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Thyroid development in zebrafish lacking Taz

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ABSTRACT

Taz is a signal-responsive transcriptional coregulator implicated in several biological functions, from chondrogenesis to regulation of organ size. Less well studied, however, is its role in thyroid formation. Here, we explored the in vivo effects on thyroid development of morpholino (MO)-mediated knockdown of *wwtr1*, the gene encoding zebrafish Taz. The *wwtr1* gene is expressed in the thyroid primordium and pharyngeal tissue of developing zebrafish. Compared to mammalian cells, in which Taz promotes expression of thyroid transcription factors and thyroid differentiation genes, *wwtr1* MO injection in zebrafish had little or no effect on the expression of thyroid transcription factors, and differentially altered the expression of thyroid differentiation genes. Analysis of *wwtr1* morphants at later stages of development revealed that the number and the lumen of thyroid follicles, and the number of thyroid follicle cells, were significantly smaller. In addition, Taz-depleted larvae displayed patterning defects in ventral cranial vessels that correlate with lateral displacement of thyroid follicles. These findings indicate that the zebrafish Taz protein is needed for the normal differentiation of the thyroid and are the first to suggest that Taz confers growth advantage to the endocrine gland.

1. Introduction

Taz (transcriptional co-Activator with PDZ binding motif), also referred to as Wwtr1 (WW-domain containing transcription regulator 1), is a transcriptional coactivator highly expressed in kidney, heart, lung, liver, testis, and placenta (Kanai et al., 2000). The Taz protein is characterized by a central WW-domain that mediates protein–protein interaction, followed by a highly conserved C-terminal sequence containing a coiled-coil domain that recruits core components of the transcriptional machinery. In addition, Taz contains a PDZ binding motif at its C-terminus, required for the transcriptional coactivator activity and that promotes Taz nuclear localization to discrete foci, and a 14–3–3 binding motif within the conserved N-terminal portion (Kanai et al., 2000; Kodaka and Hata, 2015). Taz uses different binding partners to activate distinct sets of transcriptional targets, suggesting that it may be involved in several biological processes (Wang et al., 2009; Hong and Guan, 2012; Piccolo et al., 2014). Among Taz functions are the roles in cell migration and proliferation, invasion in breast cancer cells, molecular rheostat in mesenchymal stem cells (MSC) and organ size control (Saucedo and Edgar, 2007; Zhao et al., 2008; Piccolo et al., 2013; Low et al., 2014).

Physical and functional interactions between Taz and Thyroid Transcription Factor-1 (TTF-1, also named T-EBP/Nkx2.1) in the lung epithelial cells have raised the question of whether Taz plays regulatory functions also in the thyroid (Park et al., 2004). The thyroid is one of the largest endocrine glands in mammals and is composed of two cone-like lobes or wings: *lobus dexter* (right lobe) and *lobus sinister* (left lobe) connected through the isthmus. Two types of cells are observed in the differentiated thyroid: the follicular cells producing thyroid hormones T3 (triiodothyronine) and T4 (thyroxine), and the parafollicular cells (also named C-cells) that secrete calcitonin (De Felice and Di Lauro, 2004). The thyroid follicular cell is characterized by the expression of thyroid differentiation markers: thyroglobulin (TG), sodium iodine symporter (NIS, Slc5a5), thyroperoxidase (TPO) and thyroid stimulating hormone (TSH) receptor (TSHR). To date, three transcription factors responsible for the expression of the thyroid differentiation markers have been identified: *TTF-1* (*Nkx2.1*, *nkx2.1a*), *Foxe1* and *Pax8* (Damante et al., 2001). These genes are coexpressed only in thyroid follicular cells. In particular, Pax8 seems to be the most important transcription factor for the differentiation and development of follicular cells; indeed its interaction with TTF-1 is needed to activate *Tg* transcription (Pasca di Magliano et al., 2000; Di Palma et al., 2003; Park et al., 2004). Pax8 and TTF-1 are part of a heterocomplex whose

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molecular mass is greater than the sum of the two factors, suggesting the presence of other proteins. It has been demonstrated that Taz is able to enhance the transcriptional activity of TTF-1 and Pax8 in immortalized thyroid cells. Furthermore, Taz is able to support the activity exerted by TTF-1 and Pax8 on the *Tg* promoter (Di Palma et al., 2009).

In teleosts, the thyroid follicular tissue is loosely dispersed along the ventral midline of the pharyngeal mesenchyme and it is not encapsulated by connective tissue; in addition, thyroid follicular cells and C-cells are restricted to separate organs (Raine and Leatherland, 2000; Raine et al., 2001; Wendl et al., 2002). Despite species-specific variation in thyroid morphology and morphogenetic timing, transcription factors and molecular mechanisms involved in the induction and specification of the gland are conserved between mammals and zebrafish, with nkx2.1a/Nkx2.1 and pax2a/Pax8 acting presumably in the same manner (Wendl et al., 2002; Porazzi et al., 2009; Porreca et al., 2012). Therefore, zebrafish may well contribute to elucidating the molecular basis of thyroid development in higher vertebrates.

Although MO microinjection is a widely used approach in zebrafish, it is becoming increasingly clear that the resulting knockdown phenotype is not frequently observed in the corresponding mutant (Stainier et al., 2015). However, the effectiveness of the morpholino (MO)-based *wwtr1* down-regulation was previously assessed, by demonstrating the absence of the zebrafish Taz homolog in immunoblotting (Hong et al., 2005). It was reported that MO-mediated depletion of zebrafish Taz caused lack of ossification, cardiac edema and renal cysts (Hong et al., 2005; Tian et al., 2007). Histological studies of Taz knockout mice showed cystic kidney but did not support a role in osteogenesis (Hossain et al., 2007). Further, the importance of Taz-related YAP proteins in non-mammalian vertebrates has been revealed by studies in *Xenopus* and zebrafish, where Yap1 appears to regulate tissue growth and regeneration, and to be involved in endoderm formation, nephrogenesis and hair cell differentiation (Jiang et al., 2009; Skouloudaki et al., 2009; Gee et al., 2011; Hu et al., 2013; Hayashi et al., 2014; Loh et al., 2014; Fukui, 2015; He et al., 2015).

Herein, we have used zebrafish to study the role of Taz as a candidate regulator of thyroid development. The expression of the zebrafish *wwtr1* gene is detected in different domains of the embryo including the developing thyroid. Results from whole mount in situ hybridization (WISH), Real-time quantitative PCR (qPCR) and morphometric measurements of *wwtr1* knockdown phenotype indicate that zebrafish Taz is required for the correct size of the thyroid gland. In addition to previous studies showing that Taz is a regulator of organ size (Pan, 2007) and that it promotes the proliferation of rat thyroid follicular FRTL-5 cells (De Cristofaro et al., 2011), this work results raise the possibility that Taz has a central role in thyroid growth. Further, our studies lend support to the concept that blood vessel morphogenesis is important for correct positioning of the thyroid follicles.

2. Results

2.1. Phylogeny and structure of zebrafish Taz

Zebrafish *wwtr1* gene was previously recognized based on sequence alignment and similarity (Hong et al., 2005). The last assembly of the zebrafish genome (GRCz10), as released on September 2014 (GCA_000002035.3), indicated the presence of only one *wwtr1* gene homolog, suggesting that one copy of the gene was lost after the third round of whole genome duplication that occurred at the origin of the teleost clade (3R theory) (Amores et al., 1998) (Fig. 1). Interestingly, two *Wwtr1* paralogous genes were found in the dog genome as a result of gene duplication. Here, tree topology of Taz evolution by Maximum Likelihood mirrored the traditional ordering of vertebrate classes (Fig. 1A). Likewise, phylogenetic relationships among YAP proteins, a subfamily of ancient Taz orthologs of protostome origins, fit well with general assumptions on animal evolution (Fig. 1A). We next examined changes in sequence conservation among Taz proteins using protein alignment by SIM (http://web.expasy.org/sim/) followed by graphical visualization by LalnView (Duret et al., 1996) (Fig. 1B). The evolutionary history of Taz proteins showed deep sequence conservation (>50% identity) as seen in large regions of the zebrafish Taz protein. The percentage of Taz protein identity was very high among mammals, in particular between *P. troglodytes* and *H. sapiens* (Fig. 1B).

2.2. Expression of wwtr1 in the thyroid primordium of zebrafish

Murine *Wwtr1* is expressed in forebrain, hindbrain, somites and in the thyroid primordium during late stages of embryonic development (Di Palma et al., 2009). With the aim to investigate the expression profile of zebrafish *wwtr1*, WISH was performed using an antisense riboprobe against the full length mRNA. The fish *wwtr1* gene was expressed continuously from one-cell stage through 4 days post fertilization (dpf). The message level was moderate and ubiquitously distributed until gastrula stage (Fig. 2A and B), then it increased in dorsal forebrain, hindbrain and adaxial cells (10-somite stage) (Fig. 2C–F). At 24 h post fertilization (hpf), *wwtr1* mRNA was detected again across the entire embryo, with high level in brain, eyes, lateral line primordium and otic vesicles (Fig. 2G–J). From 42 hpf onwards, mRNA labeling was seen in lens, pharyngeal area, pectoral fin mesenchyme and in the thyroid primordium (Fig. 2K). At 48 hpf, a new signal appeared in the cephalic floor plate (Fig. 2L and M). Zebrafish *wwtr1* transcription in the thyroid was further supported by the observation of follicles immunoreactive for thyroglobulin in the pharyngeal domain of *wwtr1* expression (Fig. 2N). At 4 dpf, *wwtr1* expression is detected in brain, liver, gut, pectoral fins (stronger proximal), and cartilage elements of the craniofacial (branchial arches) and neurocranial (ethmoid plate and trabeculae) areas (Fig. 2O–S). At 4 dpf larval stage, *wwtr1* mRNA in the thyroid was no longer detectable by WISH (data not shown).

2.3. Development of the zebrafish thyroid without Taz

Zebrafish embryos were injected at 1–2 cell stage with 1 ng of a MO against *wwtr1* AUG translational start site (Hong et al., 2005; Tian et al., 2007). Control embryos were injected with 1 ng of standard control-MO and found to be similar to uninjected embryos. Until 40 hpf, development of zebrafish *wwtr1* morphants was similar to that of control embryos. As previously shown, they later eventually developed heart edema typical of cardiovascular dysfunction, ventral trunk curvature and smaller head compared to controls (Hong et al., 2005). We examined the development of the thyroid gland in 48 hpf embryos by WISH experiments for thyroid transcription factors *nkx2.1a*, *pax2a* and *pax8*, and for thyroid differentiation genes *tg* and *scl5a5* (Rohr and Concha, 2000; Wendl et al., 2002; Elsalini et al., 2003). The abolition of Taz did not apparently alter the expression of the thyroid transcription factors and of the thyroid differentiation gene *scl5a5* in the thyroid primordium (Fig. 3A–F). Conversely, *tg* mRNA staining was stronger, likely due to the presence of more transcripts in the thyroid cells (Fig. 3G–J). qPCR analysis at 48 hpf partially confirmed WISH findings. Indeed, thyroid transcription factors *nkx2.1a* and *pax8* expression was not altered in *wwtr1* morphants. Conversely, *pax2a* expression was significantly reduced by qPCR, a finding not highlighted by WISH. This apparent divergence between WISH and qPCR results could be due to the wide domain of *pax2a* expression, making not easy to identify a 2-fold down-regulation of the transcript by WISH staining. Concerning thyroid differentiation genes, qPCR confirmed high *tg* expression shown by WISH in *wwtr1* knockdown embryos (Fig. 4). In particular, up-regulation of *scl5a5* likely reflected the ectopic expression seen in WISH experiments (Figs. 3I, J, 4). In addition, qPCR showed a significant up- and down-regulation of the *tpo* and *tshr* genes, which were not analyzed by WISH (Fig. 4). Of note, *tshr*, *tg*, *tpo* and *slc5a5* expression is specific to zebrafish thyroid cells, while *nkx2.1a*, *pax2a* and *pax8*