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THYROID GLAND DEVELOPMENT AND FUNCTION IN THE ZEBRAFISH MODEL

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ABSTRACT

Thyroid development has been intensively studied in the mouse, where it closely recapitulates the human situation. Despite the lack of a compact thyroid gland, the zebrafish thyroid tissue originates from the pharyngeal endoderm and the main genes involved in its patterning and early development are conserved between zebrafish and mammals. In recent years, the zebrafish has become a powerful model not only for developmental biology studies, but also for large-scale genetic analyses and drug screenings, mostly thanks to the ease with which its embryos can be manipulated and to its translucent body, which allows *in vivo* imaging. In this review we will provide an overview of the current knowledge of thyroid gland origin and differentiation in the zebrafish. Moreover, we will consider the action of thyroid hormones and some aspects related to endocrine disruptors.

INTRODUCTION

Despite some anatomical differences between fish and mammals, the hypothalamus-pituitary-thyroid axis is present also in zebrafish. As in other vertebrates, the functional unit of the teleost thyroid is the follicle, which is composed of endoderm-derived thyrocytes. However, the adult zebrafish thyroid consists of individual follicles of variable shape and diameter, lying between the first gill arch and the bulbus arteriosus, along the ventral aorta (Figure 1) (1). These follicles, filled with colloid and not organized in a compact glandular structure, produce thyroid hormones (THs): tri-iodothyronine (T3) and thyroxine (T4) (2-3). The synthesis of THs includes several steps that begin with iodide uptake and iodination of thyroglobulin at specific tyrosine residues in the follicular lumen. After coupling of iodinated tyrosines to generate iodothyronines (T3 and T4), thyroglobulin is internalized in follicular cells where T3 and T4 are removed by enzymatic digestion and are released into the blood stream. THs act by binding to specific nuclear receptors (TRs) and play important roles in embryogenesis and larval development (4-8). In humans, congenital hypothyroidism (CH) is the most common neonatal endocrine disease and may results from impaired thyroid development (thyroid dysgenesis) or defects in TH synthesis (dyshormonogenesis). To date, the pathogenesis of CH is still largely unknown and mutations in genes required for proper thyroid development, mostly identified utilizing murine models, have been found only in few patients. Since the early steps of thyroid development have been characterized in details also in the zebrafish and the main genes and molecular pathways involved in thyroid ontogenesis appear extremely conserved, the zebrafish model represents an additional and powerful tool to investigate new genes and mechanisms that may be responsible for CH.

INDUCTION AND PATTERNING

The formation of the three primary germ layers (ectoderm, mesoderm and endoderm) takes place during early gastrulation of the vertebrate embryo. The inner layer, endoderm, contributes to the digestive tract and the associated organs: liver, pancreas, lung and thyroid. A precise control of endoderm patterning is required for proper organogenesis.

The Nodal signaling pathway.

The onset of endodermal layer development has been investigated in frog, chicken, zebrafish and mouse, revealing the presence of conserved molecular pathways, among which Nodal signaling plays a pivotal role. During embryogenesis, the Nodal pathway is involved in the definition of mesoderm and endoderm from a common territory, the so called *mesendodermal* layer, as well as in positioning of the anterior–posterior axis, neural patterning and left–right axis specification. The *Nodal* gene was initially identified in mice, where it encodes an actvin-like member of the transforming growth factor β (TGF-β) family (9-10). Zebrafish possess two Nodal-related genes: *ndr1* (also known as *squint)* and *ndr2 (*previously named *cyclops)* (11). These ligands bind to a complex of the serine-threonine kinase receptors (*TARAM-A*) and the EGF-CTF co-receptor (*one eyed pinhead*: *oep*) (12-13). The signaling process is mediated by receptor-associated Smads (R-Smad), i.e. Smad2 and/or Smad3, which in turn phosphorylate and form complexes with the common mediator-Smad (co-Smad) and/or Smad4 (14-15). Subsequently, these complexes translocate to the nucleus where they are able to bind, in a sequence-specific manner, the winged-helix transcription factor *foxH1/sur* and the homeodomain protein Mixer *bonnie and clyde (bon)* (16-17). The latter finally induce the expression of MIX-like (*bon*, *mezzo*) and GATA binding protein 5 (gata5 or *fau*) transcription factors (18-19). *Bon, mezzo* and *gata5* act in parallel in a partially redundant manner, upstream of *sox32 (*previously named *casanova or cas)* transcription factors, to specify endodermal development (12). *sox32* is essential to activate the transcription of the endoderm-specific gene *sox17* and the forkhead homebox A2 (*foxa2)* (20*).* The Nodal pathway acts in a long range manner, thanks to the diffusion and stability of its ligands, and in a dose dependent manner (21-22). An example of a dose-dependent response to the level of Nodal activity is the induction of endodermal *vs* mesodermal layer, where the dose of Nodal signals required for endoderm specification is higher than in the case of mesoderm (23-24). Different mechanisms can regulate the spatiotemporal features of Nodal signaling, varying from the control of ligand activity by proteolytic processing to the inhibitory effect of the soluble ligand antagonist *lefty* (24-26).

The Nodal signaling pathway and thyroid development.

The zebrafish thyroid gland derives from precursor cells located in the anterior primitive gut (27). Fate-mapping experiments revealed the different fate of the cells that compose the endodermal layer and highlighted its regionalization, defined, for example, by the expression of specific transcription factors (28). With regard to the zebrafish thyroid, the expression of key transcription factors such as the *haematopoietically expressed homeobox* (*hhex)*, *thyroid transcription factor-1* (*nkx2.1a)* and *paired box DNA-binding domain 2.1 (pax2a)* starts prior to pharynx formation, defining the presumptive region where the primordium will develop at about 26 hpf (1, 29-30). Elsalini and Rohr showed evidence that Nodal signaling, after specifying the endoderm, is essential for the subsequent development of follicular thyroid cells in zebrafish. In *one-eyed pinhead* mutant embryos (*oep*$^{-/-}$*),* the loss of the endoderm is already evident during gastrulation, with an overall failure in the specification of mesendodermal cell fates, leading to the absence of the thyroid primordium (30-31). Furthermore, the thyroid primordium has been analyzed in the *ndr2* mutants *m294*$^{-/-}$, a strain obtained by ENU-mutagenesis. In this background, a strong reduction of pharyngeal endoderm is accompanied by the presence of a smaller thyroid primordium (reduced *nkx2.1a* and *hhex* expression from 26 hpf onward) and eventually by a reduction of the number of functional thyroid follicles, as evidenced by T4 immunostaining at about 5 dpf (30). The thyroid phenotype is more severe in the *ndr2* mutant *b16-/-,* where a gamma-ray-induced mutation caused the loss of the lower telomeric region of chromosome 12, encompassing not only *ndr2,* but also the *hhex* gene (32-33). In this case, an initial reduction of *nkx2.1a* and *pax2a* expression was noticed in the thyroid primordium but, subsequently, both markers disappeared and no thyroid follicles were detectable by T4 immunostaining, confirming the evidence that both a correct endoderm specification and the expression of thyroid-specific transcription factors are required to complete thyroid development (30). Finally, mutants of the downstream effectors of Nodal signaling *bon*, *gata5* and *sox32* completely fail to develop the thyroid primordium, as a consequence of altered endoderm organization and of the loss of the direct inductive role that these factors play in specifying the thyroid primordium (17, 30, 34-35).

Interaction between endoderm and mesoderm regulates thyroid development.

The specification of the gut region and the derived organs also depends on the cross-talk between the endoderm and the mesoderm. Recent researches highlight the importance of permissive signals sent from the mesoderm layer to promote endoderm organogenesis (i.e. during liver and pancreas development) (36-40). A recent work by Wendl and Adzic elucidates how the interaction between the mesoderm and the endoderm is fundamental for proper thyroid primordium specification and development in zebrafish (41). The knowledge of the molecular mechanisms involved in inducing the competence of endodermal cells to become thyroid cells is scarce at the moment. Nevertheless, understanding these processes appears a fundamental step to clarify some aspects of CH. The study of zebrafish *hand2* mutants suggested that, even in the presence of normal endodermal development, the lack of this transcription factor prevents thyroid specification in a non-cell-autonomous manner (41). The *hand2* locus encodes a bHLH transcription factor, named *heart and neural crest derivatives expressed transcript 2*, which is expressed in fin buds, pharyngeal arches and the anterior lateral plate mesoderm. In particular, focusing on the area where the thyroid primordium originates, *hand2* is expressed in the hearth tube, in the first pair of branchial arteries, in the precursor cells of the carotid bodies, in the neural crest mesenchyme of the pharyngeal arches and in the same pharyngeal endoderm, although at low levels (41-44). Zebrafish *hand2* mutants, *hand2*^{s6} and *hand2*^{c99}, besides defective heart, pharynx and fin development, lack a thyroid primordium (failed expression of *pax2a*, *hhex* and *nkx2.1a* at 24hpf) and differentiated follicles (absent T4 immunostaining at 7 dpf). The thyroid phenotypes is more severe in *hand2*^{s6} mutants, which have a deletion of 100 kb around the *hand2* locus and complete absence of the thyroid, than in *hand2*^{c99} mutants, in which the *hand2* gene undergoes an altered splicing, leading just to a reduction of thyroid size (41-42). Utilizing a fate-mapping approach, Wendl and Adzic demonstrated that endodermal thyroid precursor cells originate close to the lateral plate mesoderm and maintain a position proximal to the *hand2*-expressing cardiac mesoderm from somitogenesis onwards (41). It has been demonstrated that this transcription factor acts in a cell-autonomous manner in the tissues surrounding the thyroid primordium and, among them, cardiac mesoderm produces signaling factors responsible for thyroid development. The early association of the thyroid primordium with the aortic sac in the mouse, or the heart outflow tract in the zebrafish, allows

endodermal thyroid precursors to receive permissive signals from the cardiac lateral plate mesoderm (41). To date, the FGF signaling pathway is the principal mediator identified in zebrafish. In fact, zebrafish *fgf8a/ace* mutants show a small thyroid primordium with a reduced number of differentiated follicles and the same thyroid phenotype derives from a complete inhibition of FGF signaling through SU5402 (41, 45-46). Furthermore, beads soaked with recombinant FGF are able to reconstitute a proper thyroid gland in *hand2*^{s6} mutants, proving that FGF has an action parallel to or downstream of *hand2* signaling (41). A link between the FGF signaling pathway and thyroid development has been previously demonstrated also in the mouse. Mice deficient for the *FGF receptor 2-IIIb* or *FGF10* lack an adult thyroid gland, even though an initial thyroid primordium develops (47-49). In addition, Kameda et al. have recently confirming the role of FGF in promoting the development of pharyngeal endoderm derivates (such as the thyroid, ultimobranchial bodies, thymus and parathyroid glands) in the mouse (50). In FRS2α$^{2F/2F}$ murine mutant embryos, which lack the docking protein FRS2α that links FGF receptors to a variety of intracellular signaling pathways, thyroid development is severely affected and the gland, despite the formation of the thyroid diverticulum, is, in the end, absent or hypoplastic (50). Furthermore, a connection between thyroid and cardiac development is supported by the finding that infants with CH have an increased risk of additional congenital malformations (from about 1-2% to 8-10%), cardiac abnormalities representing at least half of them (51). Moreover, ectopic thyroid tissues can be found in the heart (52-53). The frequent association of thyroid and heart abnormalities stresses the importance of physical and molecular contacts between the thyroid anlage and the aortic sac. Recently, the T-box transcription factor *Tbx1* emerged as an actor in this process. Tbx1 null mutations cause thyroid hemiagenesia. Indeed, Tbx1 is required for normal development of the aortic arch and is expressed in the mesenchyme surrounding the thyroid, defining thyroid size and its final position by non cell-autonomous mechanisms (54). Tbx1, via interaction with FGF genes, has an important role in the development of the pharyngeal apparatus and the secondary heart field from which the cardiac outflow tract derives (55-56). These recent findings may explain the increased risk of thyroid dysfunction in patients affected with the Di George syndrome (57), which is characterized by a number of phenotypic features including cardiovascular defects, and is caused by 22q11 deletions that encompass the Tbx1 gene (58-59).

THYROID GLAND DEVELOPMENT AND DIFFERENTIATION IN ZEBRAFISH

During the last ten years the research on the origin of the zebrafish thyroid gland has received a considerable impulse. A detailed description of the key molecular steps of thyroid differentiation has been obtained through an integrated approach, based on cloning and gene expression studies, immunostaining, mutant fish line analysis, morphological studies and fate-mapping. In zebrafish, after specification, the growing thyroid pouch relocates and descends until it reaches a species-specific final destination, where terminal differentiation is achieved. The finding that the main signaling pathways involved in thyroid development are conserved between fish and mammals makes the zebrafish an excellent molecular tool for identifying new genes involved in early thyroid development and possibly, in the pathogenesis of CH.

Four main transcription factors, essential for thyroid primordium development, have been isolated in zebrafish: *nkx2.1a*, *hhex*, *pax2a* and *pax8*.

Thyroid transcription factor 1a: nkx2.1a.

Historically, the first transcription factor described in the zebrafish developing thyroid gland was nkx2.1a (29). *nkx2.1a* is a member of the homeodomain transcription factor family, expressed in the thyroid, lung and ventral forebrain of higher vertebrates (60-61). Zebrafish possesses two *nkx2.1* genes, *nkx2.1a* and *nkx2.1b,* evolved according to a duplication-degeneration-complementation model, but only *nkx2.1a* is expressed in the thyroid (62). *Nkx2.1* plays an important role in mammal thyroid development; indeed, null mouse embryos initially develop the thyroid primordium (E10) but it subsequently disappears (E10.5-11), supporting the role of this transcription factor in the maintenance and survival of thyroid precursor cells (49, 63). In the zebrafish**,** a domain of *nkx2.1a* expression appears at 24 hpf, in a region of the midline endoderm near to the heart tube (Figure 2A). Photoactivation of endodermal cells close to the heart primordium revealed that the cells expressing *nkx2.1a* follow the development of the lower jaw and compose the region corresponding to the thyroid bud (29). *nkx2.1a* morpholino knock-down results in failure of thyroid development at 5 dpf (absent T4 immunostaining). Morpholino-injected embryos show

the early appearance of the thyroid primordium expressing *hhex* and *pax2a* specific transcription factors, although the signal is weak and disappear**s** by 60 hpf, thus recalling the phenotype of *Nkx2.1* knockout mice (30-63). These data suggest that *nkx2.1a* is not involved in early specification of zebrafish thyroid precursor cells, but is required for primordium maintenance and perhaps subsequent growth and differentiation.

Haematopoietically expressed homeobox: *hhex.*

Hhex is a critical transcription factor involved in many aspects of vertebrate development, such as the formation of endoderm-derived organs: thyroid, pancreas, liver, lung, thymus and gallbladder (64-65). Mouse *Hhex* null embryos exhibit defects in rostral forebrain and liver, as well as thyroid dysplasia. Thyroid primordium is aplastic or hypolastic at E10.5, and is no longer detectable at E13.5 (66). These data suggest a very early function of *Hhex* in thyroid development. In zebrafish, the orthologous gene *hhex* is initially expressed in the anterior endoderm (67-68). Subsequently (from 22 hpf onward), *hhex* is present in thyroid precursor cells, colocalizing with the expression domain of *nkx2.1a* (1). Similar to *nkx2.1a* morphants, the morpholino knock-down of *hhex* gene also results in a lack of follicles and T4 immunostaining at 5 dpf. The early steps of thyroid primordium evagination and relocalization are not affected, as demonstrated by the initial presence of *nkx2.1a* and *pax2a* expression at the base of the lower jaw. However, the expression of this marker is lost at 60 hpf, similarly to *Hhex* knockout mice (30, 66). On the other hand, injecting zebrafish with *hhex* mRNA results in a gain-of-function phenotype, characterized by an increase in the number of thyroid precursor cells and precocious antero-posterior expansion (30). All together, these studies in zebrafish and mice emphasize the role that *hhex* gene plays after induction and evagination of the thyroid primordium, when it appears to control thyroid differentiation and, perhaps, growth.

The paired box genes pax2a and pax8.

The *Pax* (paired box DNA-binding domain) gene family includes nine transcription factors, important for tissue and cellular development, differentiation and proliferation. The family is divided into four groups (I-IV) based on the presence of different protein structure domains.

The group II comprises the paralogous genes *Pax2/5/8* involved in vertebrate thyroid development (69-70). In mammals, *Pax8*, besides being expressed in the thyroid, is also present in the spinal cord, midbrain-hindbrain boundary and kidney. *Pax8* knockout mice have a severe phenotype and die shortly after weaving. This is principally a consequence of thyroid defects, as other organs expressing *Pax8* have normal development, probably due to the redundant function of the paralogous genes *Pax2* and *Pax5* in these tissues. With regard to the thyroid gland, *Pax8* knockout mice initially develop a thyroid primordium, which disappears soon after evagination (E11.5-E12.0), suggesting a role for *Pax8* in the maintenance and/or proliferation of thyroid precursor cells (71). Zebrafish *pax8*, the homologous of mammalian *Pax8*, is expressed in the midbrain-hindbrain boundary region, in the eye, pronephros and nephric ducts, and in the thyroid (72). Here, its expression starts at 28 hpf, later than *nkx2.1a* and *hhex*, overlapping their domain in the thyroid primordium until 7 dpf (1, 29).

A detailed expression analysis of *pax2/5/8* paralogs in the zebrafish reveals that *pax2a* is also expressed in the thyroid primordium (1). Due to gene duplications in teleosts, the zebrafish possesses two *pax2* genes, named *pax2a* and *pax2b*. *pax2a* shows an expression pattern similar to *pax8*, and is closer than *pax2b* to mammalian *Pax2*. Neither *pax2b* nor *pax5* are expressed in the zebrafish thyroid (1, 72). The expression of *pax2a* in thyroid precursor cells starts at 24 hpf, as for *nkx2.1a* and *hhex*, before the appearance of *pax8* (Figure 2B). *pax2a* expression is detectable until 7 dpf, completely overlapping the signal of *nkx2.1a*, *hhex* and *pax8*, and subsequently labeling a small group of cells lying close to the ventral aorta (1). *noi*^{tu29} (*no isthmus*) zebrafish mutants enable the definition of the role of *pax2a* in thyroid development, since they carry a *pax2a* null allele and survive until 9-10 dpf (73). Mutants develop the thyroid primordium and express *nkx2.1a*, *hhex* and *pax2a* until 30 hpf, when the expression of these genes ceases. At 7-8 dpf *pax8* expression and T4 immunostaining are absent in *noi*^{tu29} mutants. Even in this circumstance, the described expression pattern implies an initial thyroid primordium development in absence of *pax2a*. However, the functionality of this gene is fundamental for the proper development of thyroid follicles (1). The finding that the phenotype of *noi* ^{tu29} zebrafish is similar to that of *Pax8* knockout mice, suggest that zebrafish *pax2a,* acting upstream of *pax8*, plays a similar role in thyroid development (71).

Zebrafish thyroid terminal differentiation and growth.

In addition to the transcription factors directly involved in primordium specification, differentiation markers are equally important in determining and assessing the full development and terminal differentiation of an organ. Among the known markers, *thyroglobulin (tg)* and *sodium iodine symporter (slc5a5)* have been analyzed so far. *tg* mRNA is selectively present in the thyroid starting from 32 hpf (Figure 2C) (74). Moreover, the TG precursor protein, iodinated to produce THs and stored in the follicular colloid, is present in a first single follicular structure at 55 hpf, and , later on, in a row of follicles along the pharyngeal midline. The expression of *Slc5a5*, the basolateral follicular transporter responsible for iodine uptake from the bloodstream, is detectable from 40 hpf (Figure 2D) (74).

The polarization of the follicular structure takes place at 55 hpf, preceding the massive growth of follicles along the ventral midline (74). A transplantation approach based on the induction of endodermal fate with *Tar** mRNA, combined with the biotin-dextran tracer and thyroid specific markers, suggests a model where the thyroid develops from a first anterior follicle from which isolated cells migrate posteriorly to generate the remaining follicles (74).

Like in mice, thyroid early growth, polarization and development in zebrafish appear to be independent of the thyroid-stimulating hormone (TSH) (74-76). TSH, secreted by the pituitary, binds to its receptor (*Tshr*) and regulates thyroid growth and differentiation at late developmental stages, but is not responsible for organogenesis or cell migration (75-76). In zebrafish, the mutant *lia*^{t24149}*,* lacking FGF3 signals, does not develop thyrotrope progenitors secreting TSH (77). In spite of that, it is possible to observe follicles producing T4, even if they do not elongate like in the sibling counterpart (74).

The major difference between zebrafish and mammals lies in the timing and site of TH storage. The functionality of hormone-producing follicles can be assessed by means of T4 immunostaining (2-3). In zebrafish there are different T4 immuno-positive sites. An anterior domain (usually termed T4 non-follicular domain) is localized at the second branchial arch and is not shaped like a characteristic follicle. In this area T4 labeling starts from 80 hpf, with an increasing number of T4 positive cells at 96 hpf, but there is no coexpression of the transcription factors *nkx2.1a*, *hhex*, *pax2a*. Moreover, morphants or mutants for these transcription factors do not display any impairment in the structure of the non-follicular region; it has been also demonstrated that treatment of embryos with goitrogens (chemical disruptors of TH synthesis) does not reduce T4 immunoreactivity at this level (1, 30). These findings exclude this region as a site of TH biosynthesis and rather suggest that it may represent a store of maternal T4. A more posterior follicular region producing T4 appears at 96 hpf along the ventral aorta, and the number of T4 producing follicles increases thereafter. In the latter region some key thyroid transcription factors (*nkx2.1a, hhex, pax2a*) are expressed, and *nkx2.1a* and *hhex* morphants or *noi* ^{tu29} mutants do not develop functional T4 producing follicles in this area (1, 30, 78). Zebrafish embryos are characterized by external fertilization and development; they receive both early feeding and maternal hormones through the yolk. Just after hatching**,** the yolk is gradually reabsorbed and the process is completed by 5 dpf when the larvae are self-feeding and producing hormones. Sustained T4 production at 96 hpf is able to counteract the diminished pool of maternal T4. In mammals there is a different timing of T4 production during embryo development as the supply of maternal hormone persists till the birth and the self-production of T4 starts only after a period of intense proliferation of thyroid precursor cells.

Zebrafish ultimobranchial bodies.

In mammals, the thyroid primordium merges, during its relocalization in the cervical region, with the ultimobranchial bodies to form the mature thyroid gland, which contains both follicular cells and calcitonin-producing C cells (49). The former derive from the thyroid anlage, while C cells originate from the neural crest and, after fusing, are interspersed in the interfollicular space (79-80). In fish, amphibians and birds, the ultimobranchial bodies form an independent organ that does not fuse with the thyroid (81-83). In zebrafish, the ultimobranchial bodies, which express the calcitonin related polypeptide gene (*calca*), assume by 60 hpf the shape of two groups of cells on either side of the heart, i.e. close to the muscular component of the gut and distant from the thyroid primordium (74). Differently from mammals, *nkx2.1a* is not present in zebrafish ultimobranchial bodies, which in the adult fish remain as two separate groups of cells at the transverse septum close to the sinous venosus (74, 79).

The vascular contribution to thyroid relocalization.

The final position and shape of an organ depends on interactions with the surrounding tissues, often raising from different primitive germ layers. Vascular contribution is an important factor in defining the correct localization and morphology of different organs. The cardiovascular system starts its development just before the budding of major primordia such as those of the liver and pancreas (84-85). It is now well known that the human thyroid primordium is close to the aortic sac soon after budding and during the relocalization process follows the development of carotid arteries, to end up with a bilobed gland located in front of the trachea (80). The frequent association of CH with congenital heart and thyroid malformations and the description of patients with intracardiac ectopic thyroid tissue confirm the close relationship between thyroid and vascular development (51-53). In zebrafish, the thyroid primordium expands and migrates posteriorly before dividing into scattered singular follicles along the pharyngeal midline (74). Double *in situ* hybridization experiments show the close association among thyroid primordium, ventral aorta and the first pair of branchial arteries at 55 hpf, and the development of the thyroid primordium along the extension of the ventral aorta at 120 hpf (86). Fish with alterations of pharyngeal vessel architecture (*vegfaa* and *tal1* morphants or *kdrl*^{y17} mutants) display severe thyroid defects, such as abnormal lateral expansion and misalignment of thyroid follicles, pointing to a correlation between thyroid morphology and vascular development (86). The same consequences are highlighted by the Shh-deficient mouse, in which defective cardiac rotation and asymmetric carotid artery development lead to a single-lobed thyroid gland adjacent to the delocalized carotid arteries (86-87). In addition, the creation of a mosaic of wild-type and ectopic endothelial-induced cells emphasize the role played by endothelial cells in zebrafish thyroid morphogenesis in a non-cell-autonomous manner (86). Altogether, these data support the hypothesis that, despite species-specific anatomical variations, the ventral aorta serves as a guide for follicular cell relocalization during thyroid development.

ROLE OF THYROID HORMONE ACTION IN ZEBRAFISH DEVELOPMENT

THs play fundamental roles in regulating development, differentiation and metabolism of all vertebrates (7, 88). Though their effects have been more thoroughly investigated in mammals and in amphibians, where they are required for metamorphosis, there is increasing evidence that they may play important roles also in fish development (7, 89). Indeed, high concentrations of TH of maternal origin have been measured by RIA in the eggs and larvae of several fish species (7). Moreover, exogenous TH induces premature differentiation of the zebrafish pectoral fins, which are analogous to the forelimbs of tetrapods, while goitrogens have negative and often opposite effects on zebrafish development. In particular, they inhibit the development of scales and pigment pattern, and impair the growth of both pectoral and pelvic fins (6). Finally, the proper activation of TH at tissue level, mediated by the action of the type 2 iodothyronine deiodinase (D2), is essential for TH dependent development in vertebrates. In zebrafish, it has been recently demonstrated that D2, expressed from early embryonic stages, plays a pivotal role in producing active T3, allowing an adequate availability of local and systemic T3 (90). D2 morpholino knock-down zebrafish embryos have a significant delay in development with a reduction in the otic vesicle length, head-trunk angle and pigmentation index (90).

More recently, with the introduction of molecular biology techniques, it was possible to clone the genes encoding for TRs in different fish species. The first TRs cloned in fish were from the Japanese flounder, where four different receptor transcripts, two of which corresponding to TRα and two to TRβ, were identified (91-92). Evidence suggests that the two flounder TRβ transcripts arise from a single gene and are the result of differential splicing, while two genes are present for the two flounder TRα transcripts (91). This is in contrast to the situation in mammals and chicken in which two genes, one encoding TRα and the other TRβ have been identified, and additional receptor transcripts are produced by differential splicing (93-95). The existence of two types of TRs in fish has been confirmed by subsequent cloning of TRs in other species, including zebrafish (4-5). The high level of conservation between mammals, birds and fish — sequence homology is as high as 90-95% in the domains responsible for TH binding or DNA interaction — suggests that the TRs in all these vertebrates probably bind THs, undergo dimerization and regulate gene transcription in a similar fashion.

Both TRα and TRβ mRNA were found to be present in zebrafish embryos and to respond to exogenous TH (4-8). Zebrafish genome contains one gene encoding TRß (*thrb*) and two genes encoding TRα (*thraa* and *thrab*). In addition, *thraa* gene expresses two isoforms by differential splicing (TRαA1 and TRαA1-2). However, the two types of receptor have a different temporal expression, being TRα expressed at earlier stages than TRβ (96). This finding suggests that, like in mammals, TRα and TRβ may have different functions. Most experimental work into the activity of TRs during zebrafish development has been conducted on TRα (4, 97). These important studies show that TRα overexpression results in a loss of the midbrain-hindbrain border and a severe disruption of the rostral hindbrain (96). Thus, like in mammals, THs seem to play an essential role in brain development.

Moreover, the expression of zebrafish TRαA1, TRαA1-2 and TRαB has been characterized and appears to be regulated in a stage- and tissue-specific manner (97). In fact, TRαA1 transcripts are only present in unfertilized eggs, testes and ovaries, whereas TRαA1-2 and TRαB expression levels increase during embryonic development and have an ubiquitous expression in adult tissues, with the highest expression in the eye and liver, respectively (97).

While these studies have begun to elucidate the role of TRα, little information is so far available on the specific function of TRβ. Recently, TH regulation of mRNAs encoding TRα and TRβ has been performed in the teleost fathead minnow (*Pimephales promelas*) (98). Fish fed on a T3-containing diet increase the levels of expression for TRα and TRβ in the liver and brain, while in the ovary and testis exogenous T3 elevated only the transcription of TRβ (98). Future studies will be needed to better define the temporal and tissue-specific pattern of TRs expression, to ultimately clarify their roles in zebrafish development and to highlight the similarities and differences with their mammal homologues.

Finally, in addition to the conventional TH signaling mechanism, which involves T3 binding to the specific nuclear receptor, emerging evidence supports a direct role for T4 in mediating very fast, non-genomic actions of THs. The zebrafish model has been recently employed to confirm previous data, obtained from *in vitro* studies, highlighting the role of T4 in interacting with αVβ3 integrin to initiate these rapid effects (99). In particular, thanks to this kind of mechanism, maternal T4 is able to rapidly regulates the sodium currents and the neural signaling in zebrafish early embryonic stages, promoting the development of the nervous system (99).

THYROID GLAND AND ENDOCRINE DISRUPTORS

The negative impact of pollutants on the well being of both humans and wildlife are creating an increasing public concern (100). A wide variety of health disorders have been clearly linked to the exposure to environmental contaminants which act as endocrine disrupting chemicals (EDCs) (101-102). EDCs are "exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (103). In particular, thyroid-disrupting compounds, affecting either the morphogenesis or the function of the thyroid gland (TH synthesis or metabolism), may have detrimental effects on both development and metabolism and several researchers have tried to address questions concerning EDCs by exploiting the zebrafish model.

Ammonium perchlorate is a strong oxidizer used in the manufacture of rocket propellants, fireworks, vehicle airbags and other industrial items (104). Perchlorate ions derived from ammonium perchlorate and other perchlorate salts are stable and mobile in water and can persist in the environment for many years. Increasing reports of the presence of perchlorate in ground- and surface-water sites have led to concerns about its potential effects on biotic resources and human health (105).

Perchlorate is well known to interfere with thyroid function as it competitively inhibits the uptake of iodide by thyroid follicles, thus inhibiting the production of THs (106). The perchlorate-induced reduction of TH production causes an increased secretion of TSH by the pituitary, which in turn stimulates the abnormal growth of the thyroid gland. Typical effects such as lowering of endogenous T4 and/or T3 levels and thyroidal hypertrophy are also observed in fish (107). Frequently, a reduction of growth rate and developmental retardation have been reported. The effects on reproduction or sexual differentiation observed in zebrafish during perchlorate administration are likely consequences of the inhibition of TH

synthesis (108). These results also highlight the need to consider the thyroid system in studies of gonadal and reproductive dysfunction caused by EDCs.

Arsenic induces oxidative stress in animals by modifying the antioxidant capacity. Davey et al. recently showed that arsenic can interfere with T3 action at TR level (109). Liu F et al. used zebrafish to demonstrate that hypothyroid fish were more sensitive to arsenic (110).

Perfluorooctanesulfonate (PFOS) is a persistent organic pollutant, the potential toxicity of which is causing great concern. Perfluorinated chemicals (PFCs) have been used in a variety of commercial and industrial applications, resulting in their global distribution and detection in the environment, wildlife and humans. PFOS causes growth defects and other severe developmental abnormalities in zebrafish embryos (111). PFOS was reported to induce thyroid gland differentiation at early developmental stages in zebrafish (111).

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in a wide number of synthetic applications. Losses at production sites and leaching from landfills have resulted in progressive contamination of the aquatic environment with predominant accumulation of lower pentabrominated diphenylether mixtures (PeBDE) in aquatic organisms (112). Reported effects of PBDE-exposure include modulation of the thyroid and sex steroid endocrine systems. A number of widespread tetra- and PeBDEs and commercial PeBDE showed competitive binding to both human and fish transthyretin, a major plasma thyroid hormone binding protein *in vitro* (113- 114). In zebrafish, in contrast with rodents, PBDEs produced a significant dose-dependent increase in circulating T3 and T4 levels (115).

It has recently been reported that the elimination of some pharmaceutical compounds during wastewater treatment processes is rather inefficient, and, as a result, they are found in surface-, ground- and drinking-waters (116). Fibrates are among the most frequently reported pharmaceuticals in waste- and surface-water (117). Clofibrate-exposed zebrafish larvae had disrupted thyroid gland morphogenesis associated with an impairment of ventral aorta development. Interestingly, clofibrate-exposed larvae with stronger phenotypes also had lethargic behavior as a likely consequence of hypothyroidism (118).

CONCLUSIONS

In this review we attempted to summarize the most relevant findings concerning thyroid development, TH action and thyroid disrupting chemicals that have been obtained in zebrafish. All these data highlight the great potential of this model to investigate thyroid development and the pathogenesis of CH. The current knowledge in these fields is mostly based on studies performed in rodents, but the zebrafish model can represent a valuable and powerful alternative for novel studies aimed to solve still unanswered questions concerning the organogenesis and the role of thyroid gland in vertebrate development.

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Figure legends:

Figure 1: **The thyroid of a juvenile zebrafish (20 dpf).** Thyroglobulin (TG; green) and DAPI (blue) immunofluorescence. Longitudinal section, ventral view, anterior to the left. TG labels the follicular lumen, showing a row of distinct follicles along the ventral midline (arrowhead). DAPI staining allows to appreciate the cartilaginous structures of the ceratohyale (CH), the first and the second branchial arches (cb1; cb2: ceratobranchial), basibranchial (bb), hyoid symplectic (hs) and interhyale (ih) cartilagines.

Figure 2: **Examples of marker gene expression in zebrafish thyroid primordium.** (A-B) In situ hybridization with probes specific for *nkx2.1a* (A) and *pax2a* (B) in 24 hpf zebrafish embryos. (C) In situ hybridization for *tg* in a 36 hpf embryo. (D) In siyu hybridization for *slc5a5 in a* 48 hpf larva. All images are lateral views, with anterior to the left. Bottom-right inserts display enlargements of the thyroid region. Abbreviations: T: thyroid; e; eye, mhb: midbrain-hindbrain boundary; hy: hypothalamus.

Table 1: **Comparison of thyroid development in zebrafish, mouse and humans.**
The relevant events are listed in the first column. The developmental stage is indicated as *hour post fertilization* (hpf) in zebrafish, and *day post fertilization* (dpf) in mouse and humans. These information are based on Rhor, 2000; Wendl 2002; Elsalini 2003; Alt, 2006; Macchia, 1999; De Felice and Di Lauro, 2004, Fagman, 2006.

Stage of morphogenesis	Embryonic hpf or days		
	zebrafish	mouse	humans
Thyroid early marker gene expression	24-26 hpf	E8–8.5	E20–22
Primordium at pharingeal ventral midline	32 hpf	E8–8.5	E20–22
Thyroid budding and migration	36 hpf to 45 hpf	E9.5 to E13.5	E24 to E45-50
Thyroid cells proliferation starts	72 hpf	E10.5	E24
Fusion with ultimobranchial bodies	/	E14	E45-E50
Onset of folliculogenesis	55 hpf (one first follicle)	E15.5 (many follicles)	E70 (many follilces)
Onset of T4 production	72 hpf	E15.5	E77

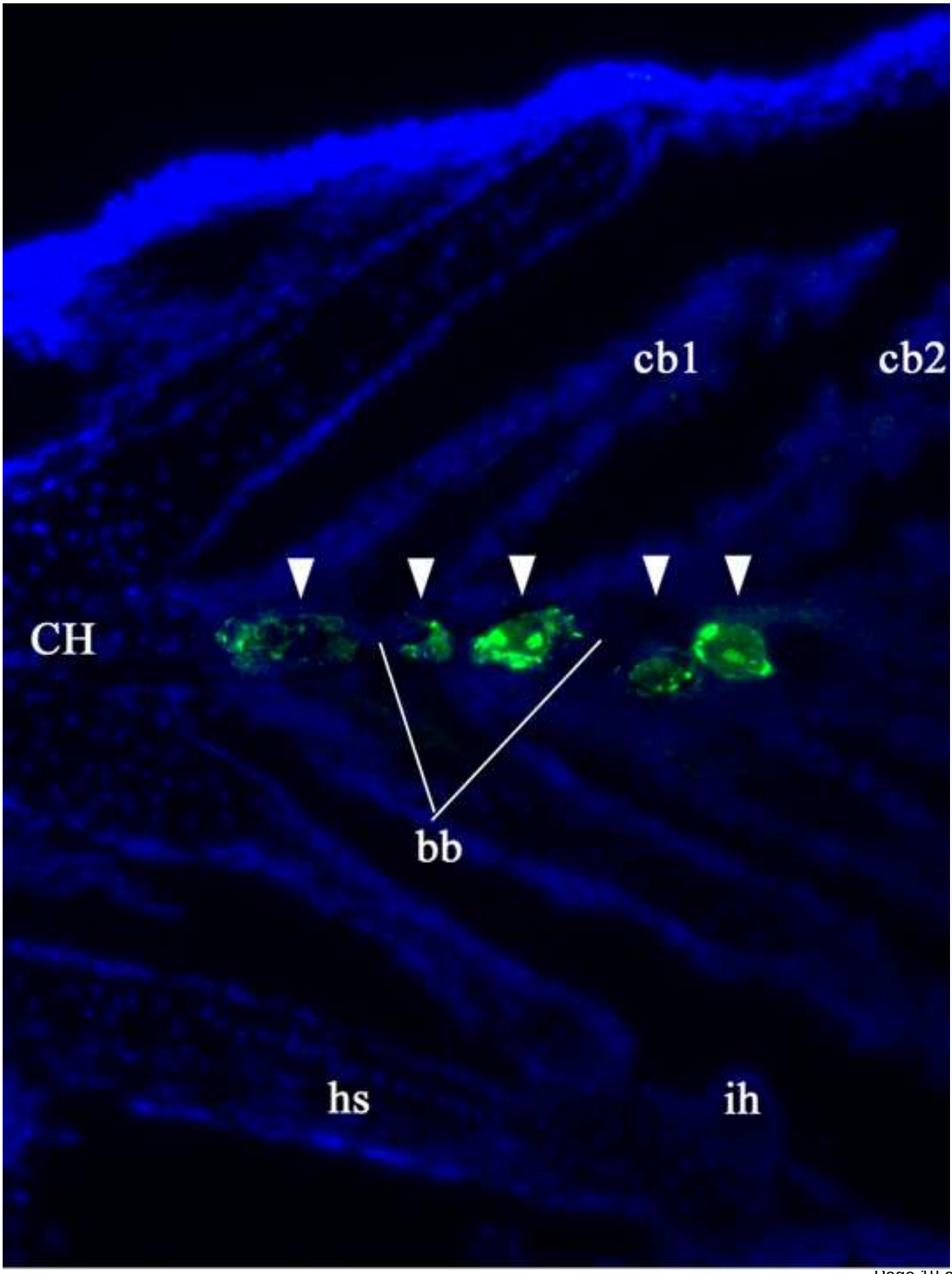
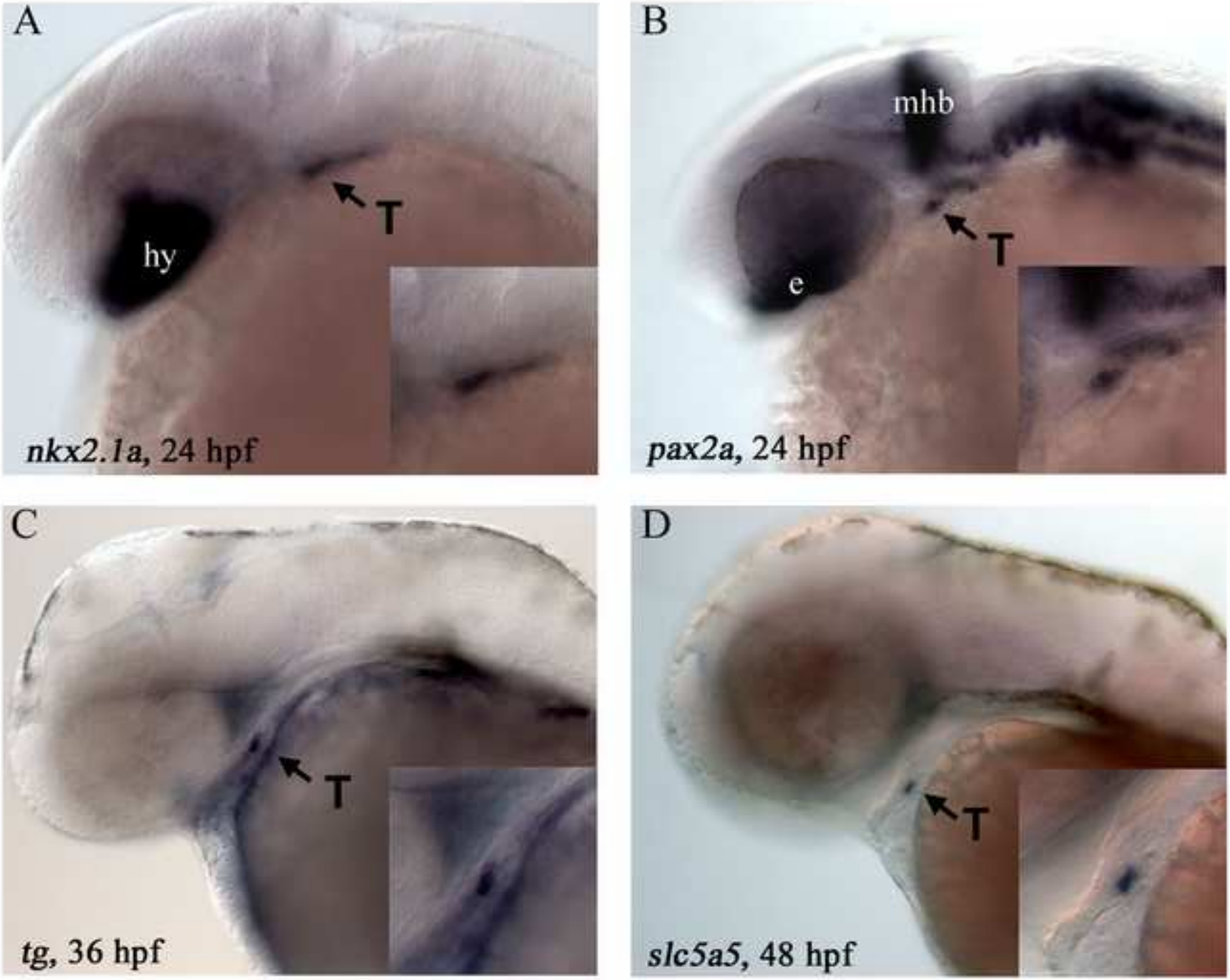


Figure 2


A
hy
T
nkx2.1a, 24 hpf
B
mhb
e
T
pax2a, 24 hpf
C
T
tg, 36 hpf
D
T
slc5a5, 48 hpf