

DEVELOPMENT AND DISEASE

pax2.1 is required for the development of thyroid follicles in zebrafish

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SUMMARY

The thyroid gland is an organ primarily composed of endoderm-derived follicular cells. Although disturbed embryonic development of the thyroid gland leads to congenital hypothyroidism in humans and mammals, the underlying principles of thyroid organogenesis are largely unknown. In this study, we introduce zebrafish as a model to investigate the molecular and genetic mechanisms that control thyroid development. Marker gene expression suggests that the molecular pathways of early thyroid development are essentially conserved between fish and mammals. However during larval stages, we find both conserved and divergent features of development compared with mammals. A major difference is that in fish, we find evidence for hormone production not only in thyroid follicular cells, but also in an anterior non-follicular group of cells.

We show that *pax2.1* and *pax8*, members of the zebrafish

pax2/5/8 paralogue group, are expressed in the thyroid primordium. Whereas in mice, only *Pax8* has a function during thyroid development, analysis of the zebrafish *pax2.1* mutant *no isthmus* (*noi*^{-/-}) demonstrates that *pax2.1* has a role comparable with mouse *Pax8* in differentiation of the thyroid follicular cells. Early steps of thyroid development are normal in *noi*^{-/-}, but later expression of molecular markers is lost and the formation of follicles fails. Interestingly, the anterior non-follicular site of thyroid hormone production is not affected in *noi*^{-/-}. Thus, in zebrafish, some remaining thyroid hormone synthesis takes place independent of the pathway leading to thyroid follicle formation. We suggest that the *noi*^{-/-} mutant serves as a new zebrafish model for hypothyroidism.

Key words: Hypothyroidism, Thyroid, *Pax2*, *Pax8*, *Nkx2.1*, *TTF1*, Evolution, Zebrafish, Mouse

INTRODUCTION

The thyroid gland is responsible for producing thyroid hormone in all vertebrates. Although thyroid hormone is best known for its role in regulating metabolism in the adult organism, it is also required during development for many processes. The thyroid gland is primarily composed of a single cell type, the thyroid follicular cell (Gorbman and Bern, 1962). These cells form colloid-filled follicles, produce thyroid hormone, store it in the colloid-filled lumen of the follicle and control the release of the hormone from the colloid into the blood stream.

Thyroid hormone production starts with the synthesis of thyroglobulin. Thyroglobulin is then secreted into the colloidal lumen of the follicle where tyrosine residues are iodinated and where it is condensed to produce tri- (T3) and tetra-iodinated thyronine (T4, thyroxine) (Frieden and Lipner, 1971). T3 and T4 remain covalently bound to thyroglobulin as long as they

are stored in the colloid. The bound forms of T3 and T4 are eventually taken up by the follicular cells and proteolytically separated from the thyroglobulin. Free T3 and T4 are then released and act as thyroid hormone.

During development, the thyroid follicular cells derive from the endoderm (Noden, 1991; Walker and Liem, 1994). Thyroid development is classically subdivided into a few distinguishable steps, primarily based on observations in mammals: first, a group of cells buds off the floor of the primitive pharynx. Second, these cells reposition dorsocaudally to reach the anterior wall of the trachea. Third, the precursor cells proliferate and, fourth, differentiate into thyroid follicular cells (Macchia, 2000). In addition, other cells, including neural crest derived C cells, merge with the group of endoderm-derived precursor cells (Manley and Capecci, 1998).

In humans, any disorder of the thyroid that leads to reduced thyroxine production at birth is called congenital hypothyroidism (reviewed by Macchia, 2000). On the one

hand, defects in genes responsible for thyroxine production can account for congenital hypothyroidism. On the other hand, such a phenotype can also be the result of earlier defects in development, such as mis-specification or mis-localisation of thyroid follicular cells. To date, work on mammalian model systems and on human diseases has revealed very few genes that are required for the development of thyroid follicular cells.

One of the few genes known to play an essential role in thyroid development is the transcription factor *Nkx2.1* (or *Ttf1*, thyroid transcription factor 1). *Nkx2.1* is expressed in the developing thyroid of mammals (Lazzaro et al., 1991), and mice that lack the *Nkx2.1* wild-type allele do not develop a thyroid gland (Kimura et al., 1996). The complete absence of any thyroid tissue, including the C-cells that normally invade the tissue, suggests a relatively early role for *Nkx2.1* in organogenesis (Kimura et al., 1996).

Pax genes, transcription factors that contain a DNA-binding paired domain, are involved in many aspects of organogenesis (reviewed by Dahl et al., 1997). *Pax8* is involved in thyroid development of mammals (Macchia et al., 1998; Mansouri et al., 1998), but in contrast to the *Nkx2.1*^{-/-} phenotype, *Pax8*^{-/-} mice still develop a thyroid gland (Mansouri et al., 1998). However, the remaining gland is much smaller than in wild type, lacks the endoderm-derived thyroid follicular cells and is composed of neural crest-derived C cells only. Hence, *Pax8* is required for late specification or differentiation of the follicular cells.

In mice, targeted or chemically induced mutations of *Pax2* result in various abnormalities that reflect the sites of its expression in midbrain, eye, ear and kidney (Favor et al., 1996; Torres et al., 1995). In zebrafish, mutations in the *pax2.1* gene lead to the *no isthmus* (*noi*^{-/-}) phenotype, which shows defects comparable with the *Pax2* mutations in mice (Brand et al., 1996; Lun and Brand, 1998; Macdonald et al., 1997; Majumdar et al., 2000). In this study, we introduce zebrafish as a model organism for thyroid development. We further demonstrate that in zebrafish *pax2.1* plays a role in thyroid development that is not conserved in mice.

MATERIALS AND METHODS

Animals

Zebrafish work was carried out according to standard procedures (Westerfield, 2000), and staging in hours post fertilisation (hpf) or days post fertilisation (dpf) refers to development at 28.5–29°C. Embryos or larvae were dechorionated manually and anaesthetised in tricaine before fixation. Some zebrafish embryos used for in situ hybridisation were treated with PTU to prevent pigmentation at young stages. As PTU belongs to a class of chemicals that have the potential to interfere with the function of the thyroid, we did not treat embryos used for immunohistochemistry with PTU and confirmed all in-situ hybridisation experiments with non-treated embryos.

Wild-type and *Pax2* heterozygous mice were mated and pregnant females later dissected at 9 dpc, 10 dpc, 11 dpc, 13.5 dpc, 14 dpc and 15 dpc. Homozygous mutant *Pax2* embryos were identified by genotyping at flanking microsatellite loci using PCR.

Preparation of specimens

Fixation of fish and mouse embryos was done overnight in 4% paraformaldehyde in PBS at 4°C for whole-mount in situ hybridisation, or in Bouin's solution at room temperature for histology and histochemistry. Paraformaldehyde fixed embryos were

then washed in PBS and stored in methanol at -20°C. Bouin fixed tissue was transferred to 70% ethanol and stored at room temperature.

Histological methods

Paraffin embedding was carried out according to standard procedures, and 8 µm sections were cut. For histology, sections were dewaxed, dried and stained. Sections of adult and larval zebrafish were stained with Giemsa, Haematoxylin and Eosin, and PAS staining in order to visualise the thyroid follicles. Sections of homozygous *Pax2* mutant mouse embryos and their littermates were stained with HE in order to compare size and shape of the thyroid gland. Giemsa stain involved 5 minutes incubation in Giemsa solution as used for blood stain, followed by two washes in tap water. Haematoxylin and Eosin staining was carried out according to Ehrlich (Fluka, catalogue number 03972): 5 minutes incubation, followed by 10 minutes washing in running tap water. PAS staining was carried out according to the manufacturer's instructions (Merck, catalogue number 1.01646). All stained slides were dehydrated and mounted in Entellan neu (Merck).

Cryosections

Mouse embryos and zebrafish larvae were fixed overnight in 4% paraformaldehyde (in phosphate buffer, PB), rinsed in PB and equilibrated in 10% and 20% sucrose in PB at 4°C over 48 hours. They were embedded in OCT compound (Agar, UK), frozen on dry ice and cut serially at 20 µm in coronal and sagittal planes. Sections were collected on Superfrost slides (BDH, UK) and stored at -70°C in sealed boxes.

Whole-mount in situ hybridisation for zebrafish and mouse embryos was carried out according to standard procedures (Westerfield, 2000). For double staining, we used Fast Red or INT (Sigma) as a second substrate.

In situ hybridisation on cryostat sections

Sections were air-dried at room temperature for 20 minutes to 3 hours and post-fixed with 4% paraformaldehyde in phosphate buffer containing 0.1 M NaCl (PBS) for 20 minutes. After three washes in PBS, sections were acetylated and incubated in 50% formamide/3×SSC. Riboprobes were diluted to 100 ng/ml in warm (60°C) hybridisation solution, (50% formamide, 5×SSC, 10 mM β-mercaptoethanol, 10% dextran sulphate, 2×Denhardt's solution, 250 µg/ml yeast tRNA, 500 µg/ml heat inactivated salmon sperm DNA). Hybridisation was carried out in a humid chamber at 58°C for 16 hours. Slides were rinsed in 50% formamide, 2×SSC at 58°C, treated with RNase A and RNase T1 at room temperature, rinsed twice with 50% formamide/2×SSC at 58°C and incubated with anti-digoxigenin antibody 1:2000. After development of the colour reaction, slides were dehydrated and mounted in DPX (BDH). We placed adjacent sections on different slides in order to analyse co-expression of molecular markers in thyroid tissue.

Immunohistochemistry

Dewaxed sections were treated with 3% H₂O₂ in methanol for 10 minutes in order to block endogenous peroxidases. Blocking and all antibody dilutions were done in 3% normal goat serum in PBT. All steps were carried out at room temperature, wash steps alternatively overnight at 4°C. As a first antibody, we used 1:4000 rabbit anti-thyroxine BSA serum (ICN biomedical, catalogue number 65-850). As a secondary antibody, we used a goat anti rabbit antibody 1:200 that is part of the elite ABC kit (Vectastain). The staining procedure followed general protocols using the Vectastain elite ABC kit (Westerfield, 2000). All experiments involving immunohistochemistry were repeated at least three times independently, and are based on *n*=4 adults, *n*>30 wild-type embryos/early larvae, and *n*=10 for both mutant and wild-type embryo siblings, respectively.

RESULTS

Adult zebrafish have a teleost-type thyroid that differs from the mammalian thyroid gland

The thyroid of teleost fishes is organised as thyroid follicles as in other vertebrates, and these follicles are presumed to produce thyroid hormone in the same way (Leatherland, 1994; Rauther, 1940). In contrast to higher vertebrates, however, the thyroid of many teleosts is not a compact single organ. Most teleost species that have been investigated have thyroid follicles loosely distributed within the mesenchyme of the ventral head area, mainly in the vicinity of the anterior aorta. Nevertheless, comparative data are scarce and there is wide variation in teleost thyroid morphology from one set of bilateral nuclei of follicles in medaka (Raine et al., 2001) to dense groups of follicles throughout the gill region in trout (Raine and Leatherland, 2000).

In order to understand development of the thyroid in zebrafish, and to compare the thyroid of zebrafish with that of other species, we first analysed the appearance of thyroid tissue in adult zebrafish. In Haematoxylin and Eosin, and Giemsa stained sections, colloid filled follicles are detected along the ventral aorta, in the ventral midline of the gill chamber (Fig. 1A-D). Periodic acid/Schiff (PAS) staining, which detects glycols and can be used to stain thyroid colloid, results in a positive staining of the follicles (Fig. 1E). The follicles appear alone or in loose aggregations of two or three embedded in connective tissue. Their shape is irregular, and all different staining methods visualise vesicles at the apical outer surface of the follicular cells (Fig. 1D,E, arrowheads). The follicles in adult zebrafish vary in diameter from 14 μm to 140 μm . Series of sections of adult zebrafish reveal that follicles are restricted in their distribution to the ventral aorta in the gill area only (Fig. 1G).

To confirm the identity of the follicles as thyroid tissue, we made use of an antibody against T4 (thyroxine). T4 is a derivative of thyronine and can normally not be detected in situ, as free T4 is highly soluble in water and alcohol. However, in thyroid tissue it is bound to thyroglobulin, and the T4 antibody has been proven to detect the bound form of T4 selectively in thyroid follicular cells of fish (Raine and Leatherland, 2000; Raine et al., 2001). By

immunostaining for T4, we find that the follicles distributed along the anterior aorta give a strong signal, confirming their identity as thyroid follicles (Fig. 1F). As described for trout and medaka (Raine et al., 2001), the follicular epithelium shows stronger immunoreactivity than the colloid.

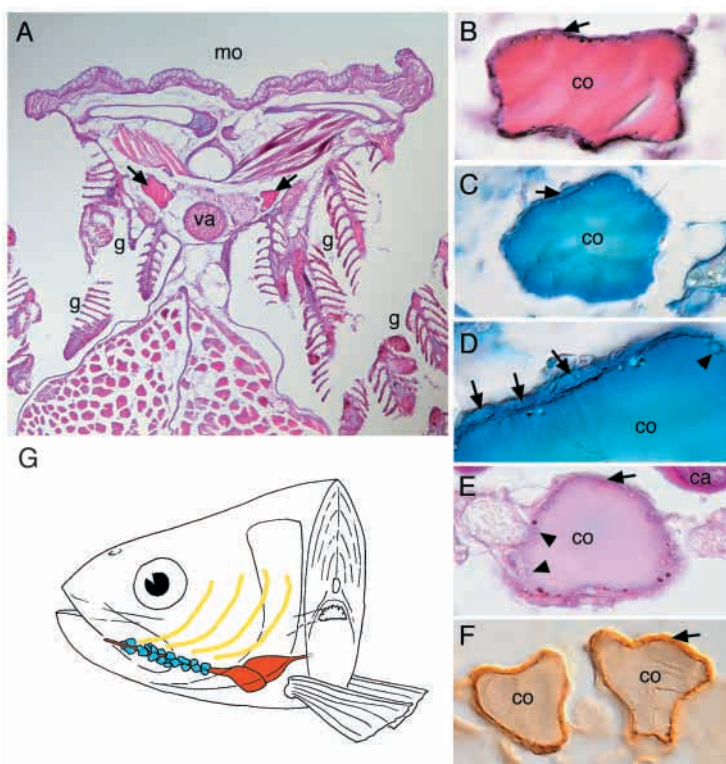
In summary, our results demonstrate that the zebrafish thyroid gland is composed of follicles that are dispersed as described in other teleosts. In zebrafish, all follicles are found close to the ventral aorta, from the first gill arch to the bulbus arteriosus.

T4 immunoreactivity indicates an early function for the larval thyroid

Thyroid hormone is required for many developmental processes and is normally provided maternally during early development, either as a maternal contribution to the yolk in fish or by maternal blood supply in mammals. To determine the onset of thyroid function in the zebrafish embryo or larva, we carried out T4 immunostaining at various stages.

We detect weak T4 immunostaining from about 80 hpf onwards in a group of cells at the base of the lower jaw, anterior to the heart. At 96 hpf, the number of T4-positive cells and the strength of the signal has increased (Fig. 2A-F). The first T4 positive cells to appear are not organised as follicles (Fig. 2C-F, H). However by 96 hpf, small T4-positive follicles appear more caudally along the anterior aorta (Fig. 2G,I). At 5 dpf, we find three to five follicles; at 7 dpf, we find six or seven follicles (see Fig. 4I,K below). Larval follicles show a colloid-filled lumen and vesicles at the apical surface of the follicular cells like thyroid follicles in adult zebrafish. They are generally round or tube-like in shape at this stage, and their size is only 5-10 μm in diameter (Fig. 2I). Tube-like follicles are oriented longitudinally along the

Fig. 1. Reconstruction of the thyroid gland in adult zebrafish. (A) Cross-section of an adult zebrafish head in the ventral gill region at the level of the second branchial arch (Haematoxylin and Eosin staining). The arrows indicate the thyroid follicles. (B-F) High magnification views of thyroid follicles of adult zebrafish. (B) Haematoxylin and Eosin staining. The arrow shows the follicular epithelium. (C,D) Giemsa staining. The arrows show the follicular epithelium in C and the nuclei of the epithelium in D, the arrowhead indicates a vesicle at the apical surface of the follicular cells. (E) PAS staining visualising the follicular epithelium (arrow) in medium pink and the colloid in light pink; the arrowheads indicate vesicles at the apical surface of the follicular cells. Cartilage is stained strongly. (F) Immunostaining with an antibody against T4 reveals strong reactivity of the follicular cells (arrow). (G) Reconstruction of the distribution of thyroid follicles in the adult zebrafish head. Thyroid follicles in blue, heart and ventral aorta in red, gill arches in yellow. ca, cartilage; co, colloid; g, gills; mo, mouth/pharynx; va, ventral aorta.



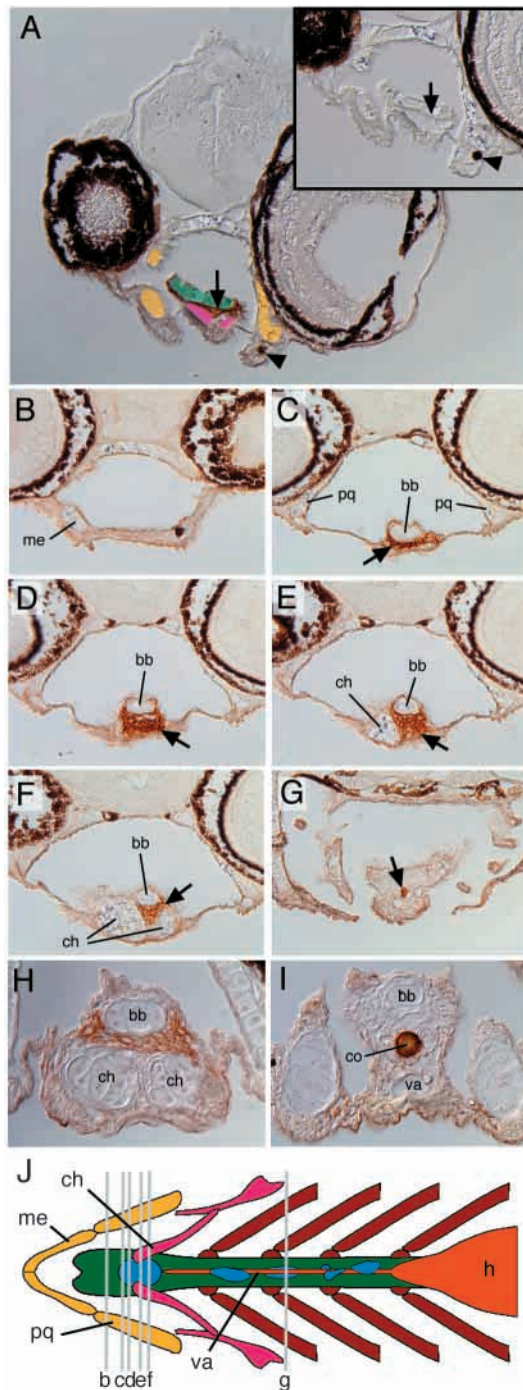


Fig. 2. T4 immunostaining in the early (4.5-5 dpf) zebrafish larva. (A-I) Selected cross-sections at 4.5 dpf, T4 immunostained (H.I, high magnification). (A) Overview. Cross-section through the head at an anteroposterior level between D and E (compare with schematic drawing in J). Cartilage is highlighted by colours as in J (yellow, first branchial arch; magenta, second branchial arch; green, basibranchial cartilage that is composed of derivatives of all branchial arches). The arrow shows immunostaining in the anterior non-follicular domain, the arrowhead a pigment cell. Insert indicates adjacent control section, processed for immunostaining without first antibody. Note absence of immunostaining (arrow). The arrowhead indicates the same pigment cell as in the adjacent section. (B-G) Selected sections from one embryo at different anteroposterior levels as indicated in J. Arrows indicate immunostaining in the thyroid. (H) Close up of the anterior non-follicular T4 domain. (I) Close up showing a follicle further posterior. (J) Schematic drawing of a zebrafish larval head of 4.5 to 6 dpf, ventral view, showing the skeleton, parts of the circulatory system and the thyroid. The approximate positions of the sections shown in B-G are indicated as grey bars. Blue, thyroid/T4 immunostaining; brown, third to sixth branchial arches; green, basibranchial cartilage; magenta, second branchial arch; orange, heart and ventral aorta; yellow, first branchial arch. bb, basibranchial cartilage; ch, ceratohyale; co, colloid; h, heart; me, Meckel's cartilage; pq, palatoquadrate; va, ventral aorta.

that develop in the zebrafish head from 50 hpf onwards (Fig. 2A,J) (Schilling et al., 1996). In sections of various stages (4 to 11 dpf) the anterior non-follicular T4 domain as well as the later appearing follicles are all located ventral to the basibranchial cartilage (Fig. 2H,I). The anterior T4 domain is localised where the anterior ends of the second branchial arch (the hyoid arch) meet ventral to the basibranchial cartilage (Fig. 2C-F,H). This T4 domain retains its position relative to the second branchial arch throughout the stages investigated (up to 11 dpf). The later appearing follicles are found further posterior (Fig. 2J), along the ventral aorta, in a similar pattern to that described for other teleost species (Rauther, 1940).

Shared expression of molecular markers in the developing thyroid of zebrafish and mammals

Although the adult zebrafish thyroid differs in its overall structure from that in higher vertebrates, morphological data suggest that the thyroid develops in the same way in all vertebrates, including fish (Rauther, 1940). Initially, cells of endodermal origin bud from the ventral midline of the pharyngeal epithelium. These cells then migrate to reach a final position in the neck of higher vertebrates, or in the ventral head region in fish. Zebrafish *nk2.1a*, an orthologue of mouse *Nkx2.1* and human *TTF1*, respectively, is an early marker that labels thyroid precursor cells during zebrafish development from about 24 hpf onwards (see Fig. 5C) (Rohr and Concha, 2000). We asked whether other genes that are expressed in the thyroid of mammals are also expressed in the thyroid of zebrafish.

In mouse embryos that lack the *Hex* gene, development of the thyroid primordium arrests early and markers like *Nkx2.1* fail to be expressed (Martinez Barbera et al., 2000). During mouse development, *Hex* is expressed in early anterior endoderm and later in the developing thyroid (Thomas et al., 1998). In zebrafish, the corresponding orthologue *hhex* starts to be expressed in the anterior endoderm as in mice (Ho et al., 1999; Liao et al., 2000). We find that from about 22 hpf through

ventral aorta and are up to 40 μ m in length. In general, immunostaining is stronger in the follicles than in the anterior domain of T4 localisation.

The early onset of T4 production shortly after hatching reflects the fast development of zebrafish. It is generally assumed that the yolk contains maternally provided thyroid hormone (reviewed by Leatherland, 1994). The larval thyroid begins to function as the yolk sac in the free swimming larva diminishes and the supply of maternally derived thyroid hormone is used up.

In order to localise the T4 immunostaining precisely, we determined its relationship to the well characterised cartilages

all stages examined (up to 96 hpf) it is also expressed in the precursors of the thyroid (Fig. 3A,D,G). Double in situ hybridisation reveals that *hhex* and *nk2.1a* expression is completely overlapping in the thyroid primordium (Fig. 3D).

The transcription factor *Pax8* is expressed in the developing thyroid of mammals (Plachov et al., 1990; Poleev et al., 1992) and is required for differentiation of thyroid follicular cells in mice (Mansouri et al., 1998). In zebrafish, *pax8* is expressed at similar sites as in mammals: in the eyes, in the midbrain hindbrain-boundary and in the pronephric ducts (Pfeffer et al., 1998). In addition, we find that from about 28 hpf, *pax8* is expressed in the developing thyroid of zebrafish (Fig. 3E,H, see also Fig. 5I below). It is expressed throughout thyroid development (tested up to 7 dpf), and double in situ hybridisation shows that *hhex* and *pax8* are expressed in the same set of thyroid precursor cells (Fig. 3E). In contrast to *hhex* (Fig. 3A) and *nk2.1a* (Fig. 5C) (Rohr and Concha, 2000), *pax8* is not expressed in the thyroid primordium at 24 hpf (Fig. 3B).

Expression of these molecular markers reveals that at around 96 hpf the single, round primordium disperses as small cell clusters along the ventral aorta (Fig. 3J). No marker gene expression was detected at the level of the second pharyngeal arch, where the non-follicular T4 domain is located (Fig. 3J). This result from whole-mount in situ hybridisation was confirmed by in situ hybridisation on cryosections. Here, small groups of cells expressing *pax8* are found only caudal to the second branchial arch, and anterior to the bulbus arteriosus (Fig. 3K). Localisation, shape and size of these sites of marker gene expression strongly suggest that they correspond to thyroid follicles as visualised by T4 immunostaining. Thus, as judged from marker gene expression, the thyroid primordium gives rise to the follicles, but not necessarily to the anterior non-follicular domain of T4 localisation.

In contrast to *pax8*, *hhex* and *nk2.1a*, we were not able to detect the zebrafish-specific paralogue *nk2.1b* (Rohr et al., 2001) in the developing thyroid. However, of all presently available zebrafish homologues to thyroid-specific genes in mammals we find at least one paralogue that is expressed in the zebrafish thyroid. Thus, common marker gene expression suggests that the basic mechanisms of thyroid development are conserved between mammals and fish.

In zebrafish, *pax2.1* in addition to *pax8* is expressed in the developing thyroid

We re-analysed the expression of other zebrafish Pax genes and found that *pax2.1* is also expressed in the thyroid primordium (Fig. 3C,F,I). Expression of *pax2.1* starts at around 24 hpf (Fig. 3C), when *pax8* expression is not yet detectable (Fig. 3B), and continues throughout development. Double in situ hybridisation shows that after onset of *pax8* expression, *pax2.1* and *pax8* overlap completely in the thyroid primordium (Fig.

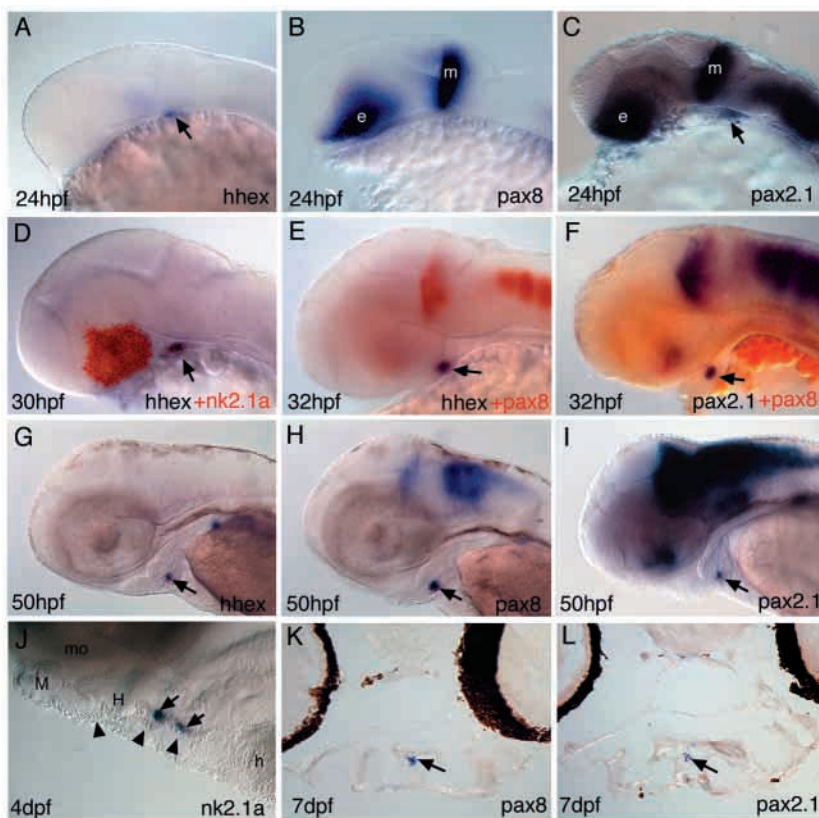


Fig. 3. Marker gene expression in the zebrafish thyroid primordium. (A,D,G) *hhex* (A,G) or *hhex* plus *nk2.1a* (D) expression in the thyroid (arrows). (B,E,H) *pax8* (B, H) or *pax8* plus *hhex* (E) expression in the thyroid (arrows). (C,F,I) *pax2.1* (C,I) or *pax2.1* plus *pax8* (F) expression in the thyroid (arrows). (J) Lower jaw area of 4 dpf wild-type larva, lateral view. Arrows indicate the *nk2.1a* expression in patches along the ventral aorta (arrowheads). (K) Cryosection of a 7 dpf wild-type larva, showing *pax8* (arrow) expression in the area of a thyroid follicle. (L) Cryosection of a 7 dpf wild-type larva, showing *pax2.1* expression (arrow) in the area of a thyroid follicle. The position of the sections in K,L corresponds roughly to the right arrow in J. e, Eye; h, heart; H, cartilage of the hyoid arch (second branchial arch); M, cartilage of the mandibular arch (first branchial arch); m, Pax gene expression in the midbrain-hindbrain boundary; mo, mouth cavity/pharynx.

3F). At 7 dpf, *pax2.1* is expressed in small groups of cells along the ventral aorta (Fig. 3L), in a pattern suggesting that *pax2.1* is expressed in the thyroid follicles like *pax8* (Fig. 3K) and *nk2.1a* (Fig. 3J). Like these other markers, *pax2.1* expression cannot be found in the area of the second branchial arch, where the non-follicular domain of T4 immunostaining is located.

In contrast to *pax2.1*, its zebrafish-specific paralogue *pax2.2* is not expressed in the thyroid or in its primordium (data not shown). As *pax2*, *pax5* and *pax8* form a closely related family of Pax genes (Pfeffer et al., 1998) we also tested whether *pax5* is expressed in the thyroid, but we were not able to detect any staining in the area of the thyroid at the stages tested (from 20 hpf to 4 dpf). Hence, in addition to *pax8*, the zebrafish thyroid expresses *pax2.1* as a second member of the *pax2/5/8* paralogue group.

Thyroid follicles are absent in *pax2.1* mutant zebrafish embryos

As *pax2.1* is expressed in the developing thyroid of zebrafish, we asked whether the lack of Pax2.1 activity leads to a thyroid-

specific phenotype. Alleles of the zebrafish *no isthmus* (*noi*) mutant carry mutations in the *pax2.1* locus, and homozygous carriers of the null allele, *noi^{tu29}*, survive up to 9–10 dpf (Lun and Brand, 1998). In 8 dpf larvae stained with HE or PAS, colloid filled thyroid follicles are clearly visible in wild type (Fig. 4A,C), but no follicles could be found in 8dpf *noi^{tu29}* homozygous larvae (Fig. 4B,D). Furthermore, we analysed thyroid function in *noi* homozygous mutant embryos by testing 7 and 8 dpf embryos for T4 immunoreactivity. All homozygous mutant embryos showed immunostaining in the anterior non-follicular domain at the level of the second pharyngeal arch essentially as in their wild-type siblings (Fig. 4E–H). Minor changes in the shape of the domain are probably a consequence of the heart oedema that develops in the mutant and that might influence jaw morphology (Fig. 4B,D,J,L). However, *noi^{tu29}* embryos completely lack T4-positive follicles (Fig. 4J,L), whereas five to seven follicles were present in all wild-type siblings of the same age (Fig. 4I,K).

We therefore conclude that *noi/pax2.1* is not required for the presence of T4 in the cells at the level of the second pharyngeal arch, but it is required for the formation of colloid-filled follicles and for their expression of T4.

We next asked whether the missing thyroid follicles in *noi^{tu29}* are reflected by marker gene expression at earlier stages. In *noi^{tu29}* embryos expression of the early markers *hhx* and *nk2.1a* is clearly visible around 24 hpf (Fig. 5A–D). Whereas *hhx* expression appears indistinguishable (Fig. 5A,B), expression of *nk2.1a* is reduced compared with wild type (Fig. 5C,D). At around 30 hpf, *hhx* expression is lost in most *noi^{tu29}* embryos or strongly reduced (Fig. 5E,F) and subsequently lost. *nk2.1a* expression is completely lost in *noi^{tu29}* by 30 hpf (Fig. 5G,H). We did not detect any *pax8* expression in *noi^{tu29}* at any stage (Fig. 5I,J). Analysis of marker gene expression in the weaker allele *noi^{th44}* and the hypomorph *noi^{tb21}* (Lun and Brand, 1998) yielded essentially the same results, with weak *hhx* expression persisting slightly longer in *noi^{tb21}* (data not shown).

We further processed *noi^{-/-}* embryos for *pax2.1* in situ staining and found early *pax2.1* expression in the thyroid primordium up to 30 hpf (Fig. 5K,L). However, at stages later than 30 hpf, *pax2.1* expression is no longer detectable in *noi^{-/-}* embryos (Fig. 5M,N).

In summary, *hhx*, *nk2.1a* and *pax2.1* expression in *noi^{-/-}*

embryos demonstrates that a thyroid primordium forms in the absence of *pax2.1*. However, the loss of expression of these markers at later stages and the absence of *pax8* expression indicates that *pax2.1* is required for the proper development of the thyroid, confirming our results with T4 immunostaining.

Pax2 does not play a role in thyroid development of mice

Murine Pax mutations frequently result in disturbed organogenesis related to the corresponding sites of Pax gene

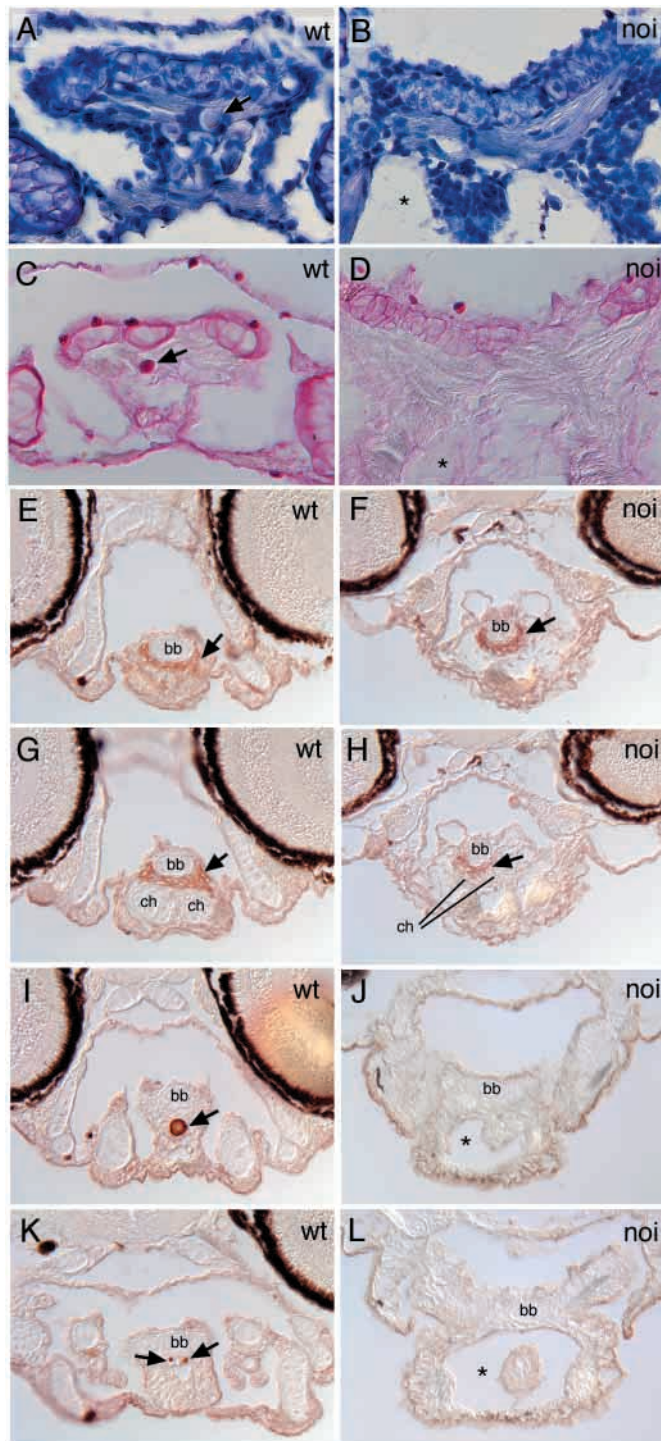


Fig. 4. Histological analysis and T4 immunostaining in wild-type and *noi^{tu29}* mutant zebrafish larvae at 7–8 dpf. (A,B) Haematoxylin and Eosin staining: selected section of a wild-type sibling (A) showing a thyroid follicle and *noi^{tu29}* larva (B), in the area where normally follicles would form. (C,D) PAS staining: selected section of a wild-type sibling (C) and *noi^{tu29}* larva (D), in the area where normally follicles would form. (E–L) T4 immunostaining. (E,G,I,K) Selected sections of a wild-type sibling with the anterior domain of T4 localisation (E,G) and three follicles out of a total of seven follicles present in this specimen (I,K). The follicles are distributed along the ventral aorta, ventral to the basibranchial cartilage (compare with Fig. 2J). (F,H,J,L) Four selected sections of a 7 dpf *noi^{tu29}* larva, showing the presence of the anterior domain of T4 localisation (F,H). No follicles were found in any of the *noi^{-/-}* mutants (J,L). Arrows point to follicles and/or T4 immunostaining, the asterisks mark the heart oedema that is forming in *noi^{tu29}*. bb, basibranchial cartilage; ch, ceratohyale.

expression (Dahl et al., 1997). For example, *Pax2* is expressed in the kidney, and correspondingly *Pax2*^{-/-} mice lack this organ (Favor et al., 1996; Torres et al., 1995).

We tested if *Pax2* plays a role in mouse thyroid development in addition to *Pax8*. The thyroid primordium develops at mouse stages E9 to E15, when genes including *Hex*, *Nkx2.1* and *Pax8* are expressed (Keng et al., 1998; Lazzaro et al., 1991; Plachov

et al., 1990). We re-analysed *Pax2* expression in wild-type embryos of different stages from E8 to E15.5 and did not detect any *Pax2* expression within the thyroid tissue (Fig. 6A-F). In addition, we analysed the thyroid of the mouse *Pax2* mutant alleles *Pax2*^{1Neu} (Favor et al., 1996) and ENU5042 (Favor and Neuhauser-Klaus, 2000) histologically. In all *Pax2*^{-/-} mouse embryos, the thyroid is normal in size and shape at an age of E11, and also at E15 when the neighbouring parathyroid glands appear (data not shown). Thus, *Pax2* in mice apparently does not function in thyroid development as it does in zebrafish.

Humans with renal-coloboma syndrome develop optic nerve colobomas and various degrees of renal abnormalities. Individuals suffering from this syndrome are heterozygous for a frame-shift mutation in the *PAX2* locus that is identical to *Pax2*^{1Neu} (Favor et al., 1996; Porteous et al., 2000). A thyroid phenotype has not been reported for *PAX2* mutations in humans, further supporting the notion that *Pax2/PAX2* plays no role in mammalian thyroid development.

DISCUSSION

The developing thyroid in zebrafish reveals conserved and divergent features compared with higher vertebrates

As in other vertebrates, the basic unit of the zebrafish thyroid is the thyroid follicle (Leatherland, 1994; Rauther, 1940). However, in contrast to most other vertebrates, the thyroid follicles of zebrafish are not organised as one compact gland encapsulated by connective tissue. Instead, they are distributed along the ventral aorta in the gill region as reported for many other teleosts. Despite this difference in morphology, the thyroid follicles develop in comparable areas

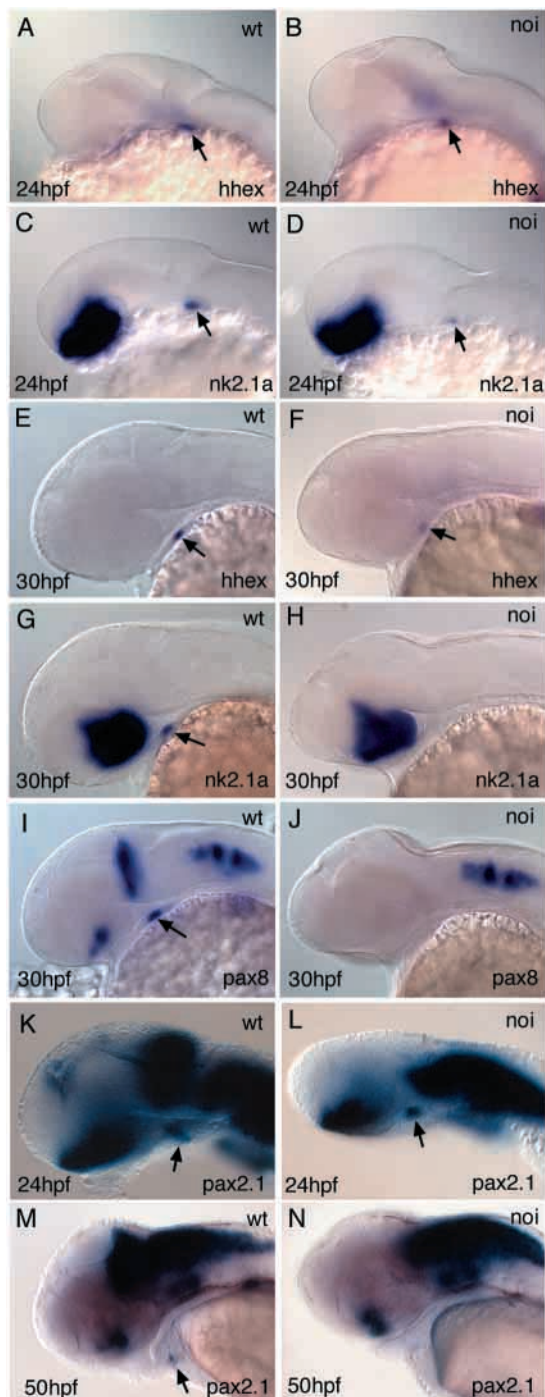


Fig. 5. Expression of molecular markers in *noi*^{-/-} mutant and sibling embryos. (A,B,E,F) *hhx* expression, (C,D,G,H) *nk2.1a* expression, (I,J) *pax8* expression, (K-N) *pax2.1* expression. Embryo age is indicated in the bottom left-hand corner. The arrows indicate the thyroid primordium.

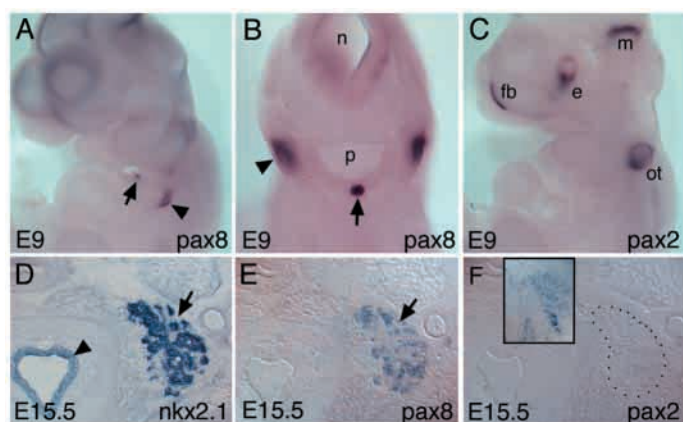


Fig. 6. Expression of *Pax2* in wild-type mouse embryos. (A-C) E9 embryos, whole-mount in situ hybridisation. (A,B) *Pax8* expression. Arrows show thyroid primordium, arrowheads indicate *Pax8* expression in the visceral arches. (A) Lateral view, (B) Cross-section at the level of the thyroid primordium. (C) *Pax2* expression, lateral view as in A. (D-F) Neighbouring sections of E15.5 wild-type embryo. (D) *Nkx2.1* expression, arrow shows thyroid gland, arrowhead *Nkx2.1* expression in the tracheal epithelium. (E) *Pax8* expression (arrow shows thyroid gland), (F) *Pax2* expression (insert shows positive staining in the CNS on the same section as a positive control). e, *Pax2* expression in the eye; fb, *Pax2* expression in the forebrain; m, *Pax2* expression in the midbrain; n, neural tube; ot, otic vesicle; p, pharynx.

in the embryos of zebrafish and higher vertebrates. In the latter, the thyroid primordium originates from the pharyngeal epithelium at the position of the second branchial arch (Noden, 1991; Shain et al., 1972). From this initial position, the thyroid primordium migrates to its final location in the neck. In zebrafish embryos, early thyroid marker gene expression can be found in the vicinity of tyrosine hydroxylase-positive cells (Rohr and Concha, 2000). These cells are associated with the second branchial arch (called 'arch associated cells') (Guo et al., 1999). Thus, the zebrafish thyroid primordium develops from the pharyngeal epithelium at the same level as in higher vertebrates, as has been suggested by classical morphological data for teleosts in general (Rauther, 1940). Furthermore, our data on expression of molecular markers suggests similar genetic regulation of development of the thyroid primordium.

Immunostaining reveals two sites of T4 localisation in the zebrafish larva: an anterior non-follicular domain at the level of the second branchial arch and more posterior dispersed groups of cells that form the common vertebrate-type of colloid filled thyroid follicles. From studies in other vertebrates, only follicular cells have been described to produce thyroid hormone. Although suggestive, T4 immunostaining does not prove that the non-follicular cells of the anterior domain actually produce T4. An alternative is that T4 may be produced elsewhere and subsequently becomes localised in these cells. It is, however, likely that the anterior domain produces T4 itself, as the first follicle only becomes apparent a few hours after T4 is detected in the anterior domain, and we have no indication that other tissues might produce T4.

Marker gene expression in *noi*^{-/-} suggests a pathway of transcription factors in thyroid development of zebrafish

noi^{-/-} mutant embryos are deficient in Pax2.1 activity (Brand et al., 1996; Lun and Brand, 1998). Corresponding to numerous sites of expression of *pax2.1*, *noi*^{-/-} mutants show abnormal development of the midbrain-hindbrain boundary, the kidneys and other structures, reminiscent of the phenotype of Pax2 deficiency in mice and humans (Favor and Neuhauser-Klaus, 2000; Favor et al., 1996; Macdonald et al., 1997; Porteous et al., 2000; Torres et al., 1995). In the present study, we find that zebrafish *pax2.1* is also expressed in the developing thyroid, and that *noi*^{-/-} embryos show a phenotype in this organ.

The missing thyroid follicles of *noi*^{-/-} embryos are reflected at earlier stages by a loss of marker gene expression in the area of the thyroid primordium. *hhex* and *nk2.1a* are initially present in the thyroid primordium of *noi*^{-/-}, suggesting that they act upstream of *pax2.1*. *pax2.1* expression starts slightly earlier than *pax8* expression, and *pax8* is never expressed in the thyroid of *noi*^{-/-} embryos. Hence, *pax8* expression functions downstream of *pax2.1*. Furthermore, *pax2.1* is expressed in *noi*^{-/-} until 30 hpf, showing that at this stage Pax2.1 is not required for its own expression. This regulation is reminiscent of the midbrain-hindbrain boundary in *noi*^{-/-}, where *pax2.1* expression is also initially present and then disappears later (Lun and Brand, 1998).

pax2.1 is required for normal thyroid development in zebrafish

As *pax8* is downstream of *pax2.1* in zebrafish, the *noi*^{-/-}

phenotype should be comparable with the *Pax8*^{-/-} phenotype in mice, perhaps more severe if *pax2.1* acts upstream of *pax8*. In mice, *Pax8* is involved in relatively late steps of thyroid development. Mice homozygous for the *Pax8* knockout allele lack all differentiated thyroid follicular cells, but initially a gland forms (Mansouri et al., 1998). Indeed our results demonstrate that the *noi*^{-/-} phenotype is comparable with the *Pax8*^{-/-} phenotype in mice, as the differentiation of follicles is affected rather than early formation of the thyroid primordium. Moreover, *Nkx2.1* is expressed in the early thyroid anlage of *Pax8*^{-/-} mice, indicating a normal early evagination of the primordium from the pharyngeal endoderm (Mansouri et al., 1998). Using *hhex* and *nk2.1a* as markers, we show that the same is the case in the zebrafish *noi*^{-/-} mutant. Whereas in *Pax8*^{-/-} mice the neural crest derived C cells populate the gland instead of the endoderm-derived follicular cells (Mansouri et al., 1998), it remains unclear whether C cells play any role in thyroid development of zebrafish, as their existence is not proven in teleosts.

The lack of morphologically visible thyroid follicles and T4 immunoreactivity in *noi*^{-/-} does not allow us to distinguish whether the thyroid follicular cells die, become mis-specified, or simply fail to secrete colloid and to produce T4. The loss of marker gene expression in the primordium supports a role of *pax2.1* in the maintenance of the primordium or the differentiation of the follicular cells. However, this does not exclude an additional later role of *pax2.1* in thyroid function.

Unexpectedly, the bound form of T4 can still be detected in the anterior non-follicular domain of *noi*^{-/-} mutant zebrafish larvae. This result suggests that in zebrafish some T4 production is independent of *pax2.1* and is not abolished by premature termination of *hhex*, *nk2.1a* and *pax8* expression in the developing thyroid. Furthermore, *nk2.1a*, *pax2.1* and *pax8* are not expressed during larval stages in the area of the second branchial arch where the non-follicular tissue is located. Nevertheless, a possible relation of the larval non-follicular domain to the embryonic thyroid primordium is suggested by the observation that initially the primordium develops at the level of the second branchial arch (Rohr and Concha, 2000). It will be interesting to investigate origin and function of the non-follicular domain in detail.

Corresponding to the mouse *Pax8*^{-/-} phenotype, mutations in the PAX8 locus in humans result in congenital hypothyroidism (Macchia et al., 1998). In *noi*^{-/-} zebrafish larvae, it is likely that the lack of thyroid follicles results in low plasma concentrations of thyroid hormone, as T4 production in the remaining non-follicular domain can probably not compensate for the missing follicles. Thus, the *noi*^{-/-} phenotype is a model for hypothyroidism, similar to *Pax8* deficiency in mice, although a variety of additional defects, such as the missing kidney and midbrain-hindbrain boundary contribute to the lethality of the young larvae.

Different members of the pax2/5/8 paralogue group play similar and redundant roles in thyroid development of vertebrates

In the present paper, we show that *pax2.1* is required for thyroid follicle differentiation in zebrafish, whereas in mice, *Pax2* has no obvious role in thyroid development. *Pax2*, *Pax5* and *Pax8* form a paralogue group of genes, like *Pax1/9*, *Pax4/6* and *Pax3/7* (reviewed by Dahl et al., 1997). Pax genes of the

same paralogue group are often expressed in the same organ systems. Despite this apparent redundancy, slight differences in their regulation might make each paralogue indispensable, thereby leading to evolutionary conservation (Force et al., 1999).

In the case of zebrafish, two genes of the *pax2/5/8* group are expressed in the developing thyroid, whereas in mice, it is only *Pax8* that plays a crucial and comparable role in the same tissue. In *Xenopus*, *pax8* is not expressed in the developing thyroid, and it is *pax2* that seems to be the only Pax gene expressed in the *Xenopus* thyroid (Heller and Brandli, 1999). *pax5* is not reported to be expressed in thyroid tissue of any vertebrate, and neither did we detect it in the developing thyroid of zebrafish. Amphioxus *pax2/5/8*, the putative common ancestor gene of this Pax paralogue group, is expressed in the endostyle, a structure that is generally believed to be homologous to the vertebrate thyroid (Kozmik et al., 1999). This finding suggests that the ancestral *pax2/5/8* gene already adopted a role in thyroid function. During evolution of tetrapods, *pax5* and either *pax2* or *pax8* have subsequently lost this function, while in zebrafish both *pax2* and *pax8* have maintained a role in thyroid development. Over time, differences in paralogue gene regulation must have occurred, as *pax2.1* expression starts earlier than *pax8* expression. Similarly, slight differences that might account for evolutionary conservation after gene duplication have also been reported for other sites of overlapping *pax2/5/8* expression in zebrafish and mice (Bouchard et al., 2000; Pfeffer et al., 1998).

Additional gene duplication is believed to have occurred in the teleost lineage (Postlethwait et al., 1998). This duplication explains the presence of a second *pax2*-type gene, *pax2.2*, within the *pax2/5/8* group zebrafish. However, his gene is not expressed in the thyroid. We speculate that this gene lost its function in the thyroid during evolution of the fish lineage. Likewise, there are two *nk2.1* genes in zebrafish, *nk2.1a* and *nk2.1b* (Rohr et al., 2001), of which only *nk2.1a* is expressed in the thyroid.

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