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Patterns of thyroid hormone receptor expression in zebrafish and generation of a novel model of resistance to thyroid hormone action

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ABSTRACT

Resistance to thyroid hormone can be due to heterozygous, dominant negative (DN) *THRA* (RTHα) or *THRB* (RTHβ) mutations, but the underlying mechanisms are incompletely understood. Here, we delineate the spatiotemporal expression of TH receptors (TRs) in zebrafish and generated morphants expressing equivalent amounts of wild-type and DN TRαs (*thraa*_MOs) and TRβs (*thrb*_MOs) *in vivo*. Both morphants show severe developmental abnormalities. The phenotype of *thraa*_MOs includes brain and cardiac defects, but normal thyroid volume and *tshba* expression. A combined modification of *dio2* and *dio3* expression can explain the high T3/T4 ratio seen in *thraa*_MOs, as in RTHα. *Thrb*_MOs show abnormal eyes and otoliths, with a typical RTHβ pattern of thyroid axis. The coexpression of wild-type, but not mutant, human TRs can rescue the phenotype in both morphants. High T3 doses can partially revert the dominant negative action of mutant TRs in morphant fish.

Therefore, our morphants recapitulate the RTHα and RTHβ key manifestations representing new models in which the functional consequences of human TR mutations can be rapidly and faithfully evaluated.

1. Introduction

Thyroid hormone (TH) is a critical regulator of many physiological and developmental processes, following its conversion from the stable pro-hormone (L-thyroxine, T4) to the short-lived active hormone, triiodothyronine (3,3′,5-triiodo-L-thyroinine, T3). In vertebrates, appropriate TH concentration is critical for promotion of intrauterine and postnatal development and growth, as well as for maintenance of metabolic pathways after development (Brent, 2012). TH are available to the embryo even before the embryonic thyroid gland is functional through placental supply or via maternal TH deposition in the egg yolk. TH act via nuclear thyroid receptors (TRs) to regulate gene expression, typically by binding thyroid response elements (TREs) in their promoter region (Cheng et al., 2010). Depending on their conformational state and the type of TRE they bind, TRs either promote or repress gene transcription (Aranda and Pascual, 2001). In humans, two separate genes (*THRA* and *THRB*) generate the T3-binding receptors TRα1, TRβ1 and TRβ2. Studies in transgenic mice have contributed substantially to our understanding of the role of each TR variant, leading to the conclusion that all of them seem to have both unique and redundant functions (O'Shea and Williams, 2002; Cheng, 2005; Flamant and Quignodon, 2010). Mutations in human TR genes result in a reduced responsiveness of peripheral tissues to TH action, causing disorders (Resistance to Thyroid Hormone alpha or beta, or RTH), typically due to dominant negative (DN) mutations in *THRB* (RTHβ) or *THRA* (RTHα) (Refetoff et al., 2014a). The phenotypic

Non-standard abbreviations and acronyms: TRs, thyroid hormone receptors (various, see nomenclature below); RTH, resistance to thyroid hormone; HPT, hypothalamic-pituitary-thyroid; hpf, hours post-fertilization; qRT-PCR, quantitative real-time polymerase chain reaction; WISH, whole-mount *in situ* hybridization; thraa, zebrafish thyroid hormone receptor alpha a; thrb, zebrafish thyroid hormone receptor beta; tg, thyroglobulin; nis, sodium-iodide symporter; tshba, zebrafish thyroid stimutating hormone beta; MOs, morphants.

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manifestations of RTHβ and RTHα are distinct, indicating isoform-dependent actions of TR mutants in different tissues *in vivo*. The cardinal features of RTHβ are elevated serum levels of free thyroid hormones with non-suppressed TSH and goitre together with variable, largely unexplained, coexistence of thyrotoxic and hypothyroid manifestations (e.g. anxiety, tachycardia, failure to thrive, impaired hearing and colour vision, attention-deficit hyperactivity disorder, low bone density) in different tissues (Refetoff et al., 2014a). Whilst several hundred families with germline *THRB* mutations have been reported since the initial description of the RTHβ, only a few kindred with germline *THRA* mutations have been reported to date, defining RTHα as an emerging genetic disorder. Patients with RTHα exhibit features reminiscent of untreated congenital hypothyroidism (CH) (e.g. short stature, skeletal dysplasia, mental retardation and chronic constipation), but without the typical biochemical signature of CH (high TSH and low circulating TH *vs* normal TSH levels and raised circulating T3/T4 ratio in RTHα patients), whose basis is not fully understood (Bochukova et al., 2012; van Mullem et al., 2012; Moran et al., 2013; van Mullem et al., 2013; Moran et al., 2014; van Guchta et al., 2014; Espiard et al., 2015; Tylki-Szymanska et al., 2015; Yuen et al., 2015). Insights into TH action have been gained either by deletion of *Thra* or *Thrb* genes or introduction of mutations homologous to human *THRA* or *THRB* defects in mouse models (Forrest et al., 1996; Rusch et al., 1998; Wikstrom et al., 1998; Abel et al., 1999a; Kaneshige et al., 2000; Al-Shaikh et al., 2001; Kaneshige et al., 2001; Ng et al., 2001a, 2001bbib_Ng_et_al_2001b; Rusch et al., 2001; Tinnikov et al., 2002; Liu et al., 2003; O'Shea et al., 2005; Quignodon et al., 2007; Vennstrom et al., 2008), but many aspects of thyroid hormone action and pathogenesis of RTHα and RTHβ remain unexplained, prompting the development of new animal models, with the potential to provide additional insights over currently available systems.

Although TH action has been studied mainly in mammals and amphibians, previous observations indicate that TH are important in fish development. Despite some anatomic differences, thyroid physiology is highly conserved, with TH levels known to be high in the eggs and larvae of several fish (Power et al., 2001). In zebrafish, almost all of the components of the thyroid axis have been identified, with their structure and function closely resembling those found in higher vertebrates (Porazzi et al., 2009, 2012; Opitz et al., 2011bib_Porazzi_et_al_2012; Heijlen et al., 2013). Additionally, thyroid status is known to be critical regulator of zebrafish embryonic growth and larval transition (Liu and Chan, 2002; Walpita et al., 2007, 2009) as well as neural development (Campinho et al., 2014; Zada et al., 2014).

Due to duplication, the zebrafish genome encodes two TRα genes (*thraa* and *thrab*), together with a single TRβ gene (*thrb*). Zebrafish *thraa* gene, homologous to human *THRA*, generates two TRα1 isoforms that showed high similarity with TRαs form other vertebrates (Darras et al., 2011). The short TRα1 (reported as TRα1A1) isoform has high homology with human TRα1, whereas the long TRα1 (reported as TRα1A1-2) isoform incorporates an additional 14-aminoacid peptide extending its carboxy-terminal "F-domain" that are not found in any other known TR (Darras et al., 2011). It has been reported that the F-domain of the TRα1A1-2 reduces the TR transcriptional activity by altering interaction with the histone acetyl transferase coactivators (Darras et al., 2011; Takayama et al., 2008). Zebrafish TRαB encoded by the *thrab* gene, is structurally similar to the short TRα isoform but lacks a large portion of the N-terminal domain and the correlated transactivation function, and is lost along evolution (Takayama et al., 2008). The TRβ isoforms, transcribed from the zebrafish *thrb* locus, are all able to bind DNA and retain the T3-dependent transactivation activity. The zebrafish TRβ1 has the typical structure of mammalian TRβs including the short N-terminal domain. More recently, a second TRβ1 isoform was identified and differs for the presence of a 9-aminoacid insertion in the ligand-binding domain (LBD) of TRβ1, a feature found in several teleost TRβs but not in the other vertebrates (Darras et al., 2011; Marchand et al., 2001). Consistent to mammals, birds and amphibians, zebrafish has a TRβ2 isoform, with a longer N-terminal, which has been reported to play important functions in mice and zebrafish retina (Jones et al., 2003; Suzuki et al., 2013). The schematic representation of zebrafish TRs (zTRs) is reported in Fig. 1, where we propose a novel nomenclature (zTRα1 short and long, and zTRαB; zTRβ1 short and long, and zTRβ2) in order to avoid possible ambiguity with the different human TRs (hTRs). *Thraa* and *thrb* derived transcripts are both maternally and zygotically expressed since 1-cell stage up to 3 days post-fertilization (dpf) (Power et al., 2001). However, apart from *thraa* expression in brain (Bertrand et al., 2007) and *thrb* expression in retina, mid- and hindbrain, little is known about the tissue distribution and role of TRs during zebrafish development (Bertrand et al., 2007; Kakizawa et al., 2007; Thisse and Thisse, 2008). Interestingly, TRα overexpression was shown to result in a loss of the mid-hindbrain border and in a severe disruption of the rostral hindbrain (Essner et al., 1999, 1997).

Since the zebrafish system enables ready access to all developmental stages and imaging of developing pathologies in real-time, we have determined ontogenic expression profiles of *thraa* and *thrb* in embryonic, early larval stage and adult tissues during zebrafish development. Then, by expressing specific morpholinos, we generated embryos harbouring endogenous DN mutations in either *thraa* or *thrb*, and evaluated the rate of embryonic development and several developmental milestones, changes in HPT axis and TH metabolism in these zebrafish models of human RTHα and RTHβ. Then, we show that zebrafish TRs can efficiently interact with their human homologues and evaluated the ability of wild-type or mutant human TRs to rescue phenotypes in zebrafish models of RTHα and RTHβ.

2. Materials and methods

2.1. Ethical statement

Current Italian national rules: no approval needs to be given for research on zebrafish embryos. Fish were maintained and raised according to EU regulations on laboratory animals (Directive 2010/63/EU).

2.2. Fish line and maintenance

Wild-type adults (AB strain) were obtained from the Wilson lab, University College of London, UK, and maintained in a flow-through system in charcoal-filtered tap water at a constant temperature (28 ± 1 °C), with a photoperiod 14:10 (light:dark). Zebrafish embryos, obtained from natural spawning, were raised and maintained according to established techniques (Fishman et al., 1997) and staged according to morphological criteria (Kimmel et al., 1995). For WISH and IHC experiments, from the 24 h post fertilization (hpf), the embryos were cultured in fish water containing 0.002% of 1-phenyl-2-thiourea (SIGMA) to prevent pigmentation and 0.01% methylene blue to prevent fungal growth. Such a low dose of 1-phenyl-2-thiourea (0.002%) does not affect TH synthesis as the thyroxine concentrations are similar in treated and untreated fish (See Suppl. Fig. 1).

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