The 22q11 deletion syndrome candidate gene Tbx1 determines thyroid size and positioning

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Thyroid dysgenesis is the major cause of congenital hypothyroidism in humans. The underlying molecular mechanism is in most cases unknown, but the frequent co-occurrence of cardiac anomalies suggests that the thyroid morphogenetic process may depend on proper cardiovascular development. The T-box transcription factor Tbx1, which is the most probable gene for the 22q11 deletion syndrome (22q11DS/DiGeorge syndrome/velo-cardio-facial syndrome), has emerged as a central player in the coordinated formation of organs and tissues derived from the pharyngeal apparatus and the adjacent secondary heart field from which the cardiac outflow tract derives. Here, we show that Tbx1 impacts greatly on the developing thyroid gland, although it cannot be detected in the thyroid primordium at any embryonic stage. Specifically, in Tbx1−/− mice, the downward translocation of Titf1/Nkx2.1-expressing thyroid progenitor cells is much delayed. In late mutant embryos, the thyroid fails to form symmetric lobes but persists as a single mass approximately one-fourth of the normal size. The hypoplastic gland mostly attains a unilateral position resembling thyroid hemiagenesis. The data further suggest that failure of the thyroid primordium to re-establish contact with the aortic sac is a key abnormality preventing normal growth of the midline anlage along the third pharyngeal arch arteries. In normal development, this interaction may be facilitated by Tbx1-expressing mesenchyme filling the gap between the pharyngeal endoderm and the detached thyroid primordium. The findings indicate that Tbx1 regulates intermediate steps of thyroid development by a non-cell-autonomous mechanism. Thyroid dysgenesis related to Tbx1 inactivation may explain an overrepresentation of hypothyroidism occurring in patients with the 22q11DS.

INTRODUCTION

The major cause of congenital hypothyroidism (CH) is developmental defects of the thyroid gland, collectively named thyroid dysgenesis, which occurs in approximately 1/4000 births. It comprises complete lack of the gland (agenesis), hypoplasia including hemiagenesis of one of the two lobes, and ectopic localization (1–4). Early diagnosis is mandatory to prevent serious and irreversible brain damage by providing adequate hormone substitution to the thyroid-deficient newborn (5). Routine thyroid hormone measurements likely identify manifest CH, but infants with a hypoplastic thyroid with an increased risk of developing hypothyroidism later in childhood may be overlooked in neonatal screening (6,7).

The molecular mechanisms of thyroid dysgenesis in humans are largely unknown; so far, genes encoding thyroid transcription factors that are required for normal thyroid development in mouse (2), i.e. Titf1/Nkx2.1 (also known as TTF-1) (8), Foxe1 (also known as TTF-2) (9) and Pax8 (10), have been found to be mutated only in few cases (3,11–13). Recent experimental observations indicate that thyroid dysgenesis may be a polygenic disease with variable penetrance depending on genetic background (14). On the other hand, twin studies suggest that epigenetic modifications, early mutations or stochastic developmental events may contribute (15).

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Patients with CH also often present other congenital malformations, of which, the most frequent are cardiovascular defects (16,17). This suggests that thyroid organogenesis may be influenced by factors that are not necessarily expressed in the thyroid itself but involved in tissue patterning in the upper thoracic and neck regions. In support of this notion, we recently showed (18) that Sonic hedgehog (Shh) regulates mouse thyroid lobulation by a non-cell-autonomous mechanism and that the thyroid phenotype of Shh homozygous mutants resembles the hemiagensis reported to occur in 2/1000 newborns (19). Although Shh deficiency in humans is very rare (20), this experimental observation offers a new explanation of how thyroid dysgenesis may develop by the influence of non-cell-autonomous factors. Conceptually, any disturbances of genes comprising Shh-signaling pathways may lead to thyroid dysgenesis.

The mouse thyroid develops in close association with large embryonic blood vessels originating from the cardiac outflow tract (21). This process comprises several steps taking place in a strict spatiotemporal fashion. First, the specification of thyroid progenitor cells at E8.5 is restricted to a portion of the foregut endoderm that apposes the aortic sac. Secondly, budding and descent of the thyroid anlage at E9.5–11.5 follow the pathway the aortic sac takes as it descends along the neck. Thirdly, bilateral growth of the midline thyroid at E11.5–12.5, constituting the start of the lobulation process, occurs along the course of the third pharyngeal arch arteries that will later form the segments of the carotid vessels located most closely to the mature thyroid lobes. This raises the possibility that vessel contacts per se or factors regulating vasculogenesis might be important for the thyroid to develop normally and, moreover, that vessel malformations directly or indirectly could contribute to or even cause an abnormal position and shape of the thyroid gland.

In this study, we provide the first evidence that vessel interactions influence thyroid size and shape and that Tbx1, a downstream target of Shh (22,23), is a candidate gene for this effect. The protein encoded by Tbx1 is a transcription factor that plays a central role in pharyngeal arch development and in the formation of their organ derivatives (24). It is well known that genetic deletion of Tbx1 in mouse reproduces many of the developmental defects found in the 22q11DS, of which, the most serious are cardiac outflow tract malformations (25). The present findings indicate that thyroid progenitor cells are specified normally in Tbx1−/− mice, but in the late budding process, the thyroid does not establish proper contact with the aortic sac and subsequently the biliobation fails, resulting in a severely hypoplastic gland. This might provide a mechanistic explanation to thyroid dysgenesis and hypothyroidism incidentally reported to occur in children with the 22q11DS (26–31).

RESULTS

Thyroid hemiagensis in Tbx1-deficient mice

The normal morphogenetic development of the mouse thyroid gland is completed at 17.5 dpc. As expected, in transverse sections through the neck, the left and right thyroid lobes (identified by E-cadherin and thyroglobulin expression) were located close to the proximal trachea in all wildtype embryos (n = 8; Fig. 1A). In contrast, in Tbx1−/− embryos of the same age, the thyroid consisted of a single lobe in all examined specimens (n = 10; Fig. 1B). The dysplastic thyroid was mostly found at the ventrolateral region of the trachea approaching one of the carotid arteries, and as the course of the vessels did not obviously differ from the normal, the thyroid position in the Tbx1−/− mutants was evidently asymmetric (Fig. 1C and D). However, as both the trachea and esophagus were malformed, i.e. with a narrow and distorted lumen, and variably displaced from the midline, the phenotype was further characterized by three-dimensional reconstructions of serial sections spanning the entire thyroid volume and surrounding tissues (Fig. 2). This proved, in comparison with the normal symmetrically bi-lobed gland (Fig. 2A and C), that the thyroid malformation present in Tbx1-deficient mice mimicked hemiagensis (Fig. 2B and D). The thyroid volume was calculated by...
morphometry to be in average 18% (range from 13 to 22%) of the total thyroid size in wildtype littermates (Fig. 3). Another difference was that the thyroid remnant had a more cranial position in the neck, i.e. at the level of the tracheoesophageal bifurcation (Fig. 2B and D). Together, this suggests that both parenchymal growth and downward translocation of the single thyroid lobe in the $\text{Tbx1}^{2/-}$ mutant are retarded.

Although $\text{Tbx1}$ haploinsufficiency is known to cause a partial developmental defect comprising, e.g. parathyroid agenesis (32), the thyroid anatomy was normal in $\text{Tbx1}^{+/+}$ mice (data not shown). As previously reported (33), the thymus and parathyroid glands were consistently missing in the $\text{Tbx1}^{2/-}$ mutants.

The hypoplastic thyroid of $\text{Tbx1}^{2/-}$ mutants consists of normally differentiated follicles but lacks C-cells of neural crest origin

As $\text{Tbx1}$ null mice die soon after birth, presumably due to cardiovascular defects, it was not possible to investigate the functional differentiation of the thyroid remnant by measuring thyroid hormone blood levels. However, immunostaining showed that thyroglobulin, the thyroid prohormone, was expressed to the same extent as in the normal gland (Fig. 1A–F). Moreover, thyroglobulin accumulated in the lumen of small follicles that typically characterized the thyroid tissue in both genotypes (Fig. 1E and F). It is likely therefore that $\text{Tbx1}$ is not required for terminal differentiation of thyroid progenitor cells into a hormone-producing follicular epithelium. In contrast, whereas calcitonin positive C-cells were numerous in the central lobe portions of the normal gland (Fig. 1G), no calcitonin immunoreactivity could be detected in the thyroid remnant of $\text{Tbx1}^{2/-}$ mutant embryos (Fig. 1H). It is previously known that C-cell precursors originating from the neural crest are brought to the thyroid by the ultimobranchial bodies (derived from the fourth pharyngeal pouches) where they fuse with the midline thyroid anlage at 12.5–13.5 dpc. Since the ultimobranchial bodies fail to develop when $\text{Tbx1}$ is deleted (32), this probably is the reason why C-cells are lacking. Whether there might be other $\text{Tbx1}$-dependent processes in early thyroid development that could explain the observed bilobation defect and poor parenchymal growth was examined next.
Thus, wildtype and Tbx1−/− influence any of the outlined steps in thyroid morphogenesis. As Tbx1 deficiency in mice is known to profoundly affect the vascular tree including the pharyngeal arch arteries (9,34), it was of obvious interest to find out whether this might affect the thyroid primordium in Tbx1-deficient embryos. We recently showed that the mouse thyroid develops stepwise in close association with large vessels derived from the cardiac outflow tract (21). First, as the thyroid placode forms between 8.5 and 9.5 dpc, the portion of the endoderm housing the progenitor cells is exclusively apposed to the aortic sac endothelium. Soon thereafter, when the placode buds and disconnects from the endoderm, the thyroid primordium temporarily loses contact with the aortic sac and becomes all surrounded by mesenchyme. One day later (at 11.5 dpc), the detached thyroid resumes a position very close to the aortic sac, after which, bidirectional growth takes place along the course of the third pharyngeal arch arteries, indicating the start of lobulation (at 12.5 dpc). In this process, Foxe1 functions as a cell-autonomous regulator of thyroid migration (9,34). As Tbx1 deficiency in mice is known to profoundly affect the vascular tree including the pharyngeal arch arteries (35), it was of obvious interest to find out whether this might influence any of the outlined steps in thyroid morphogenesis. Thus, wildtype and Tbx1−/− mice from embryonic ages between 9.5 and 13.5 dpc were collected and sections stained for Titf1/Nkx2.1 and, in some cases, for Foxe1 expression to identify the midline thyroid primordium.

The size of the thyroid placode and the number of Titf1/Nkx2.1-positive cells were not different in the Tbx1−/− mutants (data not shown), indicating that specification proceeded normally. The spatial relationship to the presumptive aortic sac was also not changed at this stage (data not shown). However, as budding and descent proceeded, the thyroid primordium in Tbx1-deficient embryos still kept contact with the endoderm. In contrast, in all age-matched wildtype embryos (7/7 examined at this time point), it had already detached and moved towards and closely apposed the aortic sac present deep in the midline mesoderm (Fig. 4A–D). Thus, the thyroid anlage failed to re-establish contact with the aortic sac at 11.5 dpc in all (7/7) Tbx1−/− mutants. The likely explanation is that closure of the budding process and the physical separation of thyroid and non-thyroid cells are disturbed when Tbx1 is lacking. Yet, at later stages, the thyroid bud evidently succeeds to detach and form a single lobe, indicating that budding merely is delayed, not blocked.

Of note, Tbx1 is apparently not required for the thyroid progenitor cells to express Foxe1 (Fig. 4A and B), the only thyroid transcription factor so far identified to play a direct role in thyroid migration during development (9). The likely explanation is that closure of the budding process and the physical separation of thyroid and non-thyroid cells are disturbed when Tbx1 is lacking. Yet, at later stages, the thyroid bud evidently succeeds to detach and form a single lobe, indicating that budding merely is delayed, not blocked.

On the basis of the examination of parasagittal serial sections, no caudal pharyngeal arch arteries could be distinguished in Tbx1−/− mutant embryos, and instead, the aortic sac was directly connected to the dorsal aorta (data not shown), which is in agreement with previous observations (35). The thyroid remnant in Tbx1−/− embryos approaches the presumptive carotid artery unilaterally during the lobulation stage

The earliest sign of thyroid lobulation in mouse is normally observed at 12.5 dpc when the midline thyroid anlage grows...
laterally in both directions in front of the proximal trachea (21). As illustrated in Figure 5A, in transverse sections capturing the lateral-most tissue portions that later will expand to form the lobes, it was not possible to simultaneously view the joining presumptive isthmus owing to the fact that the thyroid already at this stage attained a horseshoe-like shape (see also Fig. 2A and C for comparison with the final anatomy). At the presumptive lobe level, the midline thyroid anlage projected on both sides towards the paired ultimobranchial bodies that also expressed Titf1/Nkx2.1 (Fig. 5C), and 1 day later, these structures had fused to form the definitive lobes of the thyroid (Fig. 5E). In Tbx1−/− mutant embryos, no ultimobranchial bodies could be identified and the thyroid remained as a rounded or oval body in the midline with no or only modest signs of lateral expansion at 12.5 dpc (Fig. 5B). As revealed by serial sectioning, at this stage, the thyroid rudiment was fully dissociated from the pharyngeal endoderm (data not shown). However, the laryngotracheal groove representing the most proximal portion of the primitive trachea was located immediately dorsal to it and a separate esophagus lumen was also not distinguished at this level (Fig. 5B). This further indicates that there is a translocation defect in the Tbx1−/− mutant resulting in a more cranial position of the thyroid than in the corresponding wildtype embryos.

At 13.5 dpc, the localization of the thyroid in Tbx1-deficient animals was in most cases further changed to resemble more the final asymmetric phenotype, i.e. unilateral to the trachea (Fig. 5F and H). There was also a close juxtaposition between the thyroid and the carotid artery on the same side. In fact, Titf1/Nkx2.1 positive cells forming the outer contour of the thyroid were often found to almost adhere to the muscular layer of the vessel (Fig. 5I and J; as shown in Figure 5I, this pattern was occasionally found also at 12.5 dpc, indicating possible variation in the timing of thyroid lateralization in the Tbx1−/− mutant). In contrast to the normal development, the thyroid in Tbx1−/− mutants did not increase much in size between 12.5 and 13.5 dpc (Fig. 5A–H). This is most likely attributed at least in part to the lack of tissue normally contributed by the ultimobranchial bodies. However, as it is the progenitor cells of the midline thyroid that proliferate, not the ultimobranchial cells, after fusion (21), it is possible that this is also an early sign of retarded thyroid growth in the Tbx1−/− embryo.

**Tbx1 is not expressed in the developing thyroid but in adjacent mesenchyme**

Whether Tbx1 is expressed in the developing thyroid has not been previously investigated. We therefore studied this with indirect immunofluorescence, using a polyclonal antibody raised against recombinant Tbx1. Antibody specificity was confirmed by observations of Tbx1 immunoreactivity in embryonic tissues, e.g. otic vesicle epithelium, paraotic mesenchyme, teeth anlagen and hair follicles, which are known to express Tbx1 mRNA (22), the expected nuclear localization and lack of staining in Tbx1−/− embryos (data not shown).

As shown in Figure 6, Tbx1 was not expressed in the midline pharyngeal endoderm comprising the thyroid lobes of wildtype embryos (ub) co-express Titf1/Nkx2.1 and E-cadherin (E-cad). Ultimobranchial bodies and thymus (T) are present only in the wildtype embryo (A and C). Trachea (tr) and esophagus (es) are located in the midline (A and C); in the Tbx1−/− embryo, the thyroid is present at the level of the laryngotracheal groove (arrow). Size and position of carotid arteries (ca) are indicated by Pecam-1 staining (C and D). (E–H) Fusion of midline thyroid with ultimobranchial bodies at 13.5 dpc. The enlarged left and right thyroid lobes of the Tbx1+/+ embryo consist of engulfed ultimobranchial bodies with weaker Titf1/Nkx2.1 expression (E and G). The single thyroid body in the Tbx1−/− embryo deviates from the midline and approaches the carotid vessel (F and H). Gradual descent of the thyroid is indicated by its position at 13.5 dpc at a level below the division of the esophagus and trachea. Note that the isthmus portion of the thyroid joining the primitive lobes of wildtype embryos is present in other sections (more inferiorly) than shown in (A), (C), (E) and (G). (I and J) Association of thyroid remnant with ipsilateral carotid artery in Tbx1 homozygous knockout mice. (A–D) Bilateral growth of midline thyroid primordium (th) at 12.5 dpc. Both thyroid and ultimobranchial bodies (ub) co-express Titf1/Nkx2.1 and E-cadherin (E-cad). Ultimobranchial bodies and thymus (T) are present only in the wildtype embryo (A and C). Trachea (tr) and esophagus (es) are located in the midline (A and C); in the Tbx1−/− embryo, the thyroid is present at the level of the laryngotracheal groove (arrow). Size and position of carotid arteries (ca) are indicated by Pecam-1 staining (C and D). (E–H) Fusion of midline thyroid with ultimobranchial bodies at 13.5 dpc. The enlarged left and right thyroid lobes of the Tbx1+/+ embryo consist of engulfed ultimobranchial bodies with weaker Titf1/Nkx2.1 expression (E and G). The single thyroid body in the Tbx1−/− embryo deviates from the midline and approaches the carotid vessel (F and H). Gradual descent of the thyroid is indicated by its position at 13.5 dpc at a level below the division of the esophagus and trachea. Note that the isthmus portion of the thyroid joining the primitive lobes of wildtype embryos is present in other sections (more inferiorly) than shown in (A), (C), (E) and (G). (I and J) Association of thyroid remnant with ipsilateral carotid artery in Tbx1 homozygous knockout mice.
placode (Fig. 6A and E). Also, no Tbx1 immunoreactivity could be detected in the detached thyroid that had fulfilled the first step of translocation (Fig. 6B and F). At 9.5 dpc, cells present in the mesenchyme lining the wall of the aortic sac and also extending cranially towards the adjacent endoderm (but excluding the thyroid placode that faced the aortic sac endothelium directly) were weakly positive for Tbx1 (Fig. 6E). However, most strikingly, at 11.5 dpc, Tbx1 was strongly expressed in the midline mesenchyme that filled out the gap between the endoderm and the detached thyroid (Fig. 6F). On the basis of fluorescence intensity, it appeared as the expression was graded with the strongest signal in the core mesenchyme and close to the thyroid and weaker signals in the periphery and towards the endoderm proper.

Tbx1 was abundant in the endoderm of the presumptive fourth pharyngeal pouch from which the ultimobranchial bodies derive (data not shown). Tbx1 was also observed in the budding ultimobranchial bodies and the adjoining endoderm, although the expression was much weaker than in the paraxial mesoderm (Figs. 6C and G). However, when the midline thyroid was about to merge with the ultimobranchial bodies at 13.5 dpc, all Tbx1 seemed to be downregulated, including in the mesenchyme (Figs. 6D and H). Also in later stages of thyroid development (15.5–17.5 dpc), no Tbx1 could be found in the thyroid parenchyma or adjacent tissues (data not shown).

**DISCUSSION**

In this work, we show that Tbx1 null mutation causes a thyroid developmental defect that results in hemiagenesis of the gland during late mouse embryogenesis. Although the thyroid follicles appear structurally and functionally differentiated with normal size and thyroglobulin content at E17.5, the remnant tissue is severely hypoplastic constituting less than 25% of the normal gland volume. In all probability, this indicates that the total capacity to produce sufficient amounts of thyroid hormone is impaired. The clinical relevance of this phenotype is underscored by recent reports suggesting that thyroid dysfunction should be considered as a manifestation part of the 22q11DS (29,30), in which haploinsufficiency of the Tbx1 gene is responsible for the major developmental defects and features of disease (24). The prevalence of hypothyroidism is 20% in young adult 22q11DS patients (31), whereas it appears much less frequent in adolescents (28). A late onset of hypothyroidism may suggest that the disease is not congenital, but would also fit with the hypothesis of a hypoplastic thyroid with limited capability to supply thyroid hormone to the full-grown individual. Thyroid gland malformations have not been systematically investigated in the 22q11DS, but hemiagenesis, lobe hypoplasia or lack of isthmus were encountered in older autopsy materials (26,27). The present data represent the first experimental evidence indicating that the underlying cause of hypothyroidism in the 22q11DS is genetic and related to dysgenesis of the thyroid gland.

Many of the affected tissues and organs in the 22q11DS originate from the same developmental field, the pharyngeal apparatus that also contributes to the development of the thyroid gland. A thyroid phenotype in the Tbx1−/− mutant may therefore not be so surprising. However, the present findings indicate that the principal defective mechanism does not involve any known Tbx1-dependent morphogenetic event. According to current models, Tbx1, which is expressed in the pharyngeal endoderm, cell-autonomously initiates evagination of the endoderm into pharyngeal pouches, thereby participating in the segmentation of the primitive pharynx
It has furthermore been shown that Tbx1 in the endoderm affects pharyngeal arch artery formation non-cell-autonomously (36), possibly by a diffusible signal emanating from the pouch endoderm directed towards the arteries. The nature of this signal is not known, but members of the FGF family are attractive candidates given their overlapping expression with Tbx1 (38,39). Consequently, in Tbx1-deficient mice, the pharyngeal arches 2–6 and pharyngeal pouches 2–4 fail to develop (33,35), which is the main reason why aplasia of the thymus and parathyroid glands, typical features of the 22q11DS, characterize these animals. We confirm here that the ultimobranchial bodies originating from the fourth pouches are missing. As will be further discussed in what follows, lack of ultimobranchial bodies may contribute to the thyroid phenotype as they fuse with the median thyroid anlage during lobulation, but this probably does not explain the hemiagenesis. In Pax9–/– embryos, ultimobranchial bodies are not formed (40) but the thyroid still acquires a bilobed shape (Peters, personal communication).

That the thyroid succeeds to form in the Tbx1–/– mutant embryo is probably due to the fact that it is specified in the midline endoderm close to the first branchial arches that are not severely affected by Tbx1 deficiency (33,35). However, completion of the subsequent budding process was found to be much delayed, characterized by a persistent thyroglossal duct at 11.5 dpc and lack of re-association of the thyroid with the aortic sac. Failure to disintegrate the stalk of epithelial cells connecting the bud and the endoderm might hinder at a critical time point further caudal translocation necessary for the thyroid primordium to re-establish contact with the aortic sac, which concomitantly is gradually retracted along with the cardiac outflow tract towards the thoracic cavity. In the absence of Tbx1, normal remodeling of the pharyngeal epithelium might thus be disturbed, leading to persistence of the thyroglossal duct. In further support of a translocation defect, we observed that the dysplastic thyroid in late Tbx1–/– mutant embryos was located at the division of the trachea and esophagus, i.e. more cranially than the final position of the normal gland already achieved at the same time. It should be emphasized, however, that budding and detachment are eventually completed, indicating that it is not a true migration defect of the thyroid as observed in the Foxe1-deficient mouse (34). A more detailed estimation of the distance the thyroid moves in embryos lacking Tbx1 is difficult because of the developmental anomalies in neighboring structures (e.g. the larynx does not form properly), the relatively short stature of the neck (33) and that the animals are stillborn (making the postnatal position of the thyroid not possible to determine).

The mechanisms that normally regulate thyroid translocation during development are largely unknown. The only factor hitherto found to play an essential role in this process is Foxe1 acting cell-autonomously (34). Hence, the thyroid is retained and fails to dissociate and move downwards in Foxe1–/– mutant mice (9,34). Whether the embryonic thyroid progenitor cells actually migrate is controversial and therefore some authors refer the desent of the thyroid to a ‘relocalization’ process. In the present study, the expression of Foxe1 in the thyroid progenitor cells was not affected in the Tbx1 mutant embryos, and also the phenotype was clearly different from the Foxe1–/– mutant (9). We find it probable that the thyroid primordium at least partly moves by a non-cell-autonomous mechanism secondary to the influence of surrounding tissues in the neck region (41). On the basis of studies in avian embryos, it has been suggested that the thyroid descends by its own only to a minor extent and that the growing neck instead carries the evagination point in the endoderm cranially (42). We found that Tbx1 is not expressed in the thyroid at any stage, but that the mesenchyme located near the descending thyroid is strongly positive for Tbx1. Speculatively, Tbx1-dependent gene transcription may be involved in the proliferation and remodeling of mesenchyme subjacent to the midline endoderm and that this in turn is required to terminate budding and further displace the detached thyroid caudally towards the aortic sac. Also, in Tbx1–/– mutant embryos, the number of neural crest-derived cells in the mesenchyme surrounding the early thyroid bud seems to be much reduced (unpublished data). This opens the possibility that closure of the budding process and caudal translocation of the thyroid might be disturbed when neighboring cells of neural crest origin are lacking. Indeed, neural crest ablation experiments in chick embryos lead to thyroid agenesis or hemiagenesis (43), and it has therefore been suggested that neural crest-derived mesenchyme is important for thyroid bilobation. However, it could not be excluded that neural crest ablation influences thyroid morphogenesis indirectly via disturbed development of the arch arteries (44). This does not rule out that other mechanisms may operate to coordinate and guide the directed movement of the thyroid primordium. For example, the aortic sac endothelium may produce paracrine factors that serve as chemotacticants for the thyroid; if the distance gets to long, gradients of chemoattracting molecules may vanish or be inappropriate for the normal developmental process.

Recently, we showed that bilateral growth of the median thyroid anlage after it resumed contact with the aortic sac takes place along the course of the third pharyngeal arch arteries and proposed that the arch vessels function as guiding tracks for the proliferating progenitor cells to later reach the ultimobranchial bodies (21). Since the thyroid does not translocate properly, fails to contact the aortic sac at 11.5 dpc and no normal arrangement of pharyngeal arch arteries exists in the Tbx1–/– mutant (the aortic sac connects directly to the dorsal aorta), it might perhaps be expected that the lobulation process also goes wrong. That the thyroid was found to develop normally in Tbx1+/– embryos in which the third pharyngeal arch arteries are present whereas the fourth are lacking (35) further supports the notion about importance of the third arch arteries in the lobulation process. It could be argued that a symmetric lack of pharyngeal arch arteries offers no explanation to the assymmetric phenotype (hemiagenesis) recognized in Tbx1-deficient embryos at 17.5 dpc. However, a clue to this may yet come from the suggested interaction with large vessels. At the time of lobulation, the thyroid remnant present in the midline and all surrounded by mesenchyme was found to deviate unilaterally and often acquire close contact with the presumptive carotid segment of the dorsal aorta on the same side of the neck. Although we do not know the nature of the attraction signals, this likely reflects the inborn property of the thyroid
tight relationship between thyroid morphogenesis and
vascular anomalies. In different studies, 3–12% of patients
association between thyroid development defects and cardio-
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epithelium at 17.5 dpc (21). Speculatively, contact with the
small portion of the thyroid lobes consists of ultimobranchial
i.e. after the fusion with the growing median thyroid anlage,
all cells of ultimobranchial origin, for unknown reasons,
seize to proliferate, whereas cells from the midline anlage
proliferate intensively. This leads to that only a relatively
Tbx1-deficient embryos where this contact is lacking.

Taken together, we find that Tbx1 regulates mouse thyroid
development by a non-cell-autonomous mechanism that
mainly influences the growth and translocation of the
median primordium subsequent to budding of the thyroid
placode in the pharyngeal endoderm. Tbx1 deficiency results
in hemiagenesis and hypoplasia of the thyroid gland. Failure
of the embryonic thyroid to establish contact with vessels
derived from the cardiac outflow tract at a critical step neces-
sary for the proper guidance of bilateral growth and lobulation
is suggested to cause the phenotype. In a clinical perspective,
this study provides the first direct experimental evidence of an
association between thyroid development defects and cardio-
vascular anomalies. In different studies, 3–12% of patients
with CH due to thyroid dysgenesis have congenital heart
disease (16,17). Several reports of ectopic thyroid tissue
associated with the heart outflow tract (47) underscore a
tight relationship between thyroid morphogenesis and

hemoglobin, the similarities between the thyroid phenotype found in
of which is to attract the thyroid primordium. We have
recently shown that the thyroid fails to form symmetric
lobes in Shh−/− mutant embryos (18). It is previously
known that Shh positively regulates Tbx1 expression (22,23)
and that also in Shh+/− mutant embryos, the architecture
of the pharyngeal arch arteries is disturbed (23,46). Given
the similarities between the thyroid phenotype found in
Shh−/− embryos and Tbx1−/− embryos, it seems reasonable
to assume that they are both operative in a non-cell-
autonomous mechanism that regulates thyroid bilobation,
where Shh probably is upstream of Tbx1.

At 17.5 dpc, the thyroid parenchyma in Tbx1−/− embryos
comprised less than 25% of the total thyroid volume in age-
matched control littermates, suggesting that Tbx1 influenced
thyroid growth as well. The size reduction may be related to
the fact that there was no contribution of cells from the
missing ultimobranchial bodies. However, previous exper-
iments on normal embryos indicate that the ultimobranchial
bodies are of minor importance for the accelerated lobe enlarg-
gement in late embryogenesis (21). Specificially, at 13.5 dpc,
i.e. after the fusion with the growing median thyroid anlage,
all cells of ultimobranchial origin, for unknown reasons,
seize to proliferate, whereas cells from the midline anlage
proliferate intensively. This leads to that only a relatively
small portion of the thyroid lobes consists of ultimobranchial
epithelium at 17.5 dpc (21). Speculatively, contact with the
ultimobranchial bodies induces accelerated proliferation in the
midline thyroid anlage, an effect that is lost in Tbx1-deficient embryos where this contact is lacking.

The following antibodies were used for immunohistochemical
staining: rabbit pAb against calcitonin (DAKO, Glostrup,
Denmark); rat mAb against E-cadherin [ECCD-2, kindly
provided by Henrik Semb (50)]; rat mAb against Pecam-1
(Pharmingen, Stockholm, Sweden); rabbit pAb against Tgf
(DAKO); rabbit mAb against Titf1/Nkx2.1 (Biopat, Milan,
Italy); rabbit pAb against Foxe1/TTF-2 (Biopat); rabbit pAb
against Tbx1 (Zymed Laboratories, San Fransisco, CA);
biotin-conjugated donkey anti-rat and Rhodamine
Red 110-X-conjugated donkey anti-rabbit F(ab')2 fragments
(Jackson ImmunoResearch, West Grove, PA). Streptavidin-
FITC was purchased from DAKO.

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MATERIALS AND METHODS

Animals

Embryos were obtained from mating of Tbx1 +/− (32) or
C57BL/6 mice (Charles River, Sulzfeld, Germany). Embryo-
age was estimated by considering the morning when a
vaginal plug was detected as 0.5 dpc. Embryos were collected
at 9.5, 11.5, 12.5, 13.5, 15.5 and 17.5 dpc. Genotyping of
transgenic embryos was performed as previously described
(32). Animal handling and experiments were approved by
the local ethic committees at the Albert Einstein College of
Medicine and Göteborg University.

Immuneoagents

Embryos were fixed with 4% paraformaldehdye in HBS
(10 mM Hepes, pH 7.4, 150 mM NaCl) overnight at 4°C. For
cryoprotection of tissues, all samples were incubated over-
night at 4°C in 30% sucrose solution in HBS with 1 mM
CaCl2 before embedding in Tissue Tek compound (Sakura,
Zoeterwoude, The Netherlands) and freezing at −80°C.
Ten micrometre thick sections were cut on a cryostat (Leica,
Göteborg, Sweden) and collected on polylysine glass slides
(Vector, Burlingame, MA). Sections were permeabilized by
incubation in PBS with 0.1% Triton X-100 (PBS/Tx) for
20 min and blocked in PBS with 2% normal donkey serum
(Jackson ImmunoResearch) for 1 h. The sections were then
incubated overnight at 4°C with primary antibodies diluted
in blocking buffer. Secondary antibodies were added in block-
ing buffer for 60 min at room temperature. Streptavidin-FITC
was added for 30 min at room temperature. Immunolabeled
cells were counterstained with DAPI (Sigma-Aldrich,
St Louis, MO) to visualize cell nuclei. All immunoreagent
incubations were separated by washing in PBS/Tx for
3 × 5 min. Immunolabeled sections were examined in a
Nikon Microphot FXA epifluorescence microscope equipped

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with a QLC100 confocal laser scanning module (VisiTech International, Sunderland, UK). Images were processed using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD) and CorelDraw software.

Three-dimensional reconstruction and morphometry

Serial images of consecutive 10 μm sections of the neck region spanning the entire thyroid were captured by confocal microscopy. Since a single image did not cover the entire region of interest, mosaics from overlapping fields were generated using the GlueMRC software (51). Alignment of mosaicked stacks was performed by the LinkMRC software (51). Vector paths outlining organs of interest were created using Adobe Illustrator CS and three-dimensional rendering was done in Form-Z.

The relative volume of the thyroid remnant in \( Tbx1^{-/-} \) mutant embryos \((n = 4)\) or the thyroid lobes of wildtype littermates \((n = 4)\) was estimated by measuring the area of thyroid tissue in every fourth section, using the Image Pro Plus software (Media Cybernetics).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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REFERENCES


