDs but retain most of the T_3-binding domain (Plateroti et al., 2001). There are three TRβ isoforms (β1, β2, and β3) in humans, which can bind to T_3 (Williams, 2000). These TRβ isoforms share high sequence homology in the DNA and T_3-binding domains but differ in the length and amino acid sequences in the amino terminal A/B domain. Internal usage of ATG leads to the TRΔβ3 that lacks the amino terminal A/B and DBDs but retains T_3-binding activity (Cheng et al., 2010) (see Fig. 4).

Each TR isoform has a specific expression pattern that varies with the stage of development indicating the complex nature in the physiological effects of THs (Cheng, 2000; Flamant and Gauthier, 2013). TRα and TRβ differ in their expression during development (Forrest et al., 1991; Bradley et al., 1992; Sjoberg et al., 1992), tissue distribution (Schwartz et al., 1994) and ligand affinity for thyroid hormone analogs (Schueler et al., 1990). There is also isoform-specific target gene regulation (Lezoualc'h et al., 1992) and isoform-specific antagonism by mutant TRs (Zavacki et al., 1993). Among the TR isoforms, TRα1 is primarily expressed in heart, bone and brain, while TRβ1 is more abundant in liver, kidney and thyroid (O'Shea et al., 2003; Keijzer et al., 2007). The expression of TRβ2 is limited to the pituitary, hypothalamus, retina and inner ear (Hodin et al., 1989; Wondisford, 2003). It is to be noted that TRβ2 is predominantly restricted to the hypothalamic-pituitary axis, where it acts negatively to regulate thyroid TSH α- and β-subunit transcription (Rebai et al., 2012). Testis was once considered as non-target organ for TH action. Palmero et al. (1988), for the first time, demonstrated the presence of TR isoforms in prepuberal rat Sertoli cells, thereby highlighting the impact of THs on testicular development and reproduction. Consequently, the significance of THs has been ascertained by many workers using various animal models and validating the presence of TR isoforms in testis (Palmero et al., 1995; Buzzard et al., 2000; Canale et al., 2001; Wagner et al., 2008). Reverse transcriptase polymerase chain reaction and Northern blot analysis of fetal and adult human testis revealed the expression of TRα1 and TRα2, whereas TRβ1 expression could not be detected (Jannini et al., 2000).

Genomic signalling by thyroid hormones

The transcription mediated by TRs requires extensive cooperation and dynamic interplay with many nuclear receptor coregulators (McKenna et al., 1999). In general, binding of unliganded TR to DNA leads to repression of transcription, whereas binding of the T_3-liganded TR heterodimer activates transcription. The unliganded receptor homodimers/heterodimers recruit corepressors (e.g., nuclear receptor corepressor - NCoR; silencing mediator for retinoic acid and thyroid receptors - SMRT) to repress or "silence" gene transcription (Astapova and Hollenberg, 2013). Unlike androgen or progesterone receptors, the unliganded TR is not cytoplasmically anchored to heat shock proteins, and thus the NLS (located in the region of amino acid 178-185) is operative, thereby repressing the transcription of genes activated by T_3 (Koenig, 1998; Germain et al., 2006). This feature of TRs is in contrast to steroid hormone receptors, which are transcriptionally inactive in the absence of ligand (Zhang and Lazar, 2000).

In the cytoplasm, T_4 is catalyzed by 5' deiodinase to produce the active T_3, which is then translocated to the

nucleus through nuclear pores by means of specific cytosolic transporters such as CTBP (Oppenheimer and Schwartz, 1985; Suzuki et al., 2003). After gaining entry into the nucleus, T_3 binds to the TR monomer of the TR-RXR heterodimer complex. This triggers the dissociation of corepressors (relieving the repression), together with the recruitment of a complex of coactivators (e.g., steroid receptor coactivator 1 – SRC1, CREB-binding protein, and CBP-associated factor) and thereby enabling the transcription of T_3-inducible genes (Horlein *et al.*, 1995; Hu and Lazar, 1999; Rosenfeld and Glass, 2001; Lazar, 2003a) (see Fig. 5).

Interaction of TR with DNA

Though TRα and TRβ shuttle between the nucleus and cytoplasm, they primarily localize to the nucleus at a steady state level, where they either activate or repress target gene expression in response to T_3 (Bunn et al., 2001; Lazar, 2003b). Within the nucleus, TRs bind to short, consensus repeated sequences of DNA called nuclear receptor response element (NRE). Experimental studies have shown that the majority of genes which are positively regulated by TRs contain at least two hexameric half-sites consisting of the consensus sequence "AGGTCA", which constitute the core recognition sites, i.e., TRE (Cheng et al., 2010). This consensus sequence is conserved among all non-steroidal NRs like RXR and RAR, VDR and PPAR.

TREs vary in the primary nucleotide sequences of half-sites as well as their number, spacing and orientation (Williams and Brent, 1995). The two half-sites can be separated by various numbers of base pairs in between, often indicated by a number at the end of the NRE classification. For example, the IP6 class of NRE is composed of two inverted palindrome consensus sequences separated from one another by 6 bp. The half-sites may be arranged as direct repeats with a four nucleotide spacer (DR4), as tail-to-tail assembled inverted palindrome with a six nucleotide spacer (IP or F2) or as a head-to-head assembled palindrome without any spacer (Pal0) (Chen and Young, 2010) (see Fig. 6). TR–RXR heterodimers bind preferentially to elements spaced by DR4. In these complexes RXR occupies the 52 half-site while TR, located at the 32 half-site of the element, determines the specificity. RXR augments thyroid hormone mediated transactivation by increasing the affinity of TRs for binding to the cognate response elements. DRs are the most abundant TRE in natural promoters followed by the IP/F2 or Pal0 (Yen, 2001; Oetting and Yen, 2007). Homodimers of TR have been shown to prefer and bind to target gene promoters containing IP/F2 TRE over genes containing DR TRE, suggesting that IP/F2 TREs containing genes are strongly responsive to TH. TH-induced transcriptional activation through F2 TRE is conserved even in the absence of TR-RXR heterodimers (Darling et al., 1993; Velasco et al., 2007).

The core region of DBD (consisting of 66 amino acids) is the most conserved region of the nuclear receptor superfamily (>40% sequence identity) (Gronemeyer and Moras, 1995). It contains two a helices and two sets of four cysteine residues, and each set chelates a zinc ion, forming loops known as "zinc fingers", which mediate the specific sequence recognition and confer spacing specificity. A DNA recognition helix (P box) in the carboxyl terminus of the first zinc finger mediates the half-site sequence recognition by directly contacting the major groove nucleotides. In addition to the major groove contact, several members of the nuclear receptor family make additional minor groove contact through the least conserved carboxyl-terminal extension (CTE) downstream of the second zinc-containing module, as shown in the crystal structures of TR-RXR bound to DR4 repeats (Rastinejad et al., 1995). The CTE adopts a helical conformation and is essential in mediating protein-protein and protein-DNA interactions required for the cooperative DNA binding by homodimeric nuclear receptor DBDs (Lee et al., 1993). A T-box region, located at the beginning of the CTE, incorporates the residues contributing to the TR-RXR DBD heterodimers interface when bound to DR4 that establishes the downstream positioning of the TR DBD in the heterodimer-DNA complex (Katz and Koenig, 1994; Rastinejad et al., 1995; Chen and Young, 2010). There is a strict binding polarity of TR-RXR heterodimers on DR4 such that RXR occupies the upstream half site and TR occupies the downstream half site (Kurokawa et al., 1993). Thus, heterodimerization with RXR dramatically increases the binding of TRs to TREs, the responsiveness of TR to T_3, and the transcriptional activation (Lee and Privalsky, 2005).

Plasma membrane-mediated actions of thyroid hormones

While the majority of actions of THs are mediated by nuclear receptors, a few non-genomic actions of thyroid hormone are also recognized. The potential sites of non-genomic action include the plasma membrane, cytoskeleton, sarcoplasmic reticulum and mitochondria. Examples of such non-genomic actions demonstrated *in*

vitro include the relaxation of vascular smooth muscle, ion channel activation and stimulation of mitochondrial oxygen consumption. Many non-genomic actions of THs appear to contribute to basal levels of activity of a variety of proteins, including ion pumps [Ca^{2+}-ATPase (Davis et al., 2010), Na^+/K^+-ATPase (Lei et al., 2008), Na^+/H^+ antiporter (Incerpi et al., 2007)], and contribute to intracellular protein trafficking (Cao et al., 2009) and protein turnover (Carter et al., 1984).

At the plasma membrane, THs stimulate phosphatidylinositol 3-kinase (PI3K) and Rac activity, which in turn, stimulates voltage-activated potassium channels encoded by the ether-a-go-go-related gene *KCNH2* in a rat pituitary cell line (Storey et al., 2006). Interestingly, TRα1 has also been shown to interact with p85 in a T_3-dependent manner and to modulate the activity of another downstream target of Akt/PKB, endothelial nitric oxide synthase (eNOS) (Hiroi et al., 2006).

A cell surface receptor for thyroid hormone was first described in 2005 and was linked to hormonal modulation of angiogenesis (Bergh et al., 2005). A structural protein of the plasma membrane, integrin αvβ3, has been shown to contain a binding domain [Arg-Gly-Asp (RGD) recognition site] for iodothyronines, predominantly T_4 (Cody et al., 2007), which is implicated in TH-induced endothelial cell and tumor cell proliferation (Davis et al., 2006; Lin et al., 2012).

Thyroid hormones act via a plasma membrane receptor binding site on integrin αvβ3 and induce the activation of mitogen-activated protein kinase (MAPK; ERK1/2) signal transduction cascade via protein kinase C. In the cytosol, activated MAPK phosphorylates proteins such as TRβ1 (Davis et al., 2000), estrogen receptor a (Tang et al., 2004), signal transducer and activator of transcription 1α (STAT1α) (Lin et al., 1999) and tumor suppressor protein (p53) (Shih et al., 2001) and in turn translocates them to the nucleus (protein trafficking), where these proteins are transcriptionally active to induce the production of factors such as basic fibroblast growth factor and epidermal growth factor, thereby favoring angiogenesis and cell proliferation (Davis et al., 2008, 2011) (see Fig. 7).

Crosstalk with other signalling pathways

Thyroid hormones, being key metabolic regulators coordinating short-term and long-term energy needs, its effects are mediated by the potentiation or augmentation of other signal transduction pathways (Liu and Brent, 2010). TRs may also negatively regulate the transcription without binding to DNA due to interference with other transcription factors and acting as specific traps or baits for coregulatory proteins (Weitzel, 2008). TRs have been demonstrated to engage in crosstalk with a range of nuclear metabolic receptors, including RAR (Flamant and Samarut, 1998; Weston et al., 2003); PPARα (Liu et al., 2007), PPARγ (Araki et al., 2005), and liver X receptor (LXR), in metabolic regulation (Hashimoto et al., 2007); in brain cortical layering (Tan et al., 2010); adrenergic signalling in bone (Gogakos et al., 2010) and heart (Liu et al., 2003) [for a detailed review, see Brent (2012)].

Knock-out/mutation studies

Given the critical role of TRs in cellular functions, it is reasonable to expect that mutations of TRs could have deleterious effects (Cheng, 2005). The first published report of a TRα gene knock-out used a strategy of interrupting the proximal exon 2, which produced inactivation of both TRα1 and TRα2 gene products (Fraichard et al., 1997). Mutant mice phenotypes indicate that TRα1 function is important for early post-natal development, before weaning, a stage marked by a peak in the circulating level of T_3, rapid skeletal growth, intestinal epithelium remodeling, change in red blood cell populations and brain maturation. All these events are affected by T_3 deficiency, TRα1 knock-in mutations and, in a milder way, by TRα1 knock-outs (Angelin-Duclos et al., 2005; Venero et al., 2005). In adults, TRα1 function is important to maintain heart rate (Kahaly and Dillmann, 2005), muscle strength, body temperature and energy expenditure (Sjogren et al., 2007). Thus, lack of TRα1 function mimics many features of congenital and adult hypothyroidism, without changing T_3 level.

Role of thyroid hormone receptors on oncogenesis

The actions of THs mediated by TR isoforms are highly pleiotropic, affecting many tissues at different developmental stages. As a consequence, their effects on proliferation and differentiation are highly heterogeneous, depending on the cell type, the cellular context, and the developmental or transformation status. A significant number of TRs-regulated genes and proteins have been identified so far; many of them are important regulators of cellular proliferation, differentiation and apoptosis (Puzianowska-Kuznicka et al., 2006). Thus, it is not surprising that aberration in functioning of TRs result in disturbances of cell physiology. A high prevalence of

mutations of *TR*α and *TR*β genes has been identified in a Polish study on papillary thyroid carcinoma (Puzianowska-Kuznicka et al., 2002). TRα directly stimulates transcription of *β-catenin* gene in intestinal epithelial cells and may play a role in tumorigenesis in that tissue (Plateroti et al., 2006). Expression of type 3 deiodinase, which inactivates thyroid hormone, has been associated with proliferation of malignant keratinocytes in basal cell skin carcinomas (Dentice et al., 2007).

The PV model, in which animals harbor a specific truncation of TRβ, is associated with the development of thyroid cancer (Furuya et al., 2007). Mutations in TRβ have been reported to promote metastatic spread of thyroid cancer (Lu and Cheng, 2011). Furthermore, TRβ mutations have been identified to augment growth in a range of cancers including hepatocellular carcinoma, renal cell carcinoma, erythroleukemias (Chan and Privalsky, 2010; Rosen et al., 2011). Furumoto et al. (2005) have reported that TRβ mutants activate cyclin D1/cyclin-dependent kinase/retinoblastoma/E2F pathway in TSH-secreting pituitary tumors. TRβ mutants are associated with direct interaction with the regulatory p85α subunit of PI3K, which leads to activation of PI3K and increased phosphorylation of Akt and *mammalian target of rapamycin* resulting in cellular proliferation and migration (Furuya et al., 2009). For a detailed account on the role of TR isoforms in regulating cell proliferation and differentiation, the readers can refer the review of Kim and Cheng (2013).

Thyroid dysfunction

Three major categories of thyroid dysfunction have been characterized in adult humans: subclinical hypothyroidism, overt hypothyroidism, and hyperthyroidism. Subclinical hypothyroidism is defined as a slightly elevated TSH concentration and normal serum free T_3 and T_4 concentrations associated with few or no symptoms (Franklyn, 2013). Overt hypothyroidism or underactive thyroid gland is the most common clinical disorder of thyroid function. It is best defined as high serum TSH concentration and a low free T_4 serum concentration (Chakera et al., 2012). Insufficient iodine levels or low iodine intake are the major causes of overt hypothyroidism. However, in areas where iodine intake is adequate (Leung and Braverman, 2012), the most common cause of hypothyroidism is Hashimoto's thyroiditis, an autoimmune disease caused by autoantibodies to TPO (Latina et al., 2013). Hyperthyroidism (or thyrotoxicosis) is characterized by an increase in serum T_3 and T_4 and a decrease in serum TSH. The most common cause of hyperthyroidism is Graves' disease (production of antibodies to TSH receptor) (Li and Wang, 2013). Information regarding the pathologies of thyroid dysfunction is beyond the scope of this review. Interested readers can get a detailed account in the cited reviews (see Cooper and Biondi, 2012; Chiha et al., 2013; Taylor et al., 2013).

Resistance to thyroid hormone syndrome

A relatively new entity of thyroid disorder is "TH resistance syndrome", wherein inactivating TH receptor mutations or under-expression of TR is associated with hypothyroidism. RTH syndrome is characterized as an endocrine disease caused by mutations in the *TRβ* gene that impairs corepressor release in response to T_3, which in turn reflects itself in reduced sensitivity of tissues to the actions of TH (Refetoff et al., 1993). The hallmark of RTH is elevated TH titer associated with non-suppressible TSH. Other clinical signs are goiter, short stature, decreased weight, tachycardia, hearing loss, attention deficit/hyperactivity disorder, decreased IQ, and dyslexia (Weiss and Refetoff, 2000; Yen, 2003).

Indeed, shortly after the cloning of the TR genes, a tight linkage was discovered between the affected family members with RTH and the TRβ gene (Usala et al., 1988; Dumitrescu and Refetoff, 2013). The identification of a Pro453 His mutation in the TRβ gene of one kindred established that RTH is caused by mutations of the TRβ gene (Sakurai et al., 1989). The incidence of TRβ-mediated RTH is estimated to be about 1 in 40,000 (Lafranchi et al., 2003) and over 170 different beta receptor mutations, associated with a variable human phenotype, have been described to date in more than 374 families and 532 affected individuals (Refetoff and Dumitrescu, 2007). The TRβ mutant proteins identified in RTH have reduced or no T_3-binding affinity and transcriptional capacity (Yen, 2003). The central issue of how mutations of TRβ result in RTH was addressed *in vivo* after the creation of two knock-in mouse models, one harboring a C-terminal 14 amino acid frameshift mutation (PV mutation - TRβPV mouse) (Kaneshige et al., 2000), and the other harboring a D337T mutation (TRβD337T mouse) (Hashimoto et al., 2001) in the *TRβ* gene. These two knock-in mice exhibit RTH phenotypes including dysregulation of the pituitary-thyroid axis and neurological dysfunction. Consistent with phenotypes of RTH patients, TRβPV mice also exhibit growth retardation, abnormal regulation of serum cholesterol

(Kamiya et al., 2003), lower glycogen deposits (Vujovic et al., 2009), hearing defects (Griffith et al., 2002) and thyrotoxic skeletal phenotype (O'Shea et al., 2003).

Recent studies have described a homologous human disorder manifesting with some features of hypothyroidism in patients with TRα1 mutations exhibiting hypothyroid features (e.g., skeletal dysplasia, reduced intestinal motility, growth and developmental retardation, low heart rate and BMR) in tissues (bone, gastrointestinal tract, myocardium, skeletal muscle) expressing predominantly TRα, suggesting that mutant TRα1 may lead to severe resistance to hormone action. These patients showed a typical thyroid biochemical signature (low T_4/T_3 ratio, together with subnormal reverse T_3 levels, but associated with low-normal T_4 and near-normal T_3 and TSH levels (Bochukova et al., 2012; van Mullem et al., 2012; Schoenmakers et al., 2013).

Thyroid disrupting chemicals

Thyroid function is regulated by a finely tuned negative feedback mechanism of circulating THs at the hypothalamic and pituitary levels, maintaining relatively stable serum levels of THs with each individual having his or her specific set point (Feldt-Rasmussen et al., 1980). The complex system of iodine uptake, TH production, interconversion of THs, cellular uptake, cell receptor activation, and hormone degradation and elimination could be interfered by the exposure to environmental chemicals, thereby resulting in adverse effects both in the individual and in a population (Crofton et al., 2005). Due to the complex nature of the regulation of thyroid function and TH action, the consequences of exposure to such thyroid disrupting chemicals (TDCs) are also likely to be complex (Decherf et al., 2010).

Well-designed cohort studies on laboratory animals and *in vitro* models have highlighted several classes of chemicals that exhibit adverse effects on thyroid signalling mechanism. The primary environmental chemicals identified as thyroid disruptors are polychlorinated biphenyls, bisphenol A, perchlorate, tetrachlorodibenzo-*p*-dioxin and polychlorinated dibenzofuran (both commonly referred to as dioxins), pentachlorophenol, triclosan, polybrominated diphenyl ethers and tetrabrominated diphenyl ethers (commonly known as flame retardants) and naturally-occurring chemicals such as soy isoflavones and thiocyanate in cruciferous vegetables (Zoeller, 2010; Hartoft-Nielsen et al., 2011).

Among these, a few compounds are known to have a direct affinity for TRs, whereas others are able to activate receptor-dependent transcription of TH target genes by modulating upstream signalling without binding to the T_3-binding site of TRs. TDCs could also exert transcriptional effects by disrupting the recruitment/release of coactivators by TRs, by interfering with the expression of TR and their heterodimerization partner, or by interfering with the affinity between TR and TRE (Jugan et al., 2010).

The unavoidable life-long human exposure to mixtures of such TDCs raises serious concerns about their potential to adversely affect thyroid function. Subtle changes in the individual set point of thyroid homeostasis may have significant acute and long-term effects, especially if this occurs during sensitive developmental periods. Pregnant women and their foeti, premature children, infants and toddlers are particularly sensitive to permanent effects on neurodevelopment, whereas older children and adolescents may mainly exhibit adverse effects related to growth and reproductive development (Boas et al., 2012). Taking into account the complexity and toxic responses mediated by TDCs, methods to develop efficient screening and testing strategies with robust tools to identify such toxicants have drawn much scientific attention in toxicological research. Efforts on this line would make it possible to limit adverse outcomes of TDCs for future generations.

Conclusion

THs function as the major endocrine modulator of metabolic regulation, growth and development. The classical pathway of TH signalling involves the nuclear receptors - TRα and TRβ and their isoforms. TH action on target cells depends upon the bioavailability of the free hormone, presence of membrane and cytosolic T_3 transporters, expression of TR subtypes, its interactions with heterodimerization partners and corepressors/coactivators. All these events are tightly regulated to influence the transcriptional response (genomic) mediated by TH. Even though the discovery of TH-binding sites on a plasma membrane-bound protein (integrin αvβ3) appears to be a putative indirect mechanism to transduce the genomic actions of THs, we are yet to unravel the mystery completely.

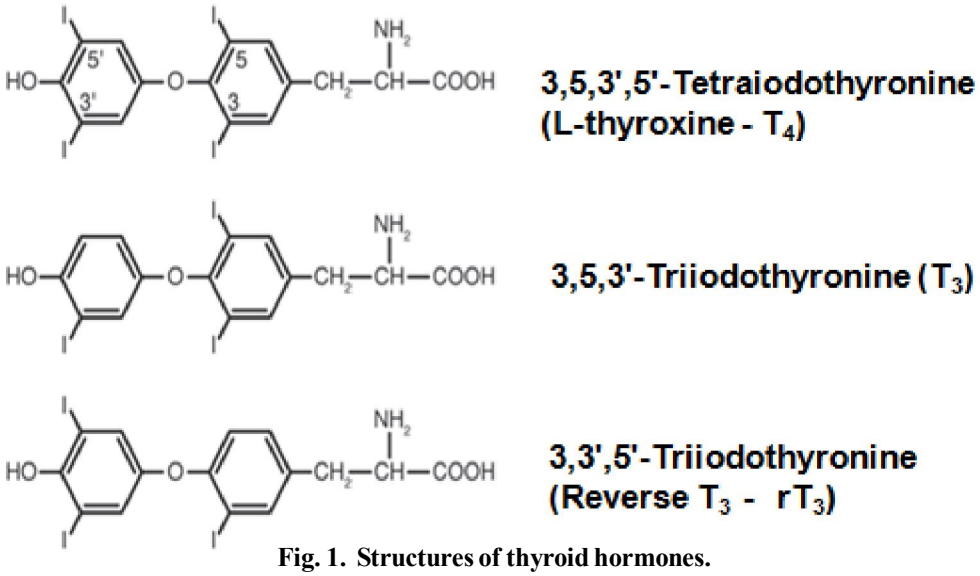

Fig. 1. Structures of thyroid hormones.

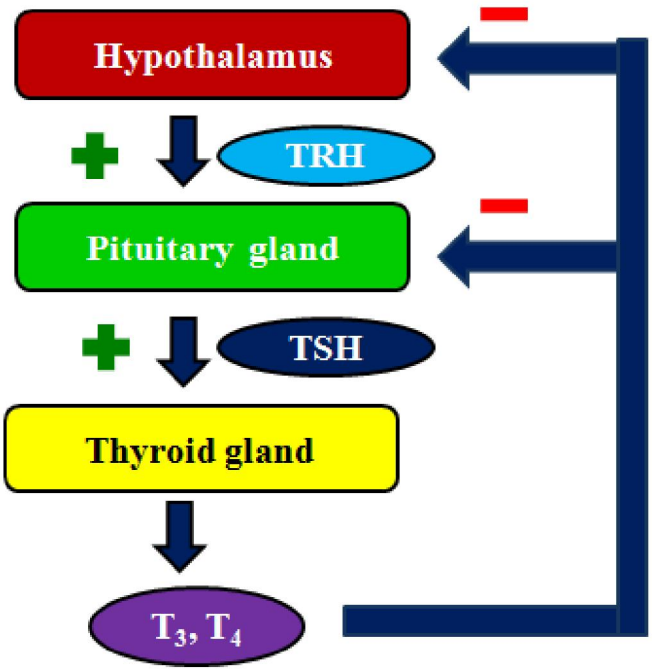

Fig..2. Schematic representation of the negative feedback regulation of hypothalamo-pituitary-thyroid axis.

TRH secreted by the hypothalamus stimulates the pituitary thyrotrophs to secrete TSH, which in turn stimulates the thyroid glands to secrete T_3 and T_4. These iodothyronines regulate their own synthesis and secretion by inhibiting at the level of both hypothalamus and anterior pituitary, thereby constituting a tightly regulated feedback loop.

TRH, Thyrotrophin releasing hormone; TSH, Thyroid stimulating hormone

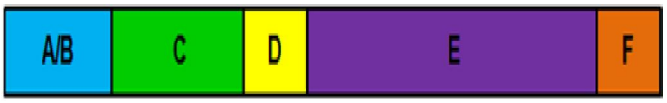

Fig. 3. Structural and functional domains of a thyroid hormone receptor.

A/B constitutes the amino terminal region and it contains ligand-independent AF-1 domain. The C domain, otherwise called as DBD, comprises of the conserved the core DNA binding and a CTE region including the T-box region. D denotes the hinge region, which connects the DBD and LBD and it contains nuclear localization signal (NLS). E indicates LBD which encompasses ligand-dependent AF-2 domain, sequences for receptor dimerization, NES and coactivator/corepressor binding regions. F indicates carboxy terminal region (Refer text for details).

AF-1, Activation function-1 domain; AF-2, Activation function-2 domain; CTE, C-terminal extension region; DBD, DNA binding domain; LBD, Ligand binding domain; NES, Nuclear export signal; NLS, Nuclear localization signal.

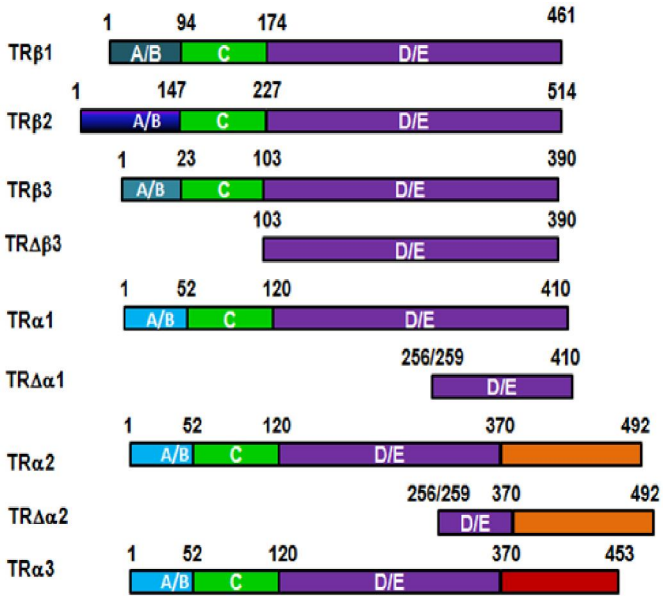

Fig. 4. Schematic view of TR subtypes (α and β) and their isoforms.

TR isoforms derived by alternative splicing are shown depicting their characteristic domains (A/B, C and D/E). Among the different isoforms, DBD and LBD share high sequence homology and the difference in color along the same domains indicates the variation in length and amino acid sequence (amino acid numbers provided) (adapted from Cheng et al., 2010).

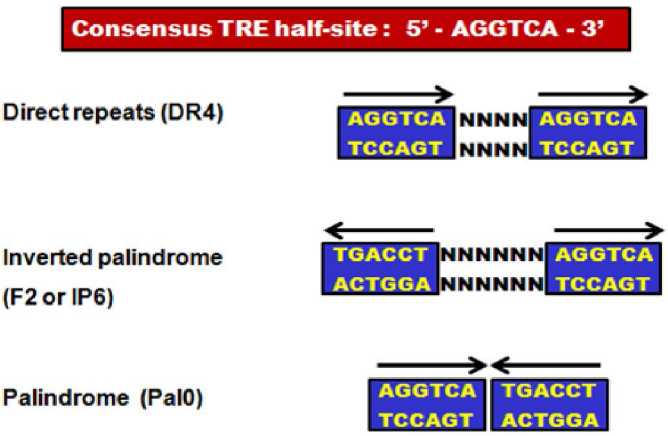

Fig. 6. A simplified overview of half sites organizations in TRE.

TRs bind to distinct thyroid hormone response elements (TREs) typically located in the upstream promoter regions of target genes. The TRE consists of two repeats of the consensus hexameric half-sites which is identified as the core recognition element having the sequence of 5'-AGGTCA-3'. The inter-half site spacings found in TR-responsive genes are 4 base pair (bp), 6 bp, and 0 bp and are designated as DR, IP6 or F2 and Pal, respectively. DRs are the most abundant TRE in natural promoters followed by IP/F2 or Pal0 (adapted from Chen and Young, 2010).

DR, Direct repeats; IP, Inverted palindrome; Pal, Palindrome.

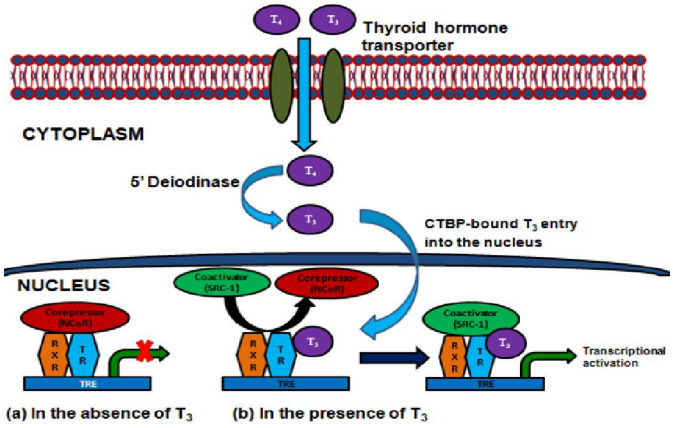

Fig. 5. Genomic signalling by thyroid hormone receptors.

In the target cells, TRs can positively and negatively regulate gene transcription. Inside the nucleus, TR remains bound to DNA as a heterodimer with RXR but exists in two mutually exclusive conformations (a & b). (a) In the absence of hormone, binding of the corepressor complex leads to chromatin inactivation and gene repression. (b) T_3 upon entry into the cell nucleus binds to the TR monomer of the TR-RXR-TRE complex which induces conformational changes in the TR enabling the dissociation of corepressor and binding of coactivator. This transition is accompanied by the opening of chromatin structure and activation of transcription machinery resulting in a T_3-specific response.

CTBP, Cytosolic T_3 binding protein; TR, Thyroid hormone receptor; RXR, Retinoid X receptor; TRE, Thyroid hormone receptor response element.

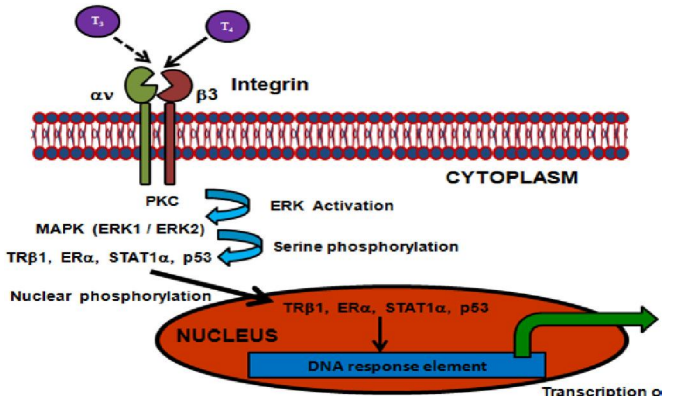

Fig. 7. Plasma membrane mediated signalling of thyroid hormones.

Thyroid hormones, specifically T_4, act via a plasma membrane receptor binding site on integrin αvβ3 to activate the MAPK/ERK signal transduction cascade via PKC. Activated MAPK phosphorylates TRβ1, ERα, p53, and STAT1α and translocates them into nucleus, which in turn results in the transcription of growth factors promoting cell proliferation. bFGF, Basic fibroblast growth factor; EGF, Epidermal growth factor; ERK1/2, Extracellular-signal-regulated kinase 1/2; ERα, Estrogen receptor α; MAPK, Mitogen-activated protein kinase; p53, Tumor suppressor protein; PKC, Protein kinase C; STAT1α, Signal transduction and activation of transcription 1α; TRβ1, Thyroid hormone receptor β1.

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