Early developmental specification of the thyroid gland depends on han-expressing surrounding tissue and on FGF signals

Development 134, 2871-2879 (2007) doi:10.1242/dev.02872

Early developmental specification of the thyroid gland depends on *han*-expressing surrounding tissue and on FGF signals

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The thyroid is an endocrine gland in all vertebrates that develops from the ventral floor of the anterior pharyngeal endoderm. Unravelling the molecular mechanisms of thyroid development helps to understand congenital hypothyroidism caused by the absence or reduction of this gland in newborn humans. Severely reduced or absent thyroid-specific developmental genes concomitant with the complete loss of the functional gland in the zebrafish *hands off* (*han*, *hand2*) mutant reveals the *han* gene as playing a novel, crucial role in thyroid development. *han*-expressing tissues surround the thyroid primordium throughout development. Fate mapping reveals that, even before the onset of thyroid-specific developmental gene expression, thyroid precursor cells are in close contact with *han*-expressing cardiac lateral plate mesoderm. Grafting experiments show that *han* is required in surrounding tissue, and not in a cell-autonomous manner, for thyroid development. Loss of *han* expression in the branchial arches and arch-associated cells after morpholino knock-down of upstream regulator genes does not impair thyroid development, indicating that other *han*-expressing structures, most probably cardiac mesoderm, are responsible for the thyroid defects in *han* mutants. The zebrafish *ace* (*fgf8*) mutant has similar thyroid defects as *han* mutants, and chemical suppression of fibroblast growth factor (FGF) signalling confirms that this pathway is required for thyroid development. FGF-soaked beads can restore thyroid development in *han* mutants, showing that FGFs act downstream of or in parallel to *han*. These data suggest that loss of FGF-expressing tissue in *han* mutants is responsible for the thyroid defects.

KEY WORDS: Thyroid, Zebrafish, *hands off* (*han*, *hand2*), *acerebellar*, Fibroblast growth factors, *fgf8*, Fate mapping, Heart

INTRODUCTION

The endoderm gives rise to pharynx and intestine, and also to thyroid, lung, pancreas and liver. Development of these organs is initiated in different areas of the primitive gut, where, after induction of different transcriptional programmes, the organ primordia bud off. The signalling mechanisms that control the early development of these endoderm-derived organs have only recently begun to be investigated. Work in mice has shown that mesenchymal cells from the lateral plate mesoderm (Kumar et al., 2003); endothelial cells (Lammert et al., 2001); and the notochord (Kim et al., 1997) are involved in induction of the pancreas from the endoderm. For both liver and lung primordia, cardiac tissue adjacent to the endodermal layer has been identified as a source of inducing signals (Gualdi et al., 1996; Jung et al., 1999; Serls et al., 2005). Understanding the processes specifying different domains of the primitive gut can provide insight into congenital defects in humans and into regenerative responses to tissue damage.

Whereas increasing details of the inductive steps in the lung, liver and pancreas are emerging, our knowledge of such early developmental processes in thyroid development is scarce. As an anterior derivative of the primitive gut, the thyroid primordium buds from the ventral midline of the primitive pharynx (reviewed in De Felice and Di Lauro, 2004). During a relocation process, the primordium loses contact with the pharynx, adopting a species-specific position in the hypopharyngeal mesenchyme. In many vertebrates, including mice and man, the primordium bifurcates in the neck area, leading to its final bilobed shape, whereas, in zebrafish and other teleosts, thyroid tissue forms an elongated strand of tissue along the ventral aorta (Wendl et al., 2002). Development of the thyroid is comparable between fish and mammals on the molecular level. The thyroid-specific transcriptional programme, including the transcription factors Nkx2.1 (also known as Nk2.1a and Titf1a/TITF1), Pax8 and Hhex, is conserved with respect to expression patterns and function between zebrafish and mouse (Elsalini et al., 2003; Wendl et al., 2002). In this study, we use zebrafish as a model to investigate the initiation of thyroid development.

In mouse development, induction of lung and liver by cardiac mesoderm was anticipated because of the close association of cardiac mesoderm with lung and liver primordia (Gualdi et al., 1996; Jung et al., 1999; Serls et al., 2005). Similarly, early mouse thyroid markers start to be expressed in the primitive pharynx adjacent to the aortic sac (Fagman et al., 2005) – the cardiac region that gives rise to the embryonic outflow tract and cervical arteries. This spatial correlation appears to be conserved in zebrafish, in which initial thyroidal *nk2.1a* expression starts, at 24 hours post-fertilisation (hpf), adjacent to the outflow tract of the heart (Rohr and Concha, 2000). Earlier in development, during the zebrafish somitogenesis stages, the anterior lateral plate mesoderm (aLPM), from which the heart later develops, as well as the endoderm, converge in parallel processes to the midline (Keegan et al., 2004; Warga and Nusslein-Volhard, 1999). Parallel development and close association of both

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Accepted 24 May 2007

tissues is reflected in a functional relationship. The endoderm is necessary for normal cardiac morphogenesis and, in its absence, the converging halves of the aLPM fail to fuse (Alexander and Stainier, 1999).

Fibroblast growth factors (FGFs) constitute a large family of signalling molecules that have been shown to act in multiple ways on endoderm-derived organ development. FGF1 and FGF2 are crucial for induction of lung and liver in mammals (Jung et al., 1999; Serls et al., 2005), and tissue explant assays suggest cardiac tissue to be the source of the signals. Moreover, in tissue-explant assays, these FGFs act in a concentration-dependent manner, with high concentrations required for lung, and lower concentrations for liver, induction (Serls et al., 2005). However, an exclusive role is not supported by the phenotype of FGF1/FGF2 double-knock-out mice, which are viable (Miller et al., 2000b).

In this study, we show that the zebrafish mutant *hands off* (*han*, *hand2*) has severe defects in early thyroid development. The *han* locus encodes the bHLH transcription factor Hand2 (Yelon et al., 2000). Research on this mutant so far has concentrated on defects correlating with known sites of *han* expression, including the cardiac mesoderm, the fin buds and the pharyngeal arches (Angelo et al., 2000; Miller et al., 2003; Yelon et al., 2000). Two *han* alleles have been isolated, han^{s6}, which has a deletion spanning a maximum of 100 kb, including the *han* locus, and han^{c99}, which has an insertion in the *han* locus. han^{s6} is a null mutation, and homozygotes exhibit a stronger phenotype than han^{c99} mutants (Yelon et al., 2000). A role of *han* in thyroid development has not been described before and represents a novel aspect in thyroid research. In grafting experiments, we show that the *han* gene is required in the surrounding tissue for thyroid development. Further studies suggest that it is *han*-expressing anterior plate mesoderm or cardiac mesoderm that is crucial for thyroid specification. We further show that FGF signalling is required for thyroid development in zebrafish, and that FGF-coated beads are able to restore thyroid development in han^{s6} mutants. Thus, our study provides a first step towards understanding the role of surrounding tissue during thyroid specification.

MATERIALS AND METHODS

Animals and preparation of specimens

Zebrafish care, in situ hybridisation, immunohistochemistry and sections were carried out as described previously (Elsalini and Rohr, 2003; Rohr and Concha, 2000; Wendl et al., 2002). We used the han^{s6}, han^{c99} (Yelon et al., 2000) and ace^{ti282a} ($fgf8^{ti282a}$) (Brand et al., 1996) alleles. The identity of homozygotes was possible based on morphology. In addition, the identity of homozygous *han* mutant embryos was always confirmed by MF20 immunostaining visualising myocardial morphology.

Embryonic manipulation

As the lineage tracer for grafting experiments, we injected biotin-dextran (10,000 M_r, 5 mg/ml; Molecular Probes) into zebrafish embryos and detected biotin-labelled donor-derived cells after in situ hybridisation using the ABC kit (Vector Laboratories). In grafted embryos, the peroxidase reaction against biotin-dextran was carried out in DAB medium containing 67 mM $NiCl_2$, resulting in black donor-derived cells. This first reaction was followed by MF20 immunostaining using normal DAB medium, resulting in brown staining of the myocardium.

For fate mapping of thyroid precursor cells, photoactivation of caged fluorescein was essentially carried out as described previously (Keegan et al., 2004). Morpholino oligonucleotides targeted against *endothelin 1* (edn1-MO) (Miller and Kimmel, 2001), *lockjaw* (*tfap2a*; 3.1-MO) (Knight et al., 2003) and *foxi1* (foxi1-MO) (Mackereth et al., 2005), as well as an unspecific control morpholino (Gene Tools), were used as described previously. Implantation of beads was performed as described (Reifers et al., 2000) in low-melting-agarose-embedded embryos. Beads (45 μm Microspheres, Polysciences) were soaked overnight in 250 μg/ml human recombinant FGF1 (Sigma, St Louis, USA) or 100 μg/ml human recombinant FGF2 (Roche, Indianapolis, USA), in both FGFs together, or in 250 μg/ml mouse recombinant FGF8b (R&D Systems, Minneapolis, USA) or 250 μg/ml BSA, all dissolved in PBS.

RESULTS

Zebrafish *hands off* mutant embryos have defective thyroid development

In zebrafish larvae, the thyroid gland can be visualised using an antibody detecting thyroid hormone (T4) at the apical membrane of follicles from approximately 60 hours post fertilisation (hpf). We found that the *hands off* mutant han^{s6} lacks the differentiated thyroid gland completely at the larval stages (Fig. 1A,B). han^{s6} has been identified based on defective heart, pharynx and fin development, and so we wondered to what extent endoderm is affected. At 24 hpf, when the thyroid starts to develop, the endoderm appeared to be normal, based on marker expression, indicating that endoderm specification was normal in han^{s6} mutants (Fig. 1C,D). Thus, han^{s6} is a good model in which to investigate the mechanisms of thyroid development.

In zebrafish, thyroid markers, such as *nk2.1a*, *hhex* and *pax2.1*, start being expressed in presumptive thyroid precursor cells in the endoderm at around 24 hpf (Elsalini et al., 2003; Rohr and Concha, 2000). In 24-28 hpf han^{s6} mutants, expression of *pax2.1* and *hhex* was always absent (Fig. 1E-H). *nk2.1a* expression was absent at this stage in most mutants, but, in about 10% of mutants, could be detected in a few endodermal cells (Fig. 1I-K), indicating that the thyroid phenotype is variable or not fully penetrant. Later, at 55 hpf, expression of *nk2.1a*, *pax2.1* and *hhex* was not detectable in any domain that would indicate a thyroid in han^{s6} (data not shown). In some clutches, occasional faint *nk2.1a* expression in few cells of the hypopharyngeal area indicated that some thyroid cells might be specified in han^{s6} mutants (barely detectable; data not shown).

The differentiation marker *slc5a5*, encoding the sodium iodide symporter (NIS), is expressed exclusively in thyroid follicle cells from about 40 hpf (Alt et al., 2006). *slc5a5* expression was usually not detectable in han^{s6} mutants at 60 hpf (Fig. 1L,M), although we found a strongly reduced expression domain in 23 out of 258 homozygous specimen (9%; Fig. 1N). Taken together, the absence of the thyroid gland in han^{s6} mutants can be explained by the lack of the early primordium in most specimens. In a small proportion of homozygous embryos, a reduced number of thyroid precursor cells were present and started the differentiation programme, but eventually failed to form a mature gland. Because it is hardly conceivable that *slc5a5* is expressed in the complete absence of thyroid-specific developmental genes, we assume that, in some mutants, remaining low levels of developmental genes are sufficient to initiate *slc5a5* expression in some cells, but not sufficient for all aspects of terminal differentiation.

To find out whether increased cell death might be responsible for the absence of the thyroid primordium in han^{s6} mutants, we carried out TUNEL assays. However, we did not observe visibly increased cell death in the pharyngeal endoderm or in the area where the thyroid would develop [tested at the 16-somite stage (ss), 20 hpf and 24 hpf; data not shown]. It should be noted that the thyroid primordium is very small, so it is possible to miss its few precursor cells undergoing cell death.

In han^{s6} mutants, the deletion might affect the expression of a neighbouring locus, and so we tested the second available, hypomorphic han^{c99} allele for thyroid defects. Here, from the

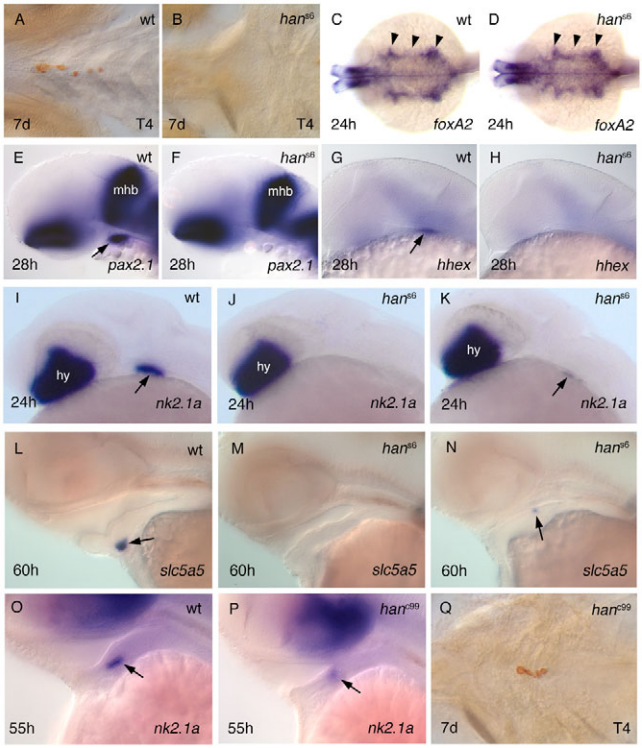

Fig. 1. Thyroid development is impaired in *hands off* mutant zebrafish embryos. Anterior is to the left. Stages are indicated bottom left, genotype top right and staining/marker bottom right. Arrows show thyroid primordium; arrowheads show pharyngeal endoderm. Ventral (A,B,Q), dorsal (C,D) and lateral (E-P) views are shown. T4 (thyroid hormone) immunostaining (A,B,Q) and in situ hybridisation (C-P). (**A**,**B**) In *han*^{s6} embryos, no T4 -producing follicles are detectable. (**C**,**D**) Pharyngeal endoderm appears to be normal in *han*^{s6} mutants. (**E-K**) Expression of thyroid developmental markers. (**L-N**) The thyroid differentiation marker *slc5a5* is expressed in approximately 10% of *han*^{s6} mutant embryos. (**O-Q**) In *han*^{c99} mutants, both primordium and differentiated thyroid are reduced. hy, hypothalamus; mhb midbrain-hindbrain boundary.

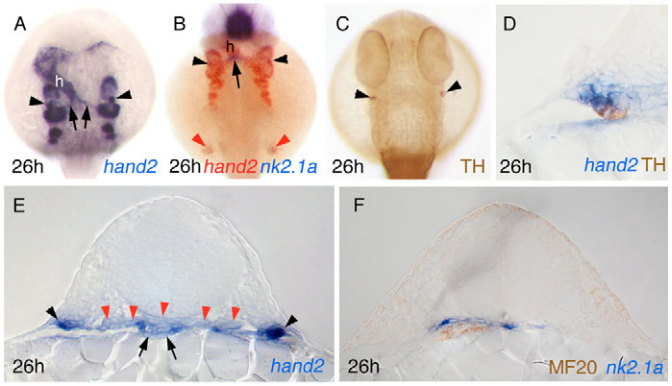

Fig. 2. *han* is expressed in tissues surrounding the thyroid primordium. (A-C) Whole-mount embryos, anterior is up; (D-F) sections. Labelling of panels is as in Fig. 1. (**A**,**B**) The thyroid primordium (arrow in B) is adjacent to the outflow tract of the heart (arrows in A). Two expression domains between the first and the second branchial arch (black arrowheads) flank the thyroid on the same anteroposterior level. Red arrowheads point to *han* (*hand2*) expression in the fin buds. (**C**,**D**) Thyroid hormone (TH) immunostaining visualises arch-associated neurons (AANs) in the putative carotid body primordium (arrowheads). Double staining of *hand2* and TH (D) shows that both expression domains are adjacent to each other. (**E**) *hand2* is also expressed in the endoderm (red arrowheads). Notice the strong expression in cells next to AANs (black arrowheads), and in the heart (arrows). (**F**) For comparison, see the thyroid marker in F (combined with the heart marker MF20). h, heart.

beginning of detectable marker gene expression, the thyroid primordium was reduced in size, albeit not absent (Fig. 1O-Q). The reduced size of the primordium persisted during development and resulted in a smaller differentiated gland at the larval stages (Fig. 1Q). The fact that thyroid development in *han*^{c99} mutants followed a similar, albeit less-severe, phenotypic trend to that of *han*^{s6} mutants confirms that it is the *han* locus in the *han*^{s6} deletion that is involved in thyroid development. Furthermore, this observation suggests a dosage-sensitive requirement for Hand2 during thyroid development.

han is expressed in tissues including and surrounding the site of initiation of thyroid development

We reinvestigated *han* expression in the area in which thyroid markers start to be expressed. Here, *han* was expressed in the heart tube and the roots of the first pair of branching arteries, and, in addition, in the neural crest-derived mesenchyme of the pharyngeal arches (Fig. 2A,B). Furthermore, strong *han* expression was detectable in a set of bilateral cells at the border between the first and second arch, on the same anteroposterior (a-p) level as thyroid marker expression, but more lateral. These cells were directly adjacent to tyrosine-hydroxylase-positive cells called arch-associated neurons (AANs; Fig. 2A-D), which are presumably the precursor cells of the carotid bodies (Holzschuh et al., 2001). It is likely that these two bilateral and distinct groups of *han*-expressing cells form part of the carotid bodies.

In addition to previously described *han* expression in cardiovascular and pharyngeal structures, we found weak expression in the pharyngeal endoderm, including in tissue that expresses early thyroid markers (Fig. 2E,F). We were not able to detect *han* expression in the thyroid after evagination from the endoderm (data not shown). Taken together, multiple tissues surrounding the site where first thyroid marker expression is initiated, including mesoderm and endoderm, express *han*. *han* expression in the posterior lateral plate mesoderm and in the fin buds is far away from the foregut endoderm and can be ignored with respect to thyroid development. In most *han*^{s6} mutant embryos, the thyroid primordium was missing from the beginning, raising the possibility that its induction or the competence of the endoderm to respond to inductive signals is impaired. We therefore addressed the question of where thyroid progenitors reside in the zebrafish embryo before the onset of earliest thyroid markers, and how does this position relate to *han* expression?

Fate mapping of thyroid precursor cells reveals their close association to the aLPM

Earlier fate-mapping studies have shown that both endoderm and cardiac mesoderm converge medially to the embryonic axis during the somitogenesis stages (Keegan et al., 2004; Warga and Nusslein-Volhard, 1999). Comparison of *han* expression with the endodermal marker *foxa3* (*fkd2*) shows that, at the 8 ss, bilateral stripes of endodermal cells are distributed with aLPM cells in a partially overlapping fashion, with some endodermal cells being closer to the

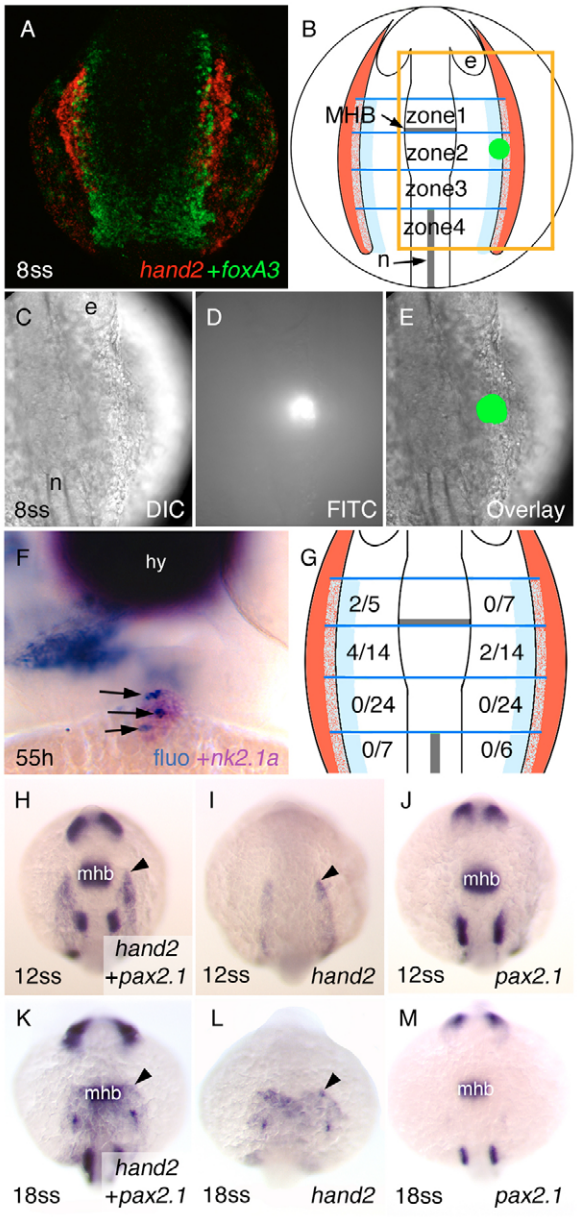

Fig. 3. Fate mapping of thyroid precursor cells reveals their close association with the lateral plate mesoderm. (**A**) Double fluorescence in situ hybridisation of *han* (*hand2*; red) and the endoderm marker *foxa3* (green). Dorsal view, anterior is up. (**B**) Subdivision of the region of interest (light blue: potential overlap between lateral plate mesoderm and endoderm) into four zones according to landmarks. Red: lateral plate mesoderm; green, see D,E. The orange square corresponds to the section shown in C-E. (**C-E**) Example of photoactivation: (C) Nomarski view, (D) after photoactivation, (E) overlay. (**F**) Example of an embryo (frontal view) in which photoactivated cells (fluorescein, dark blue, arrows) are detectable in the thyroid primordium (pink). Photoactivated cells are also present in pharyngeal cells further away from the midline. (**G**) Numbers of embryos in which photoactivated cells contributed to the thyroid. Given are numbers of such embryos/total numbers of uncaging experiments at the corresponding anteroposterior level. Compare with B. Notice that only photoactivation in zone 1 and 2 resulted in contributions to the thyroid. (**H-M**) Comparison of *hand2* expression with the midbrain-hindbrain boundary (MHB) marker *pax2.1*. Arrowheads point to the anterior border of *hand2* expression. e, eye; hy, hypothalamus; mhb, midbrain-hindbrain boundary; n, notochord; ss, somite stage.

midline (Fig. 3A). We wanted to know where prospective thyroid precursor cells are located in relation to the *han* expression in the aLPM.

For this fate-mapping approach, we injected caged fluorescein into embryos at the one-cell stage and photoactivated the fluorescein dye at around the 8 ss. This stage was chosen because it is when the endoderm has not yet reached a position ventral to the neural tube during its convergence movements and is therefore accessible for photoactivation. As landmarks along the a-p axis, we used the posterior end of the eye, the midbrain-hindbrain boundary (MHB) and the anterior tip of the notochord to subdivide the aLPM into four zones (Fig. 3B). Nomarski optics allowed for visualisation of the aLPM edge. During photoactivation, we targeted cells along the medial border of the aLPM in zone 1 to zone 4 (Fig. 3C-E) and probed their contribution to the thyroid at 55 hpf.

In total, 224 embryos were uncaged in 20 sets of experiments. In 100 embryos (45%), cells contributed to the pharynx epithelium, showing that the medial border of the aLPM is also the area where the anterior endodermal cells reside, as predicted from *han*/*foxa3* double staining. Photoactivated cells contributed to the thyroid primordium in eight embryos (Fig. 3F). This low number reflects the small size of the thyroid primordium in comparison to the pharyngeal endoderm. In all of these eight embryos, photoactivated cells were derived from areas within zone 1 or zone 2 (Fig. 3G). By contrast, cells derived from zone 3 or zone 4 never contributed to the thyroid. Taken together, endodermal thyroid precursors are, at the 8 ss, at the medial border of the aLPM, on the a-p level of the MHB. The thyroid primordium is also associated with the MHB at 24 hpf, when the first thyroid markers start to be expressed (Wendl et al., 2002).

To get a rough estimation of *han* expression in relation to the MHB during subsequent somitogenesis stages, we compared the expression of *han* with that of *pax2.1*, a MHB marker. *han* expression in the aLPM had its anterior border on the level at the MHB throughout the somitogenesis stages (Fig. 3H-M). Thus, *han* expression in the cardiac mesoderm is always ventral to the MHB. Even if thyroid precursors are only roughly associated with the MHB along the a-p level, it is likely that the *han*-expressing cardiac mesoderm is continuously close to thyroid precursors throughout the somitogenesis stages.

Grafted wild-type cells can restore thyroidal *nk2.1a* expression in *han*^{s6} mutants in a non-cell-autonomous manner

To find out whether *han* is cell-autonomously required in the endoderm for initial thyroid development or is required non-cell-autonomously in adjacent structures, we created genetic mosaics by the transplantation of wild-type donor cells into *han*^{s6} mutant hosts. We first tested whether wild-type grafted cells are capable of expressing *han* in the *han*^{s6} environment. Embryos were fixed at the 12 ss, when *han* is broadly expressed in the anterior lateral plate mesoderm. In *han*^{s6} mutant embryos, *han* expression was completely missing because of the deletion, but single wild-type cells ending up in the region of the anterior lateral plate mesoderm expressed *han* (Fig. 4A-C).

For analysis of thyroid development, embryos were fixed at 55 hpf and processed for *nk2.1a* in situ hybridisation. Homozygotes were identified by MF20 immunohistochemistry visualising heart muscle, which is strongly reduced in *han*^{s6} mutants. Unequivocal identification of mutants was possible because grafted wild-type cells did not restore *han*^{s6} heart morphology. Out of 87 homozygous *han*^{s6} hosts (from 327 hosts in total) that received grafted wild-type cells, 75 did not show

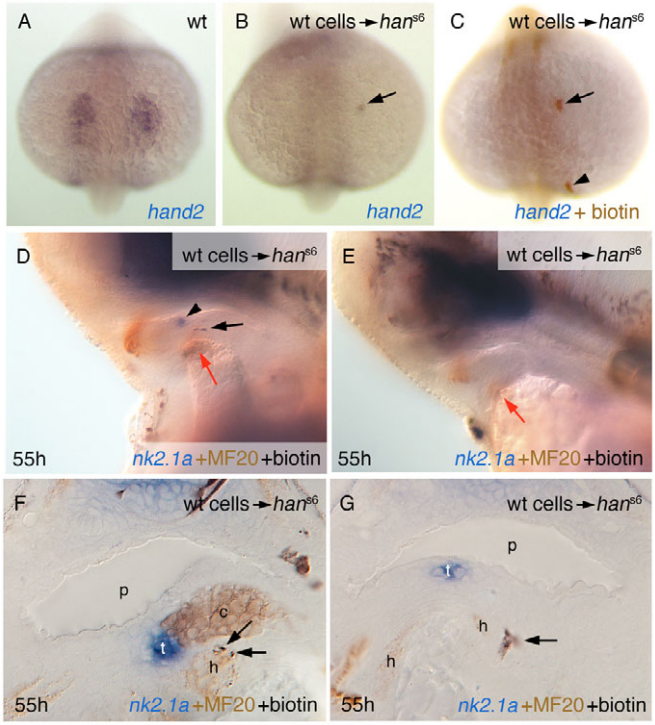

Fig. 4. Grafted wild-type cells can restore *nk2.1a* expression in *han*^{s6} mutant embryos. Labelling of panels is as in Fig. 1. Anterior up (A-C) or to the left (D,E), and sections (F,G). (**A-C**) Grafted wild-type cells express *han* (*hand2*) in the mutant background. Arrows point to a single grafted cell that contributed to the anterior lateral plate mesoderm. The arrowhead in C indicates a grafted cell that contributed to other tissue, not expressing *hand2*. (**D-E**) Examples of hosts fixed at 55 hours post fertilisation (hpf). D shows an embryo (corresponding to #4 in Table 1) that has restored *nk2.1a* expression (arrowhead), an occurrence that was never seen in siblings. The black arrow points to grafted wild-type cells, the red arrows to the heart rudiment. E shows a *han*^{s6} embryo without restored thyroidal *nk2.1a* expression. (**F**,**G**) Cross sections of specimens of *han*^{s6} embryos with restored *nk2.1a* expression. Grafted cells in the embryo shown in F (corresponding to #5 in Table 1) are adjacent to the thyroid in cartilage, but also in heart tissue (arrows). Note also that such a large thyroid as in F was never observed in untreated *han*^{s6} embryos. Often, donor cells clearly belong to the pharyngeal mesenchyme, but are also close to the heart rudiment (G, embryo corresponds to #3 in Table 1). c, cartilage precursor cells; h, heart rudiment; p, pharynx; t, thyroid.

any sign of a thyroid at 55 hpf. However, 12 embryos (13.8%) showed a strong *nk2.1a* expression domain in the pharyngeal epithelium or in the pharyngeal mesenchyme (Table 1, Fig. 4D-G). The position of these *nk2.1a* domains resembled normal *nk2.1a* expression in the thyroid primordium. In the remaining homozygotes from the same clutches, which were fixed as controls, thyroidal *nk2.1a* expression was consistently not detectable at 55 hpf. As mentioned earlier, we occasionally observed faint *nk2.1a* expression in homozygotes of other clutches, but expression was weaker and restricted to a smaller domain. We therefore conclude that wild-type cells can restore *nk2.1a* expression in *han*^{s6} mutants, or can increase weak levels of expression that are otherwise below detection, or can prolong initially present expression to unusually late time points.

Biotin detection revealed that grafted cells were always close to the restored *nk2.1a* expression domain in *han*^{s6} mutant embryos. In none of the 12 embryos could wild-type cells be found in the domain of pharyngeal *nk2.1a* expression itself (Table 1). Thus, for restoration of *nk2.1a* expression, it is sufficient to bring wild-type cells into the surrounding tissue of the place where the thyroid would develop in *han*^{s6} mutants. This means that *han* is required in cells other than thyroid cells for *nk2.1a* expression in the thyroid primordium.

han encodes a transcription factor, and so its cell non-autonomous action in thyroid development depends on its cell autonomous activity in surrounding tissue. In embryos with restored *nk2.1a* expression, grafted cells were rarely found to have contributed to the pharyngeal epithelium (Table 1). Thus, lack of *han* expression in the surrounding endoderm cannot be responsible for the loss of thyroidal *nk2.1a* expression in the mutant. Between the early steps of thyroid development and the time point of fixation at 55 hpf, morphogenesis of both heart and pharyngeal arches is highly abnormal in *han*^{s6} mutants (Miller et al., 2003; Trinh et al., 2005; Yelon et al., 2000). Therefore, grafted cells cannot be unequivocally classified as belonging to either of these structures. To determine whether cardiac or branchial arch tissue is required for thyroid development, we eliminated *han* expression in the pharyngeal arches and putative carotid bodies by morpholino knock-down of upstream genes.

Thyroid development is independent of *han* expression in pharyngeal arches and arch-associated cells

As was previously demonstrated, *han* expression in the branchial arches depended on the expression of *endothelin 1* (*edn1*) and *tfap2a* (Knight et al., 2003; Miller and Kimmel, 2001; Miller et al., 2000a;

Table 1. Grafted wild-type cells restore *nk2.1a* expression in *han*^{s6} mutant embryos

han^{s6} embryo with restored *nk2.1a* expression (No.)	Donor cells in or directly adjacent to heart rudiment as visualised with MF20 staining	Donor cells in pharyngeal mesenchyme	Donor cells in pharyngeal endoderm (entire anterioposterior axis)	Donor cells in thyroid primordium
1	+	–	+	–
2	+	+	+	–
3	–	+	–	–
4 (Fig. 4D)	+	+	–	–
5 (Fig. 4F)	+	+	–	–
6	+	+	+	–
7	+	+	+	–
8	+	+	+	–
9	+	–	–	–
10	+	+	–	–
11	–	+	+	–
12	–	+	+	–

The position of wild-type donor cells relative to *nk2.1a* expression in rescued *han*^{s6} mutants.

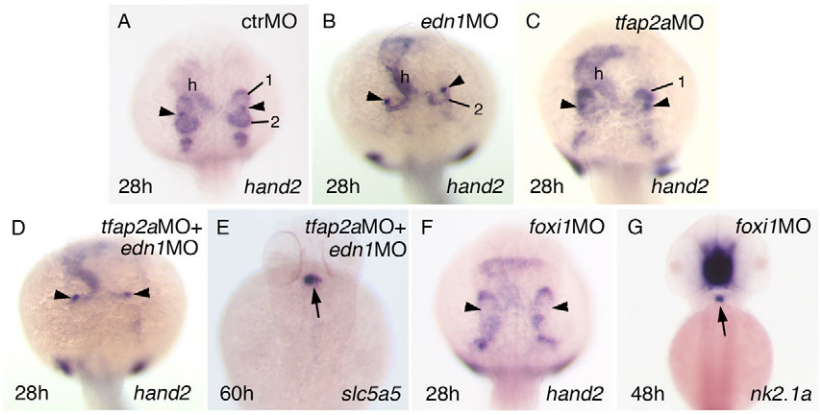

Fig. 5. Elimination of *han* expression sites in the pharyngeal area does not influence thyroid development. Labelling is as in Fig. 1. (**A-D**) In *edn1* morphants (B), *han* (*hand2*) expression in the first (1) and third branchial arches is eliminated. In *tfap2a* (*low*, *lockjaw*) morphants (C), *hand2* expression in the second branchial arch (2) is missing. In double morphants (D), *hand2*-expressing cells are only present in arch-associated cells (arrowheads), as well as some weak expression in the endoderm (confirmed on sections, data not shown). (**E**) The thyroid (arrow) appears normal in *edn1*/*tfap2a* double morphants. (**F**) In *foxi1* morphants, *hand2* expression in arch-associated cells (arrowhead) is specifically ablated. (**G**) Normal expression of *nk2.1a* in *foxi1* morphants (arrow). h, heart.

Piotrowski et al., 2003). Correspondingly, double morpholino knock-down of these two genes eliminated *han* expression in the pharyngeal arches completely (Fig. 5A-D). By contrast, *han* expression in the putative carotid bodies, heart and endoderm was unaffected in these double morphants. Thus, *edn1*/*tfap2a* double morphants can serve as a model for thyroid development in the absence of pharyngeal arch *han* expression.

nk2.1a and *slc5a5* expression is essentially normal in *edn1* and *tfap2a* single morphants as well as in *edn1*/*tfap2a* double morphants (Fig. 5E, and data not shown), indicating that thyroid development does not depend on *han* expression in the pharyngeal arches.

To analyse whether *han* expression in the arch-associated cells (adjacent to the tyrosine hydroxylase-positive AANs) is required for thyroid development, we analysed *foxi1* morphants. It has been shown that the *foxi1* (*no soul*) gene is required for specification of the AANs (Guo et al., 1999). We found that, in *foxi1* morphants, not only the AANs, but also the adjacent *han*-expressing cells located between the first and second pharyngeal arches are missing (Fig. 5F). Nevertheless, *foxi1* morphants had a normal thyroid (Fig. 5G), indicating that *han* expression in arch-associated cells is not required for thyroid development. These data suggest that the remaining other site of detectable *han* expression, the cardiac mesoderm, is required for thyroid development. To date, it is not possible to ablate this tissue specifically, and so its role in thyroid development awaits further experimental confirmation.

FGFs are candidate signalling factors in thyroid development

We focused on FGFs as putative downstream factors of *han* in thyroid development, because they have been shown to act downstream of *han* in tooth development (Abe et al., 2002) and are known to play roles in cardiac development (Reifers et al., 2000). In zebrafish *ace* mutant embryos, the *fgf8* gene is disrupted (Reifers et al., 1998). In this mutant, a reduced size of the thyroid primordium at early stages (Fig. 6A-F) and a reduced number of follicles after differentiation (Fig. 6G,H) indicates that *fgf8* is required for normal thyroid development.

FGFs constitute a large family of signalling molecules (Ornitz and Itoh, 2001; Thisse and Thisse, 2005), and the loss of a specific FGF might be compensated for by the overlapping expression of other family members. The FGF-receptor blocker su5402 is an excellent tool to eliminate FGF signalling completely in specific time windows, and has also been used to narrow down the temporal requirement of FGF in zebrafish heart development (Reifers et al., 2000). We treated zebrafish embryos at various stages with 10 μM su5402 for a time window of 2 hours and tested at 36 hpf for *nk2.1a* expression and at 60 hpf for *slc5a5* expression. Higher concentrations of su5402 lead to severe malformations, making analysis of thyroid development questionable, so that we confined our analysis to 10 μM concentrations only.

In general, su5402 treatment during the somitogenesis stages was sufficient to eliminate thyroidal *nk2.1a* and *slc5a5* expression in around 70% of embryos (Fig. 6I-M). The uniform result for different time windows (Fig. 6M) suggests that washing su5402 out after 2 hours of treatment was probably inefficient, or that FGF signalling is continuously required for thyroid development. Treatment starting at 30 hpf, after the thyroid primordium is induced, affects the thyroid less efficiently, but still eliminates the gland in about 20% of embryos. This could be the result of a reduced influence of FGFs on later thyroid development, but could also be due to a limited potential of the chemical to diffuse into older embryos. su5402 treatment at the somitogenesis stages did not visibly affect endoderm development on the level of *foxa2* (*axial*) expression at 30 hpf (data not shown), indicating that it is not a severe reduction of endoderm that causes the absence of the thyroid. In conclusion, su5402 treatment did not enable precise definition of the time window in which FGF signalling is required for thyroid development. Nevertheless, the drug treatments confirm that FGF signalling is required for thyroid specification and for subsequent differentiation. Furthermore, these data indicate that FGF signalling is not only required during the early steps of thyroid development, as indicated by the reduced early thyroid primordium in *ace* mutants, but also during later steps, after 30 hpf.

FGFs restore thyroid development in *han*^{s6} mutants

To test a possible role of FGFs downstream of *han* in thyroid development, we implanted beads soaked with recombinant FGF protein into *han*^{s6} embryos and analysed subsequent thyroid differentiation based on the level of *slc5a5* expression. We chose recombinant mouse FGF8 and, in addition, recombinant human FGF1 and FGF2. FGF1 and FGF2 signals from the cardiac mesoderm are suspected to be involved in liver induction in mice (Jung et al., 1999; Serls et al., 2005) and are therefore also good candidates as having a role downstream of *han*. Using the MHB as a landmark, beads were embedded into the embryo within proximity to the endoderm. Implantation was carried out at the 14-18 ss, because the reduced size of the early thyroid primordium in *ace* mutants suggests a specific role of FGFs in thyroid development before or around the onset of thyroid marker expression. Numbers of untreated *han*^{s6} mutants with residual *slc5a5* expression were not significantly different compared to *han*^{s6} mutants that received BSA-soaked control beads (Fig. 7A,B). The

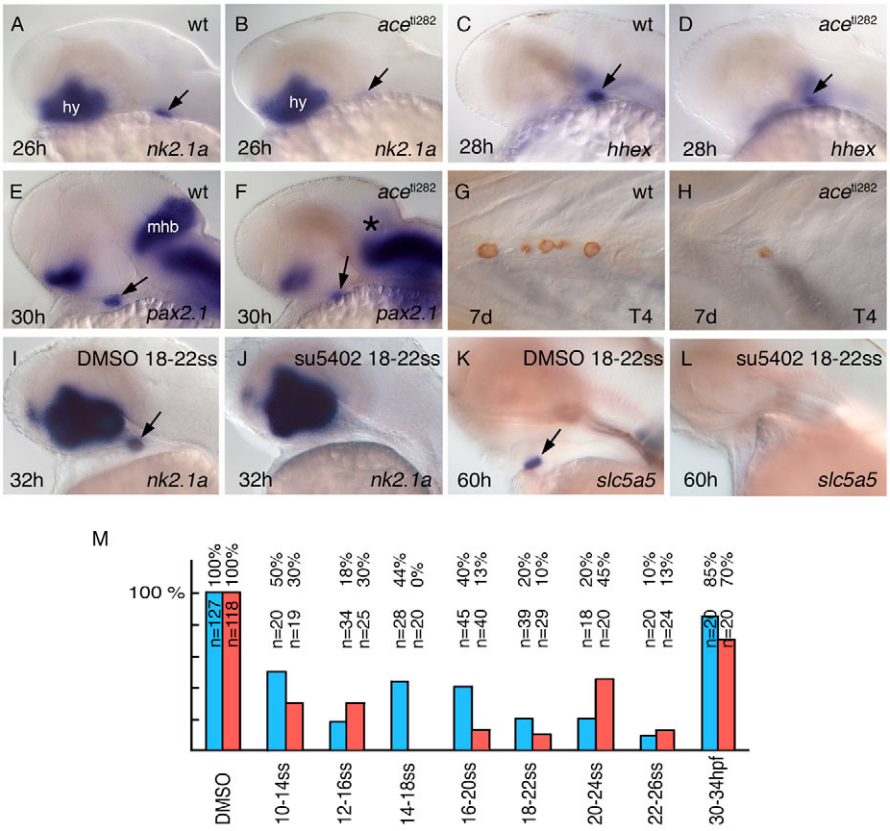

Fig. 6. FGF signalling is required for thyroid development. Labelling is as in Fig. 1. Anterior is to the left. Lateral (A-F,I-L) and ventral (G,H) views are shown. (**A-F**) Expression of thyroid markers in *ace* mutants and in wild-type (wt) siblings. (**G**,**H**) *ace* larvae have a strong reduction in thyroid gland size. (**I**,**K**) In DMSO-treated control embryos, the thyroid appears normal. (**J**,**L**,**M**) Following different stages of su5402 treatment, the thyroid is completely lost. (J,L) Example embryos without thyroid; (M) the complete data set. Blue bars, embryos with thyroidal *nk2.1a* expression; red bars, *slc5a5* expression. Arrows point to the thyroid primordium; the asterisk indicates the absent midbrain-hindbrain boundary (MHB) in *ace* mutants.hy, hypothalamus; mhb, midbrain-hindbrain boundary, ss somite stage.

implantation of beads soaked in FGF proteins, however, resulted in significantly increased numbers of *han*^{s6} embryos expressing *slc5a5* (BSA control compared to FGF8: X^2=3.99, P=0.046; FGF1: X^2=4.49, P=0.034; FGF2: X^2=5.03, P=0.024; FGF1+FGF2: X^2=8.00, P=0.004). Interestingly, all three FGFs were able to restore *slc5a5* expression to a similar percentage. Thus, on the protein level, these different FGFs and probably also other members of the family can replace each other functionally. In summary, recombinant FGF protein is able to rescue the thyroid in *han*^{s6} mutants by restoration of *slc5a5* expression, showing that FGFs act downstream or in parallel to *han* in the differentiation of this gland (Fig. 7C).

DISCUSSION

Early thyroid development depends on *han* expression in the surrounding tissue

Until now, research on the genetics of thyroid development has mainly concentrated on transcription factors expressed in thyroid precursor cells, such as Nkx2.1 (Nk2.1a), Pax proteins and Hhex. In both zebrafish and mice these factors are required for the differentiation of follicle cells (De Felice and Di Lauro, 2004). By contrast, the genetics of thyroid specification are poorly understood. Neither the factors involved in induction, nor in defining the competence of endodermal cells to become thyroid have been identified. Our grafting experiments show that *han* is required for thyroid development in a cell non-autonomous manner. Because *han* encodes a transcription factor, it is conceivable that the Hand2 protein is necessary for the development of certain tissues (most probably lateral plate mesoderm or heart) in the vicinity of the endoderm. Thyroid development, in turn, depends on the proper development of these surrounding structures. Such an indirect role could also account for the incomplete penetrance of the *han*^{s6} phenotype at early stages of thyroid development, and for the *han*^{c99} phenotype, in which both heart and thyroid are less severely affected. If the development of adjacent tissue is impaired, local sources of signalling molecules, such as FGFs, are not necessarily completely abolished. Interestingly, final thyroid differentiation leading to hormone (T4) production always failed in *han*^{s6} mutants, despite initial *nk2.1a* expression in some embryos, and despite occasional later expression of the differentiation marker *slc5a5*. It is possible that reduced *nk2.1a* levels are not sufficient for normal differentiation. Alternatively, the severely abnormal heart and pharyngeal arches in *han*^{s6} mutants might influence later thyroid development independently of the early steps.

The cardiac mesoderm contains a potential signalling centre for early thyroid development

Our *edn1*/*tfap2a* and *foxi1* morpholino experiments strongly suggest that *han*-expressing pharyngeal arch mesenchyme and arch-associated cells, respectively, are dispensable for thyroid specification. It remains unresolved which other *han*-dependent tissue exerts such a function. We discovered so-far unnoticed *han* expression in the endoderm. However, in *han*^{s6} embryos, in which grafted wild-type cells restored *nk2.1a* expression, these cells only contributed in some cases to the pharyngeal epithelium, indicating that *nk2.1a* expression is not dependent on *han*-expressing neighbouring endoderm. Therefore, the most likely candidate of *han*-expressing tissue to act on thyroid development is the aLPM or cardiac tissue.

Because it is unknown exactly when *han*-expressing tissue is required for thyroid specification, at least two different scenarios are possible. Our fate mapping indicates that aLPM is continuously close to thyroid progenitors in the converging endoderm and, in *han*^{s6} mutants, normal expansion of the aLPM fails during the somitogenesis stages. Thus, one possible model is that the aLPM

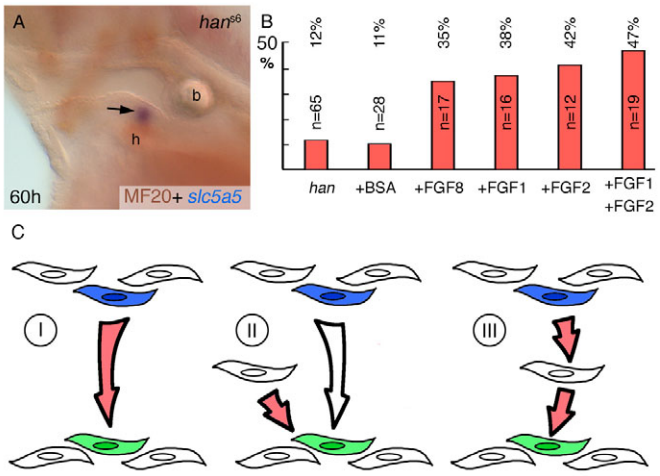

Fig. 7. FGFs act downstream of or in parallel to *han*. Labelling is as in Fig. 1. Grafting of FGF-soaked beads significantly increases the number of *han*^{s6} mutant embryos with detectable *slc5a5* expression. (**A**) Example of a *han*^{s6} mutant implanted with an FGF1-soaked bead. Implantation of FGF-soaked beads significantly increased the probability that *han*^{s6} mutants expressed detectable thyroid markers (arrow). Counterstaining against MF20 enables the identification of *han*^{s6} mutant homozygotes. (**B**) Diagram showing the complete data set. (**C**) Schematic drawing, summarising scenarios of how FGF signalling and Hand2 might influence thyroid development. *han*-expressing cells (blue) influence early thyroid development either directly (I,II) or indirectly (III). (I) Hand2-expressing tissue signals directly to the thyroid precursor cells (green) via the FGF pathway (arrow) to promote the development of the precursor cells. (II) Hand2-expressing tissue has a direct influence on thyroid development (not via FGF signalling), with neighbouring tissue providing additional FGF signals. In this scenario, increased levels of FGFs can compensate for the missing influence of Hand2-expressing cells. (III) In the case of indirect influence of Hand2-expressing tissue on thyroid development, FGF signals can be involved at different levels (i.e. on the level of the upper or the lower arrow, or on the level of both arrows). Green cells: thyroid precursor cells; blue cells: Hand2-expressing cells; red arrows: potential contribution of FGF signals. b, bead; h, heart.

signals to the endoderm, specifying thyroid precursors early during the somitogenesis stages. Alternatively, because cardiac development is severely disrupted in *han*^{s6} mutants, it could also be that cardiac structures such as heart muscle or the outflow tract are required for thyroid specification. In this model, interactions between heart and pharyngeal endoderm would occur later than in the first model, just before the onset of thyroid markers, at around 24 hpf. Because the aLPM is considered to give rise to cardiac structures, both models are similar in that they support a central role of heart development in thyroid specification. Moreover, in both *han* and *ace* mutants, defects in cardiac morphogenesis (Reifers et al., 2000; Yelon et al., 2000) are correlated with a similar thyroid phenotype.

Interactions between cardiac and thyroid development are further supported by human syndromes. In DiGeorge (22q11) syndrome, caused by variable deletions on chromosome 22 in humans, congenital heart defects occur. In conjunction, an increased risk of congenital thyroid defects has been described (Bassett et al., 2005). Similarly, in human patients suffering from congenital hypothyroidism, an increased incidence of congenital heart defects has been observed (Olivieri et al., 2002). Furthermore, ectopic thyroid tissue can occasionally be found in human cardiac tissue (Casanova et al., 2000).

FGF signalling is required for thyroid development in zebrafish

Our su5402 experiments indicate that FGF signals are required for thyroid development, and the *ace* mutant phenotype shows that Fgf8 is involved in this process. *fgf8* was not expressed in the thyroid primordium at visible levels (data not shown), suggesting that Fgf8 acts in a non-cell-autonomous manner in thyroid development. This is supported by the observed effects of FGF-soaked beads in *han*^{s6} mutants. *fgf8* is expressed in the aLPM (Reifers et al., 2000), tissue that is continuously close to thyroid precursors and therefore a candidate for being the source of FGF signals. A search for FGFs acting together with *fgf8* and probably redundantly in thyroid development has not been successful as yet (T.W., D.A. and K.B.R., unpublished observations). For instance, morpholino knock-down of other zebrafish FGFs (Fgf1, Fgf2, Fgf3) in conjunction with Fgf8 did not lead to a more-severe thyroid phenotype.

Genes encoding downstream factors or modifiers of the intracellular signalling cascade of Fgf8, such as *spry2*, *spry4* or *sef* (also known as *il17rd* – Zebrafish Information Network) (Furthauer et al., 2002), were not found to be expressed at visible levels in the thyroid or in the endoderm at corresponding stages (T.W., D.A. and K.B.R., unpublished observations), suggesting that Fgf8 is unlikely to signal directly to the pharyngeal endoderm. It is therefore possible that further, unknown factors link FGF signalling and thyroid development.

FGFs have been implicated to play a role in thyroid development previously. In mouse embryos deficient for the FGF receptor 2 IIIb, multiple defects in organogenesis occur, including dysgenesis of the thyroid (Revest et al., 2001). A similar phenotype of FGF10 knock-out mice suggests that FGF10 is a major ligand acting via FGF receptor 2 IIIb (Ohuchi et al., 2000). However, initial thyroid development still occurs in the absence of the receptor 2 IIIb isoform (De Felice and Di Lauro, 2004), suggesting that FGF activity via this isoform is not responsible for early specification of the thyroid. Taken together, it can be anticipated that several FGFs act at different time points in thyroid development.

han and FGFs: a novel link in thyroid development

In *han*^{s6} mutants, FGF proteins are able to restore thyroid differentiation, therefore acting downstream or in parallel of *han* in thyroid development (Fig. 7C). *fgf8* expression in the aLPM appears to be normal in *han*^{s6} mutants (J.J.S. and D.Y., unpublished data), indicating that here *fgf8* expression does not depend on Hand2. Thus, *fgf8* rather acts in parallel to Hand2 in thyroid development, and it is possible that morphogenetic changes in *han*^{s6} mutants alter the temporal or spatial relation of FGF-expressing tissue to pharyngeal endoderm.

In the bead-implantation experiments, the thyroid was never restored at the wrong level along the a-p axis. This argues against inductive activity of FGFs, which would be likely to cause ectopic primordia. In particular, because *slc5a5* expression is seen in a percentage of untreated mutants, we would expect a second thyroid in some FGF-bead-implanted embryos, which was not the case. Alternatively, we favour the possibility that FGFs act permissively in thyroid development, together with other signals. Our su5402 data, as well as the abovementioned mouse data, suggest that FGFs are also, and probably continuously, required for later thyroid differentiation, at which point structures in addition to the aLPM or cardiac tissues might act as a source.

Taken together, *han* and *ace* mutants represent two models that shed light on the role of the surrounding tissue in thyroid specification. Our study identifies the aLPM or cardiac structures to

be key in this process. It will be interesting to analyse the role of Hand transcription factors as well as FGF signals and their downstream pathway components with respect to congenital thyroid defects in humans, in particular in those cases where they are associated with congenital heart defects.

We thank Didier Stainier and the members of his laboratory for support during the initiation of the project; our colleagues from the zebrafish community for plasmids and morpholino samples; and the members of the Brand, Rohr and Yelon labs for discussion and support. We thank Alexander Picker for advice on bead implantation, Tom Shilling for helpful information and Julia von Gartzen for excellent technical assistance. T.W., D.A. and K.B.R. were supported by DFG: SFB 572.

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