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Animal Model

Genetic Deletion of *Sonic Hedgehog* Causes Hemiagenesis and Ectopic Development of the Thyroid in Mouse

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Thyroid dysgenesis encountered in 85% of patients with congenital hypothyroidism is a morphologically heterogeneous condition with primarily unknown pathogenesis. Here we identify *sonic hedgehog* (*Shh*) as a novel regulator of thyroid development. In *Shh* knockout mice the thyroid primordium is correctly specified in the pharyngeal endoderm, but budding and dislocation are slightly delayed. In late development the thyroid fails to form a bilobed gland. Instead a single thyroid mass is found unilaterally and mostly to the left of the midline. Thyroid-specific transcription factors (TTF-1 and TTF-2) and thyroglobulin are expressed indicating terminal differentiation. Strikingly, TTF-1- and TTF-2-positive cells aberrantly develop in the presumptive trachea of *Shh*−/− embryos. The ectopic tissue buds ventrolaterally into the adjacent mesenchyme, and less extensively into the tracheal lumen, forming follicle-like structures that accumulate thyroglobulin. Shh mRNA is not expressed in the thyroid precursor cells at any developmental stage. The results indicate that Shh signaling indirectly governs the symmetric bilobation of the thyroid during late organogenesis. Shh also seems to repress inappropriate thyroid differentiation in non-thyroid embryonic tissues. This study provides clues to the molecular mechanisms that might be dysregulated in thyroid hemiagenesis and development of ectopic thyroid tissue outside the thyroglossal duct. *(Am J Pathol 2004, 164:1865–1872)*

Congenital hypothyroidism (CH) affects 1 in 3000 to 4000 infants and is one of the most common preventable causes of mental retardation.[1] Developmental anomalies of the thyroid gland, thyroid dysgenesis, account for 85% of all cases with CH. This condition is phenotypically heterogeneous and includes thyroid agenesis or hemiagenesis, thyroid hypoplasia, thyroglossal duct cysts, and ectopic thyroid tissue.[2] Monozygotic twins are generally discordant for thyroid dysgenesis suggesting that this malformation most often is due to postzygotic events such as epigenetic modifications, early somatic mutations, or stochastic developmental events.[3] However, a recently identified familial occurrence of CH associated with thyroid dysgenesis suggests the involvement of hereditary factors.[4] Evaluation of asymptomatic first-degree relatives of children with CH has revealed a high frequency of different thyroid developmental abnormalities.[4,5] This suggests that the thyroid phenotype may be variable despite a common genetic alteration. Therefore, characterization of master genes involved in thyroid morphogenesis is likely to provide clues to the development of CH.

Specification of the thyroid gland in the floor of the primitive pharynx can first be distinguished in the mouse embryo at 9 days post coitum (dpc).[6] The primordium buds off from the foregut endoderm and descends at 10 to 15 dpc to the sublaryngeal position after which the cells start to organize into follicles. At this time the expression of proteins involved in thyroid hormone biogenesis, ie, thyroglobulin (Tg), thyroperoxidase, and sodium-iodide symporter, is initiated.[7] Recent results on cadherin expression during thyroid development suggest that the translocation of the thyroid anlage to its final position probably does not involve active migration of individual cells but might rather be secondary to the growth and expansion of surrounding tissues.[8] Indeed, earlier studies have suggested that the developing thyroid after bud-

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ding and detachment is carried along with the caudal movement of large vessels.[9] It is known that the developmental process of the thyroid is governed by the anteroposterior polarity transcription factor Hoxa-3[10] as well as by the conjoint action of the thyroid transcription factors (TTF)-1 and TTF-2 and Pax8.[11–13] Interestingly, murine knockout models of these genes produce phenotypes resembling CH. Mice deficient in TTF-1 are athyreotic,[11] and TTF-2 knockouts either lack a thyroid gland or retain an ectopic, sublingual thyroid rudiment.[12] In Pax8-null mutant mice thyroid follicular cells are lacking whereas C cells develop normally.[13] However, extensive search for germline mutations in the homologous genes in humans with thyroid dysgenesis has identified very few cases of TTF-1, TTF-2, or Pax8 mutations.[14–16] The molecular defects underlying CH because of impaired thyroid development thus remain primarily unknown, and it is likely that other transcription factors and effector molecules are involved.

Members of the hedgehog family are soluble ligands for Patched (Ptc) receptors and act as key regulators of embryogenesis. A hedgehog gene was originally identified as a determinant of segment polarity in *Drosophila*.[17] However, the activities of vertebrate hedgehog homologues are known to orchestrate a wide spectrum of developmental processes ranging from left-right axis determination of the embryo to tissue patterning and organogenesis including cells derived from all three germ layers.[18] *Sonic hedgehog* (*Shh*) is of particular interest because it has been found to be essential to foregut development.[19] Specifically, separation of the esophagus and trachea and further branching of the distal airways fail in *Shh*−/− mice.[19,20] The morphogenesis of other endodermal derivatives like the pancreas is also influenced by *Shh* signaling.[21,22] Whether Shh has a role in thyroid development has not previously been investigated.

In this study, we show that Shh-deficient mice display distinct features of thyroid dysgenesis. Whereas the thyroid anlage buds normally during early morphogenesis, the gland fails to separate into two distinct lobes and instead forms a single thyroid mass that is located unilaterally to the presumptive trachea. In addition to hemiagenesis, ectopic thyrocytes expressing Tg aberrantly develop from the primitive respiratory epithelium.

Materials and Methods

Animals

Embryos were obtained from natural matings of *Shh* +/− mice.[23] Embryonic age was estimated by defining the morning when a vaginal plug was detected as 0.5 dpc. After cervical dislocation of pregnant animals, embryos were collected at 9.5, 11.5, 12.5, 15.5, and 17.5 dpc. Five or more mutant embryos were investigated at each developmental stage. *Shh* knockout pups were stillborn and could not be investigated because of rapid cannibalization.[23] Animal handling and experiments were approved by the local ethic committee at Göteborg University.

Immunoreagents

The following antibodies were used for immunohistochemical staining: rabbit polyclonal antibody against TTF-1 (Biopat, Milan, Italy),[24] rabbit polyclonal antibody against TTF-2 (provided by Prof. Roberto di Lauro, Stazione Zoologica Anton Dohrn, Naples, Italy),[25] rat monoclonal antibody against E-cadherin (ECCD-2) (provided by Prof. Henrik Semb, Department of Medical Biochemistry, Göteborg, Sweden),[26] rabbit polyclonal antibody against Tg (DAKO, Glostrup, Denmark),[24] and rhodamine red-X-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-rat IgGs (Jackson ImmunoResearch, West Grove, PA).

Immunohistochemistry and in Situ *Hybridization*

Embryos were fixed with 4% paraformaldehyde in HBS (10 mmol/L Hepes, pH 7.4, 150 mmol/L NaCl) overnight at 4°C. For cryoprotection of tissues, samples were incubated overnight at 4°C in 30% sucrose solution in HBS with 1 mmol/L $CaCl_2$ before embedding in Tissue Tek compound (Sakura, Zoeterwoude, The Netherlands) and freezing at −80°C. Ten-μm-thick sagittal or transversal sections were cut on a Micron cryostat and collected on polylysine glass slides (Vector, Burlingame, CA). Sections were permeabilized by incubation in phosphate-buffered saline (PBS) with 0.1% Triton X-100 for 20 minutes before being blocked in PBS with 1.5% fetal bovine serum for 60 minutes at room temperature. The sections were then incubated overnight at 4°C with primary antibody diluted in blocking buffer. Secondary antibodies were added in Tris-buffered saline-Ca^{2+} supplemented with 1.5% fetal bovine serum for 60 minutes at room temperature. The sections were examined and photographed in a Nikon Microphot FXA epifluorescence microscope equipped with a QLC100 confocal laser-scanning module (VisiTech International, Sunderland, UK).

For *in situ* hybridization, embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight and then processed for paraffin embedding. Sections were prepared with ^{35}S-UTP-labeled anti-sense riboprobes as described.[27] A *Hind*III-linearized 642-bp mouse *Shh* template in pBSK[28] and a *Bam*HI-linearized 841-bp mouse *Ptc1* template in pBSK[29] were transcribed with T3 RNA polymerase to generate anti-sense probes. For generation of sense riboprobes, the *Shh* and *Ptc1* templates were linearized with *Xba*I and *Hind*III, respectively, and transcribed using T7 RNA polymerase. Probes were used at 60,000 cpm in hybridization buffer consisting of 60% formamide, 300 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8, 5 mmol/L ethylenediaminetetraacetic acid, 10% dextran sulfate, 0.5 mg/ml yeast tRNA, and 1× Denhardt's solution (added from a 50× stock: 10 mg/ml bovine serum albumin, 10 mg/ml Ficoll, and 10 mg/ml polyvinylpyrrolidone). Sections were hybridized overnight at 56°C. After posthybridization washes, the slides were dipped in Kodak NTB2 emulsion (diluted 1:1 v:v with distilled water). After a 2-week exposure time, the slides were developed in Kodak D19 developer and fixed with Kodak

sodium fixer, according to the manufacturer's instructions, and counterstained with Richardson's azure II-methylene blue. Dark-field and bright-field photographs were taken with a Nikon FX-350DX camera mounted on a Nikon Optiphot-2 microscope. The micrographs were digitalized and color adjustment of the dark-field micrographs generated red-colored silver grains indicating the signal. As expected from these well-characterized probes, sections hybridized with the sense probes did not display signal above background levels.

Results

Key Events in Early Thyroid Morphogenesis Are Initiated in Shh−/− Mice

Specification of the thyroid primordium is first characterized by the onset of TTF-1 and TTF-2 expression.[7] The thyroid precursor cells then form a caudally directed protrusion that becomes completely dissociated from the pharyngeal endoderm at 11.5 dpc (Figure 1, A and B, and inset). In *Shh*−/− embryos, the thyroid was also found to be correctly specified (Figure 1, C and D) and the anlage appeared as a single bud similar to that of control mice. However, at 11.5 dpc the bud was still connected with the overlying epithelium by a narrow stalk of TTF-1-positive cells (Figure 1C), probably representing the thyroglossal duct that already had regressed in control animals of the same age (Figure 1B). At 12.5 dpc, the thyroid cell mass in *Shh*−/− animals had dislocated from the site of origin in the pharyngeal floor (Figure 1, E and F). However, the distance of caudal movement was notably shorter than that found in age-matched controls and also when compared to 11.5-dpc wild-type embryos (compare Figure 1, E and F, with inset in B). As previously reported,[8] the early developing thyroid was located close to a vessel presumably corresponding to the aortic outflow tract (Figure 1; A to F), that might be involved in downward thyroid migration. The spatial relationship between this vessel and the thyroid was essentially maintained in mice lacking Shh, although some mutants showed a slightly thicker zone of interfacing mesenchyme (Figure 1; C to F). No alterations in thyroid morphogenesis could be detected at these or later stages in *Shh* +/− embryos (data not shown).

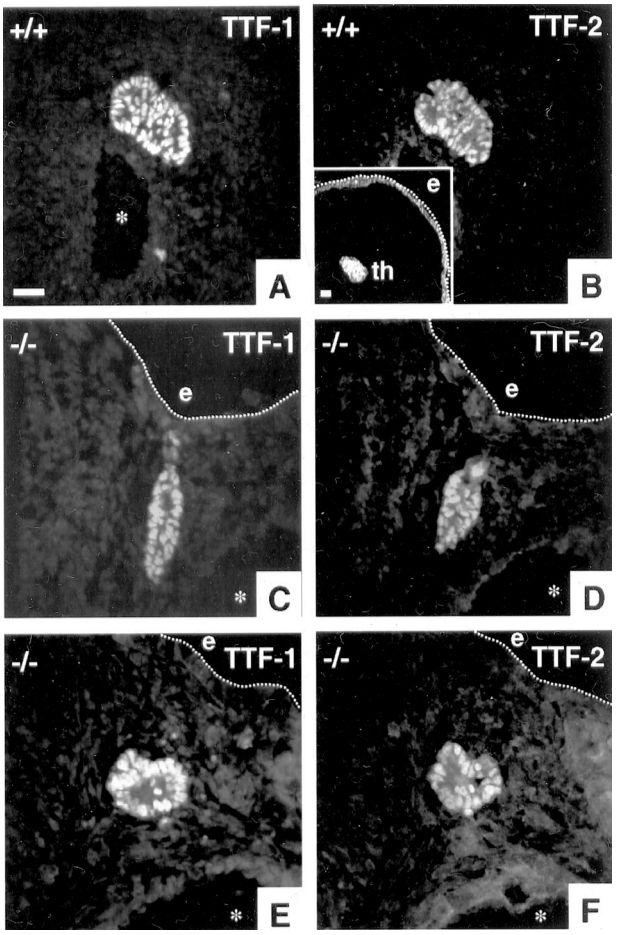

Figure 1. TTF-1 and TTF-2 expression in early thyroid development in normal and *Sbh*−/− mouse embryos. Immunofluorescent staining of TTF-1 (**A**) and TTF-2 (**B**) on serial sagittal sections of an 11.5-dpc control embryo showing the thyroid anlage closely apposed to the aortic outflow tract. E-cadherin immunostaining of the same region in lower magnification (**B**, **inset**) illustrates the distance to the overlying endoderm. TTF-1 (**C**, **E**) and TTF-2 (**D**, **F**) immunostaining showing budding and dislocation of the thyroid primordium in *Sbh*−/− embryos at 11.5 (**C**, **D**) and 12.5 dpc (**E**, **F**). The apical side of the foregut endoderm is indicated by **broken lines**. e, foregut endoderm; th, thyroid primordium. Lumen of blood vessel is indicated by an **asterisk**. Scale bar, 50 μm.

The Thyroid Primordium Fails to Form Two Lobes in Shh−/− Mice

In 15.5-dpc control embryos, the developing thyroid had separated into two lobes of equal size located on the lateral sides of the proximal trachea (Figure 2A). Closer examination showed that the lobes were connected by a very thin pretracheal isthmus (data not shown). At this stage functional differentiation of the precursors to Tg-producing thyrocytes was also evident (Figure 2B). At 17.5 dpc the thyroid lobes of control embryos had increased in size (Figure 2E) and small follicles containing Tg in the central lumina were formed (Figure 2F). In contrast, a thyroid consisting of two symmetric lobes was never detected in *Shh*−/− mice. In 15.5-dpc mutant embryos, the thyroid still appeared as a single midline tissue mass (Figure 2C), whereas 2 days later it was found to be displaced laterally and most often (six of seven examined embryos) to the left side of the presumptive trachea (Figure 2G). The size of the lateralized thyroid was similar to that of a single lobe in normal mice (compare Figure 2, F and H). Despite these gross morphogenetic defects the *Shh*−/− thyrocytes developed normally into a polarized epithelium, as indicated by the presence of E-cadherin in the basolateral plasma membrane and formation of follicles (Figure 2G, inset). Moreover, the cells readily produced Tg (Figure 2, D and F) that was accumulated in the follicle lumina (Figure 2H, inset). Another striking observation was the appearance of large aberrant vessels located closely to the thyroid tissue (Figure 2, G and H). These vessels were often found to protrude deep into and passing through the unilateral thyroid cell mass (Figure 3).

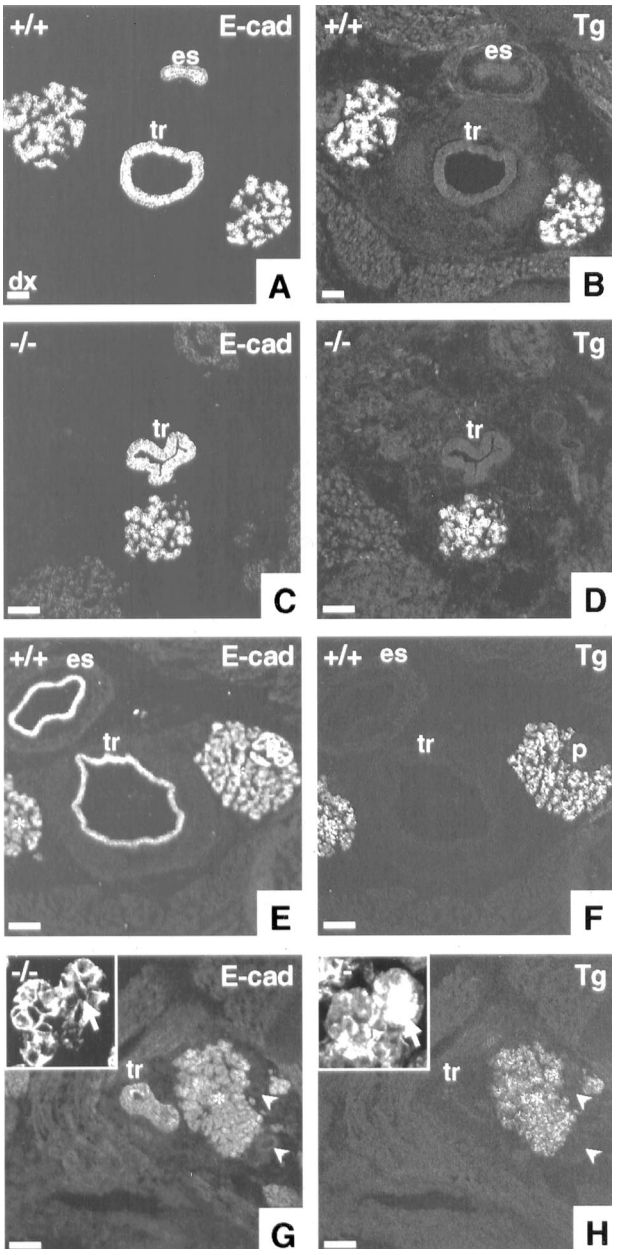

Figure 2. E-cadherin and Tg expression in late thyroid development in normal and *Shh*−/− embryos. Immunofluorescent staining of E-cadherin (E-cad) (**A**, **C**, **E**, **G**) and Tg (**B**, **D**, **F**, **H**) on serial transversal sections of 15.5-dpc *Shh*+/+ (**A**, **B**), 15.5-dpc *Shh*−/− (**C**, **D**), 17.5-dpc *Shh*+/+ (**E**, **F**) and 17.5-dpc *Shh*−/− (**G**, **H**) embryos. High-magnification **insets** in **G** and **H** indicate follicle-like structures with accumulation of Tg in lumen (**arrow**). Large vessels closely associated with the thyroid tissue in 17.5-dpc *Shh*−/− embryo are indicated by **arrowheads**. dx, right side of embryos; es, esophagus; tr, trachea. Thyroid tissue is indicated by an **asterisk**. Scale bars, 100 μm.

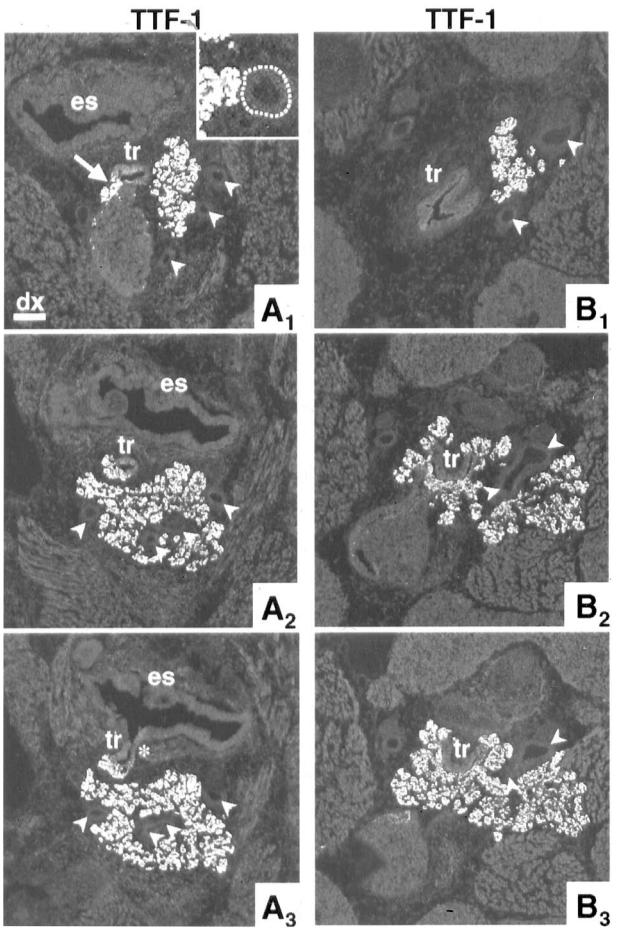

Figure 3. TTF-1 immunostaining of transversal sections at three levels along the cranio-caudal axis (**1** to **3**) of the thyroid primordium in two (**A** and **B**) 17.5-dpc *Shh*−/− embryos. **A_1 to A_3:** TTF-1-positive cells of the thyroid are seen mainly to the left of the presumptive trachea. Buds of TTF-1-positive cells originating from the pharyngotracheal epithelium and not making contact with the thyroid primordium are noted (**arrow**). Tracheo-esophageal fistulation is indicated by an **asterisk**. **B_1 to B_3:** TTF-1-positive cells of the thyroid are seen mainly to the left of presumptive trachea, whereas the esophagus cannot be clearly distinguished. Numerous buds of TTF-1-positive cells extend from the pharyngotracheal epithelium and make contact with the thyroid lobe. Blood vessels passing through the thyroid tissue are indicated (**arrowheads**). High-magnification **inset** in **A_1** shows a large aberrant blood vessel (**broken lines**) close to thyroid cells. dx, right side of embryos; es, esophagus; tr, presumptive trachea. Scale bar, 100 μm.

Budding of TTF-1-Positive Cells from the Presumptive Trachea in Shh−/− Mice

In *Shh* knockouts at 17.5 dpc a restricted number of epithelial cells lining a segment of the presumptive trachea were found to express TTF-1 (Figure 3). A similar expression pattern was not observed in the proximal airways of wild-type embryos (data not shown), which previously are known to express TTF-1 only in early development.[30] The aberrant TTF-1-positive cells were mainly located at the same level as the unilateral thyroid. Because of the lack of a distinct laryngeal border in Shh-deficient mice, it was difficult to precisely define the proximal limitation of the trachea (which therefore also is referred to as the pharyngotracheal tube). Strikingly, the TTF-1-positive cells protruded into the adjacent mesenchyme, and often formed buds of tightly apposed cells that appeared to be pinched off from the primitive respiratory epithelium (Figure 3). Serial sectioning revealed that such budding was mostly confined to the ventral or ventrolateral aspects of the trachea (Figure 3). In cases in which ectopic budding was less pronounced, the TTF-1-positive cells originating from the trachea were clearly separated from the thyroid lobe (Figure 3A). However, in several *Shh*−/− embryos the budding from the trachea was so extensive that it

often coalesced with the caudal pole of the single thyroid lobe close to the midline (Figure 3B).

Shh-Deficient Pharyngotracheal Cells Differentiate into Ectopic Thyrocytes

Sagittal sections of 15.5- and 17.5-dpc Shh-deficient embryos showed that the aberrant budding of TTF-1-positive cells extended over a considerable length of the pharyngotracheal tube (Figure 4C). Interestingly, double immunostaining revealed that these cells co-expressed TTF-2 (Figure 4, B and D), which normally is not expressed in the developing airways.[25] That the TTF-1- and TTF-2-positive cells indeed had differentiated into thyrocytes was indicated by Tg immunostaining. The cells growing into the mesenchyme as well as those integrated within the pharyngotracheal epithelium were found to express Tg (Figure 4; E to H). This notion was further supported by the observation that the ectopic buds often formed small follicle-like structures (Figure 4, F and H). TTF-1- and TTF-2-positive cells sometimes also protruded into the lumen of the presumptive trachea (Figure 4, A and B).

Shh Expression Is Excluded from the Thyroid Primordium

The localization of Shh in the normal developing thyroid and adjacent embryonic tissues was investigated by *in situ* hybridization. In 9.5-dpc embryos Shh was found to be present throughout the endoderm except in the stretch of TTF-1-expressing cells (Figure 5, A and B). At 11.5 dpc the thyroid precursor cells had moved caudally and were, in contrast to the overlying endoderm, still negative for Shh (Figure 5, C and D). In 15.5-dpc embryos, Shh was undetectable in the thyroid, whereas Shh transcripts were readily detected in the mucosal lining of the trachea and esophagus (Figure 5, E and F). Shh activity within these epithelia was further corroborated by Ptc1 *in situ* hybridization; a strong Ptc1 signal was observed in the subepithelial mesenchyme of both trachea and esophagus (data not shown), confirming previous findings.[19] In contrast, no signal above background level could be detected in the mesenchyme surrounding the thyroid at any developmental stage (data not shown).

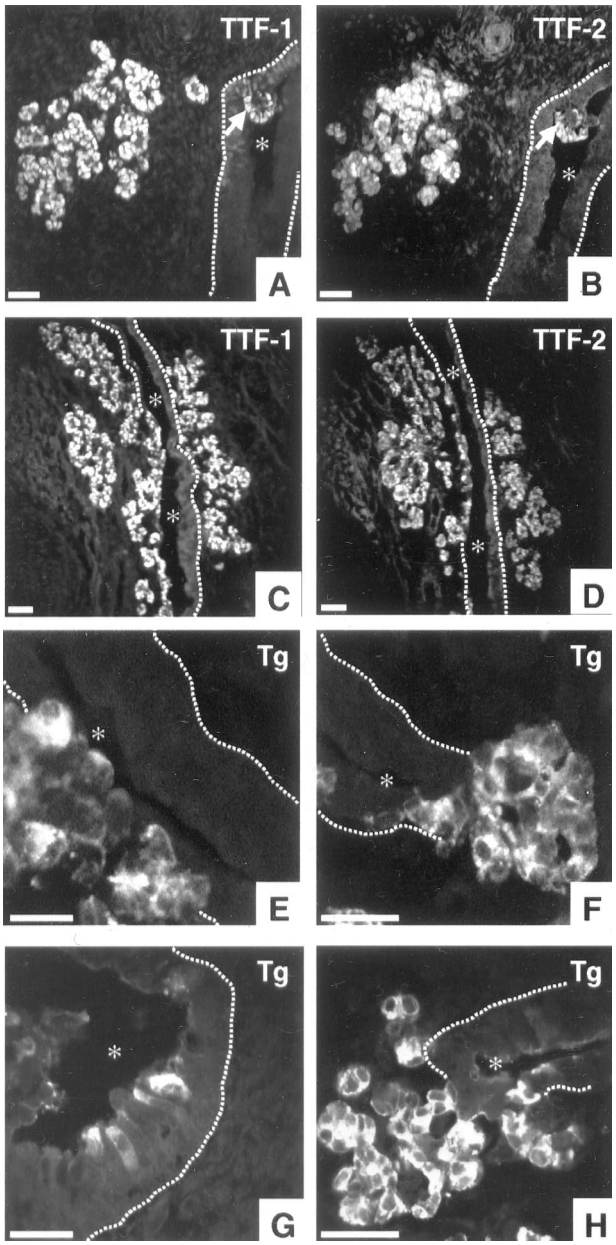

Figure 4. TTF-1, TTF-2, and Tg expression in the presumptive trachea of 15.5- and 17.5-dpc *Sbh*−/− embryos. Images are from immunostaining of serial sagittal (**A** and **B**, **C** and **D**) or transversal (**E** and **F**, **G** and **H**) sections. **A** and **B:** Buds of cells positive for TTF-1 and TTF-2 protrude into the pharyngotracheal lumen (**arrows**) of 15.5-dpc embryos. **C** and **D:** Cells positive for TTF-1/TTF-2 are present within the tracheal epithelium of 17.5-dpc embryos and protrude into the surrounding mesenchyme. **E** to **H:** Cells expressing Tg are located within the primitive respiratory epithelium of 15.5- and 17.5-dpc embryos (**E**, **G**) and protrude into the surrounding mesenchyme (**F**, **H**). Lumen of the presumptive trachea is indicated by an **asterisk**. The basal side of the tracheal epithelium is indicated by **broken lines**. Scale bars, 50 μm.

Discussion

Genetic deletion of *Shh* in transgenic animals causes severe organ malformations.[18] Early foregut defects are prominent features, eg, the trachea and esophagus do not separate correctly but communicate via a fistula or share a common lumen.[19] Late development of foregut derivatives may also be disturbed in Shh-deficient animals, as illustrated by a reduced branching of the distal airways and lack of alveolar maturation.[20] It has previously not been investigated if Shh plays a role in thyroid morphogenesis. In this work, we show that thyroid specification and the subsequent budding of the thyroid primordium into the surrounding mesenchyme proceed without major disturbances in *Shh*−/− mice. However, instead of being divided into two lobes connected by a thin pretracheal isthmus, the thyroid of Shh-deficient mice maintains the shape of a single tissue mass throughout development. Moreover, the final position of the gland is always unilateral, most often to the left side of the presumptive trachea, and its size equals that of a single lobe in age-matched control embryos. Another remarkable

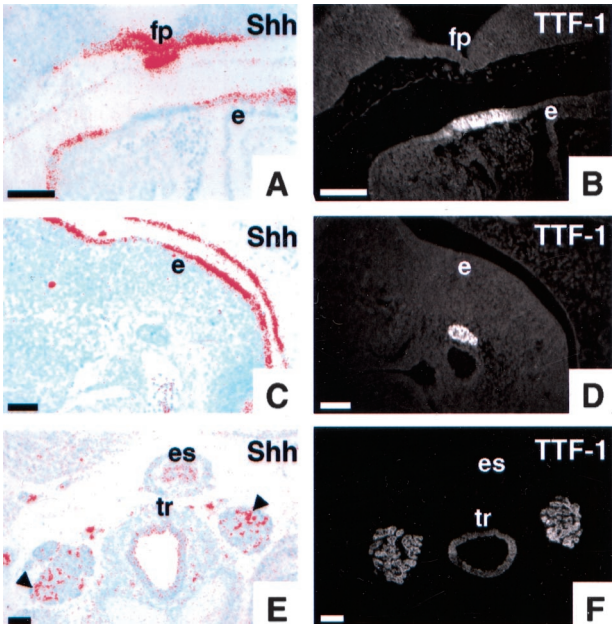

Figure 5. *In situ* hybridization for Shh in 9.5- to 15.5-dpc wild-type mouse embryos (**A**, **C**, **E**). Thyroid precursor cells were identified by TTF-1 immunostaining on parallel sections (**B**, **D**, **F**). **A** and **B:** Shh transcripts are detected in the entire foregut endoderm except for the thyroid anlage at 9.5 dpc. Shh transcripts are also undetectable in the thyroid anlage at 11.5 dpc (**C**, **D**) and 15.5 dpc (**E**, **F**). The signal in the thyroid at 15.5 dpc (**arrowheads** in **F**) is artifactual and because of refractile properties of red blood cells. e, foregut endoderm; es, esophagus; fp, floor plate; tr, trachea. Scale bars, 200 μm.

feature of *Shh*-null mutants is that ectopic thyroid tissue aberrantly develops from the primitive respiratory epithelium of the presumptive trachea.

Both of the thyroid malformations (hemiagenesis and ectopia) observed in mice lacking Shh have clinical counterparts in man. Hemiagenesis of the thyroid is encountered in 2 of 1000 births, mostly affecting the left lobe,[31] and may be associated with CH.[2] Ectopic thyroid tissue within the respiratory tract is probably more infrequent, but there are several reports in the literature that highlight this condition as a rare but important cause to severe airway obstruction.[32–34] Interestingly, both hemiagenesis and ectopic thyroid tissue resembling the present findings may occur in the same patients.[35–37] The possible contribution of impaired Shh activity to this phenotype in humans remains to be investigated. However, there are pieces of circumstantial evidence suggesting that this might be the case. A single-lobed thyroid was part of the section findings in a stillborn child[38] that in addition had several developmental defects, eg, tracheo-esophageal fistualtion, pulmonary hypoplasia, and holoprosencephaly, typical of Shh deficiency.[19,23] Further tentative connections between impaired Shh signaling and thyroid dysgenesis in man are suggested from recent studies on Williams syndrome, a complex genetic disorder with multiorgan involvement caused by a deletion at 7q11.23.[39] Thyroid hemiagenesis with or without CH has been described in several cases of Williams syndrome,[40,41] pointing out the possibility that genes involved in thyroid development may be contiguous to the deleted chromosomal region. Interestingly, genes implicated in Shh signaling as *Frizzled* and *bHLH* have been mapped to the 7q11.23 deletion.[42,43] Moreover, combined microdeletions of both 7q11.23 and 7q36, which harbors the *Shh* gene, have been reported.[44]

In situ hybridization showed that Shh expression was lacking in the portion of the pharyngeal endoderm from which the thyroid develops. In fact, a complete overlap between TTF-1-positive and Shh-negative cells was obvious when evaluating hybridized and immunostained serial sections. This is strikingly similar to the Shh distribution in the foregut-midgut transitional zone, in which Shh is ubiquitously expressed in the endoderm apart from the restricted areas that specify the two pancreas primordia.[21] Inappropriate expression of Shh in the posterior stomach results in hypoplasia of the dorsal pancreas.[45] Conversely, in Shh-deficient mice the pancreas is proportionally larger than in wild-type animals.[22] Taken together, this indicates that absence of Shh in the endoderm is a prerequisite for normal pancreas development. Although we did not observe any obvious differences in size of the early thyroid primordium between *Shh*−/− and *Shh*+/+ animals, it is reasonable to assume that Shh contributes to a molecular boundary also for thyroid development in the foregut. However, other factors are likely to be required to induce thyroid specification in the region of Shh-deficient endoderm. In this respect, recent observations that blood vessels provide inductive signals necessary for pancreatic organogenesis are of particular interest.[46] We have previously shown that the cells in the pharyngeal floor that express thyroid transcription factors at 9.5 dpc are in close contact with the endothelium of the early cardiac outflow tract.[8] Also at later stages the thyroid primordium is in close proximity to the developing aorta, or one of its major branches, which seems to guide the migration of the thyroid anlage into the subjacent mesenchyme.[9] Here, we found that the spatial relationship between this vessel and the budding thyroid was primarily maintained in the *Shh*-null mutant. If this interaction is of importance to thyroid induction it might explain why the specification site is restricted also in *Shh*−/− embryos.

The mechanisms that separate the thyroid into left and right lobes, thus determining the final shape of the gland, are hitherto unknown. The present study provides first evidence of a regulatory step in late thyroid morphogenesis. As neither Shh nor its receptor Ptc were found to be expressed in or close to the thyroid at any developmental stage, it is likely that the influence of Shh is indirect and that the thyroid phenotype of Shh-deficient mice may be secondary to a disturbed morphogenetic patterning of adjacent Shh-regulated tissues in the head and neck region. A characteristic abnormality of the hemithyroid in these animals was the spatial relation to large vessels that frequently passed through the thyroid tissue. The possibility that such aberrant development of vessels might be related to thyroid dysgenesis gains support from clinical conditions, eg, DiGeorge syndrome in which congenital anomalies of the heart and great vessels is a hallmark.[47] In fact, recent attention has been drawn to an association of DiGeorge syndrome with CH,[48] and numerous autopsy reports have documented thyroid abnor-

malities including right- or left-sided hemiagenesis.[49,50] Experimental findings also suggest a possible link between the pathogenesis of DiGeorge syndrome and Shh. The molecular etiology of DiGeorge syndrome is related to defects in the *Tbx1* gene.[51] Early embryos deficient of Shh or Tbx1 have similar vascular defects in the prospective head/neck region.[52] Interestingly, Tbx1 is positively regulated by Shh and its expression is abrogated in *Shh*−/− mice.[52] Further experimental support for an association between abnormal development of vessels and thyroid dysgenesis comes from studies on ablation of the cardiac neural crest in chick embryos, which results in cardiac outflow anomalies associated with thyroid hemiagenesis or hypoplasia.[53]

Despite abnormal thyroid organogenesis, the histogenetic patterning and functional differentiation of thyroid precursor cells, as evidenced by Tg production and accumulation in follicular lumina, did not differ between *Shh*−/− and wild-type embryos. Rather, the unexpected finding that cells expressing thyroid transcription factors (TTF-1 and TTF-2) and Tg were aberrantly located in the presumptive trachea in 15.5- to 17.5-dpc *Shh*-null mutants suggests that Shh may act as a repressor of inappropriate cytodifferentiation. Although budding of ectopic thyroid tissue from the trachea was most pronounced close to the developing hemithyroid, serial sectioning revealed that thyrocytes could appear as integrated parts of the primitive respiratory epithelium without any spatial connections to the thyroid proper, indicating their true ectopic origin. Why thyroid aberrantly develops in the trachea and not in other surrounding tissues is unknown, but might be related to the fact that cells of both tissues depend on TTF-1 transcriptional activity in early morphogenesis.[11] In normal lung development, TTF-1 expression in the proximal airways is silenced when distal branching of the respiratory tree progresses.[30] Inappropriate re-expression of TTF-1 in a segment of the trachea, as presently observed in *Shh*−/− mice, might thus be a key event in the pathogenesis of ectopic thyroid formation at this location. Transcriptional mechanisms that control TTF-1 expression are yet poorly defined, but members of the fibroblast growth factor (FGF) family of growth factors have been inferred a role.[54] Interestingly, targeted misexpression of FGF10 to the esophagus was recently found to induce TTF-1 and subsequent budding of the esophageal epithelium.[54] Because FGF10 is known to be negatively regulated by Shh in lung parenchyme,[55] it is possible that a similar unrestricted activity of FGF or other growth factor(s) influenced by Shh may be involved in ectopic thyroid development.

In conclusion, our results demonstrate that several steps in mouse thyroid development are controlled by Shh. Firstly, Shh in co-operation with hitherto unknown factor(s) creates a molecular boundary that defines the foregut territory for thyroid specification. Secondly, Shh signals indirectly govern the bilobation of the thyroid gland in late organogenesis. Thirdly, loss of Shh gives rise to thyroid hemiagenesis and ectopic thyroid in the respiratory tract, both of which have clinical counterparts in man. Shh-deficient mice thus promise to be useful in the further elucidation of molecular mechanism(s) responsible for thyroid dysgenesis related to developmental anomalies in nearby embryonic tissues.

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References

1. New England Congenital Hypothyroidism Collaborative: Effects of neonatal screening for hypothyroidism: prevention of mental retardation by treatment before clinical manifestations. New England Congenital Hypothyroidism Collaborative. Lancet 1981, 2:1095–1098
2. Kopp P: Perspective: genetic defects in the etiology of congenital hypothyroidism. Endocrinology 2002, 143:2019–2024
3. Perry R, Heinrichs C, Bourdoux P, Khoury K, Szots F, Dussault JH, Vassart G, Van Vliet G: Discordance of monozygotic twins for thyroid dysgenesis: implications for screening and for molecular pathophysiology. J Clin Endocrinol Metab 2002, 87:4072–4077
4. Castanet M, Lyonnet S, Bonaiti-Pellie C, Polak M, Czernichow P, Leger J: Familial forms of thyroid dysgenesis among infants with congenital hypothyroidism. N Engl J Med 2000, 343:441–442
5. Leger J, Marinovic D, Garel C, Bonaiti-Pellie C, Polak M, Czernichow P: Thyroid developmental anomalies in first degree relatives of children with congenital hypothyroidism. J Clin Endocrinol Metab 2002, 87:575–580
6. Romert P, Gauguin J: The early development of the median thyroid gland of the mouse. A light-, electron-microscopic and histochemical study. Z Anat Entwicklungsgesch 1973, 139:319–336
7. Missero C, Cobellis G, De Felice M, Di Lauro R: Molecular events involved in differentiation of thyroid follicular cells. Mol Cell Endocrinol 1998, 140:37–43
8. Fagman H, Grande M, Edsbagge J, Semb H, Nilsson M: Expression of classical cadherins in thyroid development: maintenance of an epithelial phenotype throughout organogenesis. Endocrinology 2003, 144:3618–3624
9. Hilfer SR, Brown JW: The development of pharyngeal endocrine organs in mouse and chick embryos. Scan Electron Microsc 1984, (Pt 4): 2009–2022
10. Manley NR, Capecchi MR: The role of Hoxa-3 in mouse thymus and thyroid development. Development 1995, 121:1989–2003
11. Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, Gonzalez FJ: The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev 1996, 10:60–69
12. De Felice M, Ovitt C, Biffali E, Rodriguez-Mallon A, Arra C, Anastassiadis K, Macchia PE, Mattei MG, Mariano A, Scholer H, Macchia V, Di Lauro R: A mouse model for hereditary thyroid dysgenesis and cleft palate. Nat Genet 1998, 19:395–398
13. Mansouri A, Chowdhury K, Gruss P: Follicular cells of the thyroid gland require Pax8 gene function. Nat Genet 1998, 19:87–90
14. Macchia PE, Lapi P, Krude H, Pirro MT, Missero C, Chiovato L, Souabni A, Baserga M, Tassi V, Pinchera A, Fenzi G, Gruters A, Busslinger M, Di Lauro R: PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat Genet 1998, 19: 83–86
15. Clifton-Bligh RJ, Wentworth JM, Heinz P, Crisp MS, John R, Lazarus JH, Ludgate M, Chatterjee VK: Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia. Nat Genet 1998, 19:399–401
16. Krude H, Schutz B, Biebermann H, von Moers A, Schnabel D, Neitzel H, Tonnies H, Weise D, Lafferty A, Schwarz S, DeFelice M, von Deimling A, van Landeghem F, DiLauro R, Gruters A: Choreoathetosis, hypothyroidism, and pulmonary alterations due to human NKX2–1 haploinsufficiency. J Clin Invest 2002, 109:475–480

17. Ingham PW, McMahon AP: Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001, 15:3059–3087
18. McMahon AP, Ingham PW, Tabin CJ: Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 2003, 53:1–114
19. Litingtung Y, Lei L, Westphal H, Chiang C: Sonic hedgehog is essential to foregut development. Nat Genet 1998, 20:58–61
20. Pepicelli CV, Lewis PM, McMahon AP: Sonic hedgehog regulates branching morphogenesis in the mammalian lung. Curr Biol 1998, 8:1083–1086
21. Apelqvist A, Ahlgren U, Edlund H: Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. Curr Biol 1997, 7:801–804
22. Hebrok M, Kim SK, St. Jacques B, McMahon AP, Melton DA: Regulation of pancreas development by hedgehog signaling. Development 2000, 127:4905–4913
23. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA: Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function. Nature 1996, 383:407–413
24. Postiglione MP, Parlato R, Rodriguez-Mallon A, Rosica A, Mithbaokar P, Maresca M, Marians RC, Davies TF, Zannini MS, De Felice M, Di Lauro R: Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland. Proc Natl Acad Sci USA 2002, 99:15462–15467
25. Dathan N, Parlato R, Rosica A, De Felice M, Di Lauro R: Distribution of the titf2/foxe1 gene product is consistent with an important role in the development of foregut endoderm, palate, and hair. Dev Dyn 2002, 224:450–456
26. Shirayoshi Y, Hatta K, Hosoda M, Tsunasawa S, Sakiyama F, Takeichi M: Cadherin cell adhesion molecules with distinct binding specificities share a common structure. EMBO J 1986, 5:2485–2488
27. Wilkinson DG, Bailes JA, Champion JE, McMahon AP: A molecular analysis of mouse development from 8 to 10 days post coitum detects changes only in embryonic globin expression. Development 1987, 99:493–500
28. Echelard Y, Epstein DJ, St.-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP: Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 1993, 75:1417–1430
29. Motoyama J, Takabatake T, Takeshima K, Hui C: Ptch2, a second mouse patched gene is co-expressed with sonic hedgehog. Nat Genet 1998, 18:104–106
30. Lazzaro D, Price M, de Felice M, Di Lauro R: The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. Development 1991, 113:1093–1104
31. Shabana W, Delange F, Freson M, Osteaux M, De Schepper J: Prevalence of thyroid hemiagenesis: ultrasound screening in normal children. Eur J Pediatr 2000, 159:456–458
32. Richardson GM, Assor D: Thyroid tissue within the larynx. Case report. Laryngoscope 1971, 81:120–125
33. Donegan JO, Wood MD: Intratracheal thyroid—familial occurrence. Laryngoscope 1985, 95:6–8
34. Brandwein M, Som P, Urken M: Benign intratracheal thyroid: a possible cause for preoperative overstaging. Arch Otolaryngol Head Neck Surg 1998, 124:1266–1269
35. McLean R, Howard N, Murray IP: Thyroid dysgenesis in monozygotic twins: variants identified by scintigraphy. Eur J Nucl Med 1985, 10:346–348
36. Hsu CY, Wang SJ: Thyroid hemiagenesis accompanying an ectopic sublingual thyroid. Clin Nucl Med 1994, 19:546
37. Huang SM, Chen HD, Wen TY, Kun MS: Right thyroid hemiagenesis associated with papillary thyroid cancer and an ectopic prelaryngeal thyroid: a case report. J Formos Med Assoc 2002, 101:368–371
38. Gilbert-Barness E, Debich-Spicer D, Cohen Jr MM, Opitz JM: Evidence for the "midline" hypothesis in associated defects of laterality formation and multiple midline anomalies. Am J Med Genet 2001, 101:382–387
39. Nickerson E, Greenberg F, Keating MT, McCaskill C, Shaffer LG: Deletions of the elastin gene at 7q11.23 occur in approximately 90% of patients with Williams syndrome. Am J Hum Genet 1995, 56:1156–1161
40. Cammareri V, Vignati G, Nocera G, Beck-Peccoz P, Persani L: Thyroid hemiagenesis and elevated thyrotropin levels in a child with Williams syndrome. Am J Med Genet 1999, 85:491–494
41. Stagi S, Bindi G, Neri AS, Giovannucci-Uzielli ML, Lapi E, Galluzzi F, Salti R: Thyroid hypoplasia of the left lobe in two girls affected by Williams syndrome. Clin Dysmorphol 2003, 12:267–268
42. Wang YK, Samos CH, Peoples R, Perez-Jurado LA, Nusse R, Francke U: A novel human homologue of the Drosophila frizzled wnt receptor gene binds wingless protein and is in the Williams syndrome deletion at 7q11.23. Hum Mol Genet 1997, 6:465–472
43. Meng X, Lu X, Li Z, Green ED, Massa H, Trask BJ, Morris CA, Keating MT: Complete physical map of the common deletion region in Williams syndrome and identification and characterization of three novel genes. Hum Genet 1998, 103:590–599
44. Wouters CH, Meijers-Heijboer HJ, Eussen BJ, van der Heide AA, van Luijk RB, van Drunen E, Beverloo BB, Visscher F, Van Hemel JO: Deletions at chromosome regions 7q11.23 and 7q36 in a patient with Williams syndrome. Am J Med Genet 2001, 102:261–265
45. Kim SK, Hebrok M, Li E, Oh SP, Schrewe H, Harmon EB, Lee JS, Melton DA: Activin receptor patterning of foregut organogenesis. Genes Dev 2000, 14:1866–1871
46. Lammert E, Cleaver O, Melton D: Induction of pancreatic differentiation by signals from blood vessels. Science 2001, 294:564–567
47. Momma K, Matsuoka R, Takao A: Aortic arch anomalies associated with chromosome 22q11 deletion (CATCH 22). Pediatr Cardiol 1999, 20:97–102
48. Scuccimarri R, Rodd C: Thyroid abnormalities as a feature of DiGeorge syndrome: a patient report and review of the literature. J Pediatr Endocrinol Metab 1998, 11:273–276
49. Robinson Jr HB: DiGeorge's or the III-IV pharyngeal pouch syndrome: pathology and a theory of pathogenesis. Perspect Pediatr Pathol 1975, 2:173–206
50. Conley ME, Beckwith JB, Mancer JF, Tenckhoff L: The spectrum of the DiGeorge syndrome. J Pediatr 1979, 94:883–890
51. Merscher S, Funke B, Epstein JA, Heyer J, Puech A, Lu MM, Xavier RJ, Demay MB, Russell RG, Factor S, Tokooya K, Jore BS, Lopez M, Pandita RK, Lia M, Carrion D, Xu H, Schorle H, Kobler JB, Scambler P, Wynshaw-Boris A, Skoultchi AI, Morrow BE, Kucherlapati R: TBX1 is responsible for cardiovascular defects in velo-cardio-facial/DiGeorge syndrome. Cell 2001, 104:619–629
52. Yamagishi H, Maeda J, Hu T, McAnally J, Conway SJ, Kume T, Meyers EN, Yamagishi C, Srivastava D: Tbx1 is regulated by tissue-specific forkhead proteins through a common sonic hedgehog-responsive enhancer. Genes Dev 2003, 17:269–281
53. Bockman DE, Kirby ML: Dependence of thymus development on derivatives of the neural crest. Science 1984, 223:498–500
54. Sakiyama J, Yamagishi A, Kuroiwa A: Tbx4-Fgf10 system controls lung bud formation during chicken embryonic development. Development 2003, 130:1225–1234
55. Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL: Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 1997, 124:4867–4878