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"Gene expression profiles during thyroid embryonic development"

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1. Abstract

Congenital Hypothyroidism (CH) is commonly due to structural defects of thyroid gland, collectively known as thyroid dysgenesis. Defects in growth and/or differentiation of the thyroid primordium can result in an absent (athyreosis) or hypoplastic thyroid; an impaired migration of thyroid precursor cells causes an ectopic gland (*Van Vliet G, 2003*). The clinical picture of thyroid dysgenesis thus suggests that defects of the specification, survival and movement of thyroid precursor cells are the key aberrations in CH. Understanding the regulation of early thyroid morphogenesis is thus important to elucidate the pathogenesis of CH. Murine models with target inactivation of the transcription factors Nkx2.1, Pax8, Foxe1 and Hhex expressed in thyroid progenitor cells and in the adult gland have demonstrated their important functions in thyroid development (*De Felice M and Di Lauro R, 2004*). Even though the thyroid is specified in each of these models it later disappears suggesting a role of these transcription factors also in the survival and expansion of the thyroid progenitors cell population. However, very few germ-line mutations have been detected in the corresponding genes of human patients with thyroid dysgenesis (*Al Taji et al., 2007*) suggesting the importance of additional genes. The aim of this thesis has been to discovery these genes by an unbiased search for transcripts enriched in the early thyroid primordium.

Mouse thyroid primordia at the bud stage (E10.5) were isolated by laser capture microdissection (LCM). In parallel, RNA from whole embryos was obtained. RNA was amplified and labelled; samples were hybridized to Affymetrix microarrays. Bioinformatic analysis tools identified over 3000 transcripts as significantly enriched in the thyroid bud as compared to expression in the whole embryo. Such an enrichment is expressed as Fold Change (FC), 450 transcripts out 3000 displayed a FC >5. High and restricted expression of several of these in the E10.5 thyroid bud was confirmed experimentally by in situ hybridization on mouse embryos. This preliminary analysis indicates that the list has a high degree of validity. By this approach I have identified a large number of transcripts enriched in the embryonic thyroid bud with currently unknown functions in its development.

This list will be an important resource in further efforts to elucidate the genetic networks that govern thyroid morphogenesis and might underlie CH.

2. Introduction

The thyroid gland in mammals

General aspects

The thyroid gland, a component of the endocrine system, derives its name from the Greek words 'thyreos' meaning 'shield', and 'eidos' meaning 'form'. In humans, the thyroid gland is located in front of the neck (*Figure 1 A*). In women, it is slightly heavier than in men and enlarges in pregnancy.

The thyroid gland, in all vertebrates, is responsible for producing thyroid hormone, known for its role in regulating metabolism in adults and is also required for many developmental processes.

The human thyroid gland is a brownish-red organ, in most cases having two lobes connected by an isthmus; normally weighs about 28 g and consists of cuboidal cells arranged to form epithelial follicles (*Figure 1 B*), supported by connective tissue that forms a framework for the entire gland. In the normal thyroid gland, the follicles are usually filled with a colloid substance containing the protein thyroglobulin enclosing the main thyroid hormone thyroxine (or tetra-idothyronine, T_4). Another hormone, triiodothyronine (T_3), is present in much lesser amounts in thyroglobulin even though it will be the main hormone peripherally, where it is produced by enzymatic de-iodination of T_4. Thyroid hormone production starts with the synthesis of thyroglobulin, which is then secreted into the colloidal lumen of the follicle with the iodination of tyrosine residues and where it is condensed to produce tri- (T_3) and tetra-iodinated thyronine (T_4, thyroxine) (*Frieden and Lipner, 1971*). T_3 and T_4 remain covalently bound to thyroglobulin as long as they are stored in the colloid. The bound forms of T_3 and T_4 are eventually taken up by the follicular cells and proteolitically separated from the thyroglobulin. Free T_3 and T_4 are then released and act as thyroid hormones.

Although the thyroid gland constitutes about 0.5 percent of the total human body weight, it holds about 25 percent of the total iodine in the body, obtained from food and

water in diet. Iodine usually circulates in the blood as an inorganic iodide and is concentrated in the thyroid to as much as 500 times the iodide level of the blood.

The amounts of T_3 and T_4 secreted by the thyroid are controlled by the thyroid-stimulating hormone (TSH) of the pituitary gland and TSH, in turn, is regulated by thyroid-stimulating hormone releasing factor (TRF), secreted by the hypothalamus.

The functions of the thyroid gland include: regulation of normal body growth in infancy and childhood, regulation of metabolism, regulation of body temperature, maintenance of skeletal maturation and regulation of protein, fat and carbohydrate metabolism. These functions are dependent upon the serum levels of T_4.

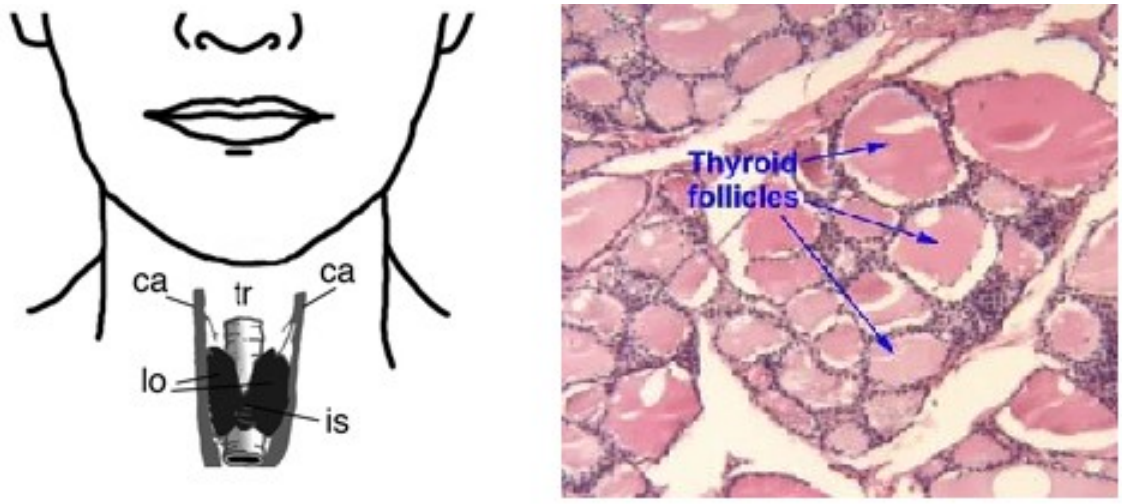

Figure 1 The human thyroid gland. (A) Drawing showing the thyroid position and related structures in the neck region. (B) Cross section of the thyroid showing structure of the thyroid follicles. Ca: Carotid artery; Is: Isthmus; Lo: Lobes Tr: Trachea.

The development of the thyroid gland

The adult thyroid gland in mammals is assembled from two different embryological structures. This composite origin reflects the dual endocrine function of the gland. The thyroglobulin-producing follicular cells (TFCs) are derived from a small group of endodermal cells of the primitive pharynx (the thyroid anlage) whereas the calcitonin-producing parafollicular cells are the neural crest-derived cells contained in the ultimobranchial bodies, the transient embryonic structures originated from the fourth pharyngeal pouch. The thyroid anlage and the ultimobranchial bodies migrate from their respective sites of origin, reaching their final position in front of the trachea and fuse to form the definitive thyroid gland and thus disappear as individual structures. Cells derived from the thyroid anlage begin to form thyroid follicles, whereas the C-

cells scatter within the interfollicular space. After this early ontogenetic phase, the thyroid function commences but remains at basal level; the lateral differentiation of hypothalamic nuclei and the organization of the pituitary-portal vascular system guarantee the maturation of the thyroid-system function (*Fisher et al., 1977*).

The morphogenesis and differentiation of the thyroid have been extensively studied in rodents. On the contrary, data on thyroid organogenesis in humans are scarce. However, the formation of the initial primordium, the differentiation of follicular cells and the folliculogenesis probably follow the same developmental pattern in all mammals. In addition recent studies on patients affected by congenital hypothyroidism with thyroid dysgenesis have confirmed that identical genetic mechanisms are involved in thyroid organogenesis both in humans and mice. The morphological and molecular aspects of thyroid development in mice are described in details as follows.

Morphological aspects of mouse thyroid development

After the gastrulation, the endoderm layer forms the primitive gut tube that is a cylindrical cavity running along the antero-posterior axis of the embryo. The anterior and posterior portions of this tube are called foregut and hindgut respectively. In the beginning, the primitive gut appears as a homogeneous tube, which shortly, through the effects of signalling molecules and specific transcription factors, "regionalizes" in different districts, undergoes different developmental programs and gives rise to different organs (*Shivdasani, 2002; Fukuda and Kikuchi, 2005; Grapin-Botton and Melton, 2000*). For example, the epithelial components of thyroid, thymus, lungs, stomach, liver, pancreas, intestine and bladder derive from the endodermal cells of the gut (*Hogan and Zaret, 2002*). The thyroid is the anterior-most organ that derives from the foregut. Mechanisms responsible for thyroid specification, by which a group of endodermal cells are recruited to establish the thyroid anlage, are still unknown. Undifferentiated endodermal cells could be "specified" toward their thyroid fate as a result of inductive signals from the mesenchyme or from adjacent heart mesoderm. Factors such as Nodal, FGF and members of GATA or Sox family could be involved in thyroid specification. In mice, thyroid does not develop in the absence of Fgf10 (*Ohuchi et al., 2000*); whereas in zebrafish, Bon and Gata5, the two transcription factors downstream of Nodal signalling, seem to be specifically relevant to the early step of thyroid bud specification (*Elsalini et al., 2003*).

In the mouse (gestation period-19 days) the thyroid anlage is first visible at embryonic day (E) 8-8.5 as a midline endodermal thickening in the ventral wall of the primitive pharynx (*Kaufman and Bard, 1999*) (Figure 2). This thickened bud first forms a small endodermal pit and then an outpouching of the endoderm that is apposed to the distal part of the outflow tract of the developing heart. The close contact between thyroid anlage and developing heart suggests an inductive role of myocardial cells on thyroid morphogenesis. There are no data proving a direct influence of the developing heart on thyroid organogenesis. However alterations in the foregut have been demonstrated as a consequence of an impaired heart development (*Cai et al., 2003*). Furthermore, cardiac malformations represent the most frequent birth defects associated with thyroid dysgenesis (*Olivieri et al., 2002; Roberts et al., 1997*).

As soon as the thyroid anlage is visible as an endodermal thickening in the midline of the floor of the primitive pharynx, the precursors of TFCs acquire a specific molecular signature and can be distinguished by their co-expression of four transcription factors Hhex (*Thomas et al., 1998*), Titf1 (*Lazzaro et al., 1991*), Pax8 (*Lazzaro et al., 1991*) and Foxe1 (*Zannini et al., 1997*). It is worth noting that each of these transcription factors is expressed also in other tissues but such a combination is a unique hallmark of both differentiated TFCs and their precursors (*Damante et al., 2001*). Studies in animal models have shown the relevance of these factors for thyroid development.

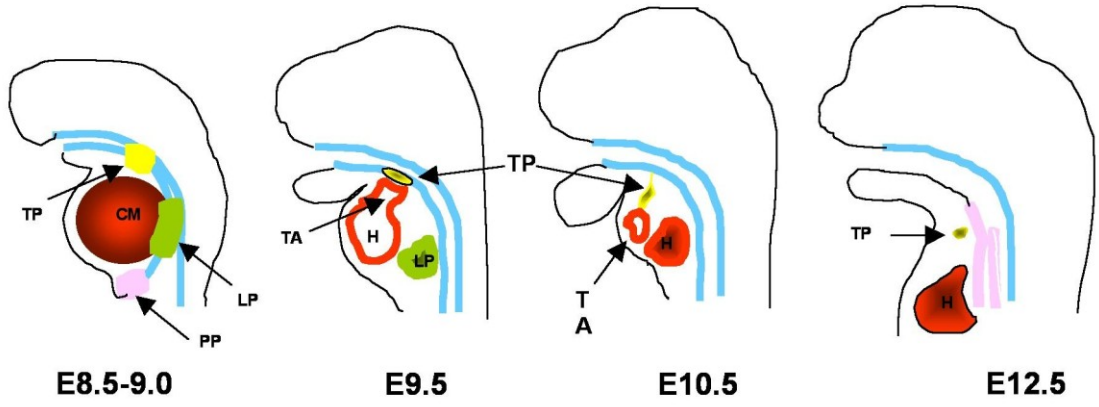

Figure 2 Early stages of thyroid development. CM: Cardiac Mesenchyme; H: Heart; LP: Liver Primordium; PP: Pancreatic Primordium; TA: Truncus Arteriosus; TP: Thyroid Primordium.

By E9 the thyroid anlage projects into the surrounding mesenchyme and forms the thyroid bud that rapidly becomes an endodermal-lined diverticulum. The thyroid primordium begins to descend towards its final position (*Figure 2*), a process that lasts for almost four days (*Kaufman and Bard, 1999*). The molecular mechanisms involved in the translocation of the thyroid primordium are still matter of debate (*Fagman et al., 2003*). Budding and translocation from the gut tube is a developmental process shared by many endoderm-derived organs (*Hogan and Zaret, 2002*). In the case of thyroid, the definitive location is rather distant from the site of primitive specification and any existing connection between the gland and the gut tube disappears. Whereas the development of other organs, such as the lung, involves a process of branching morphogenesis (*Cardoso and Lu, 2006*), thyroid development requires the migration of TFC precursors. Cell migration is a common phenomenon during embryogenesis. During many processes, such as gastrulation, neural crest migration and heart formation, the migrating cells lose epithelial phenotype and acquire mesenchymal features (*Thiery and Sleeman, 2006*). This epithelial-mesenchymal transition is a hallmark with an increased expression of N-cadherin and the down-regulation of E-cadherin, a molecule relevant for cell-cell contacts. In contrast, TFC precursors seem to use a different and yet unidentified pathway to move because they maintain their epithelial phenotype through their "journey" towards the trachea (*Fagman et al., 2003*). The expression of transcription factors such as either Hhex or Pax8 or Titf1 is not sufficient for thyroid migration, while Foxe1 plays a crucial role because the presence of this factor in the thyroid bud is required to allow the cells to move (*De Felice et al., 1998; Parlato et al., 2004*). Thus, in TFC precursors, Foxe1 controls the expression of key molecules required for migration, though it has been supported that the translocation of thyroid primordium towards the sublaryngeal position not only depends on cell autonomous events but could also be driven by the movements of other surrounding tissues of the neck region. (*Hilfer and Brown, 1984*).

At E10.5, the thyroid primordium caudally migrates into the mesenchyme but is still connected to the floor of the pharynx by a narrow channel, the thyroglossal duct, which gradually undergoes atrophy and at E11.5 the thyroid primordium loses all connections with the pharyngeal floor. One day later the thyroid primordium starts expanding laterally and by E 13-14 reaches its destination - in front of the trachea, where it merges with the ultimobranchial body-derived cells, which have completed their ventro-caudal migration (*Kaufman and Bard, 1999; Cordier et al., 1980*).

Once the final location has been reached, the thyroid lobes expand considerably (E15-16) and the gland exhibits its definitive shape: two lobes connected by a narrow isthmus (*Figure 3*). The mechanisms controlling thyroid growth and formation of the lobes are unknown. It is worth noting that while TSH signalling is required for the growth of the adult thyroid cells, it is not relevant for the growth of the foetal gland. Since mesenchymal signals have been proved to be necessary for the morphogenesis of several endoderm-derived organs (*Zaret, 2002; Grapin-Botton and Melton, 2000; Lammert et al., 2001; Matsumoto et al., 2001*), an inductive role of surrounding tissues can be hypothesized in thyroid development too, mainly the vessels located close to the thyroid tissue. This is observed in mice in which, when either Sonic hedgehog gene (Shh) (*Fagman et al., 2004*) or TBX1 (*Fagman et al., 2006*) has been disrupted, correct patterning of the vessels is disturbed; the lobulation process is impaired and the thyroid gland assumes the shape of a single midline mass located lateral to the trachea. In the same way, the localization of growing thyroid tissue along the antero-posterior axis in zebrafish is linked to the development of the ventral aorta; ectopic vascular cells influence the localization of the thyroid tissue non-autonomously, showing that vessels provide guidance cues in zebrafish thyroid morphogenesis (*Alt and Elsalini et al., 2006*).

Figure 3 Thyroid gland morphogenesis. *In situ* hybridization with Pax8 antisense probe reveals the location of the thyroid.

Between E14.5 and E16.5 a series of events lead the thyroid primordium towards a functional thyroid gland able to produce and release hormones (*Figure 4*). The final differentiation of TFC is featured by the expression of a number of genes required for thyroid function such as thyroglobulin (Tg), thyroid peroxidase (TPO), TSH receptor (Tshr), sodium/iodide symporter (NIS), thyroid oxidase (Thox's) and pendrin (PDS). The final differentiation program of TFC requires almost three days. Tg

appears around E14 (*Lazzaro et al., 1991*) while thyroxine is first detected at E16.5 (*Meunier et al., 2003*). The expression of TPO as well as NIS, the two key enzymes involved in the process of Tg iodination, is absolutely dependent on the pathway activated by the binding of TSH to its receptor Tshr (*Postiglione et al., 2002*). Notably, the normal final location of TFCs in front of the trachea is not an essential requirement for functional differentiation, since the sublingual thyroid expresses thyroglobulin in human patients (*Hartzband et al., 1984*) as well as in mutated mice (*De Felice et al., 1998*).

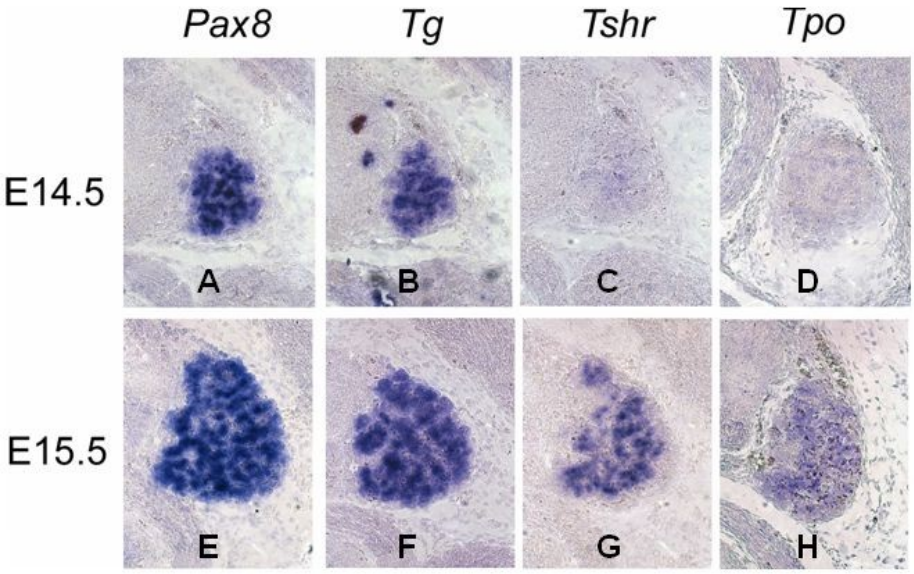

Figure 4 Onset of the functional differentiation in developing mouse thyroid. Serial sagittal sections of E14 (A-D) and E15 (E-H) reveal the hybridization with different antisense probes, as indicated.

In the mouse, small thyroid follicles begin to appear by E15.5 and a day later the gland displays an evident follicular organization. However the regulation of the growth and function of the thyroid by the hypothalamic-pituitary axis is fully active only after birth. In humans, the establishment of the characteristic histological organization lasts several weeks and can be divided into three phases: the precolloid, the beginning of colloid and the follicular-growth, which occur at 7-10, 10-11 and after 11 weeks of gestation, respectively (*Fisher et al., 1997*). In the precolloid phase small intracellular canaliculi develop as an accumulation of colloid material. These small canaliculi enlarge and the colloid organizes itself into extracellular spaces. In the last phase

primary follicles are clearly visible and the foetal thyroid is able to concentrate iodide and synthesize thyroid hormones. In variation with mice, in humans at mid-gestation (18-20 weeks), the hypothalamic-pituitary thyroid axis begins to develop and hormone production increases.

Molecular genetics of thyroid development

The discovery that *Titf1*, *Foxe1*, *Pax8* and *Hhex* transcription factors, relevant for the expression of genes specific of mature TFCs, are also expressed in the thyroid primordium, offered a useful tool for the exploration of the genetic basis of the developmental process of the thyroid gland. These four factors remain expressed all life long as a hallmark of differentiated TFCs (*Table I*) and their expression can be down-regulated only after transformation of the cells (*Francis-Lang et al., 1992b*). The hypothesis that the expression of these four factors is required at early stages of thyroid morphogenesis has been confirmed by studies on both animal models and patients affected by thyroid dysgenesis. Titf1 (*Kimura et al., 1996; Kimura et al., 1999*), Hhex (*Martinez Barbera et al., 2000*) and Pax8 (*Mansouri et al., 1998*) are required for the survival of the TFC precursors, whereas in the absence of Foxe1 the thyroid primordium either disappears or remains in a sub-lingual ectopic position (*De Felice et al., 1998*). These data indicate that Titf1, Hhex, Pax8 and Foxe1 play individual roles in the organogenesis of the gland; however functional interaction among these factors has been demonstrated in the developing thyroid (*Figure 5*). Actually, Titf1, Hhex and Pax8 are linked in a complex regulatory network because each of them controls the maintenance of expression of the other factors. The simultaneous presence of these three factors is required for the expression of Foxe1, suggesting that Foxe1 is located downstream in the thyroid regulatory network (*Parlato et al., 2004*). However, the presence of these genes is not sufficient to guarantee a correct organogenesis of the gland. Mutations in other genes too, both thyroid-enriched and ubiquitous, have been demonstrated to impair the development of the thyroid.

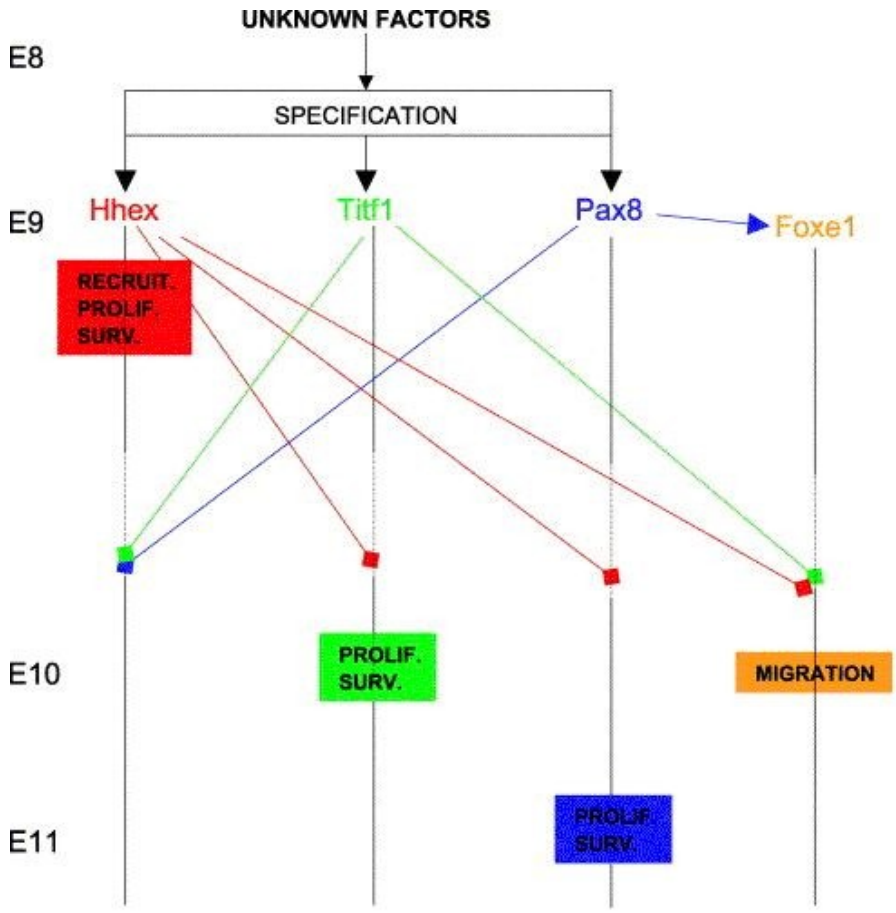

Figure 5 Functional interaction among Hhex, Titf1, Pax8 and Foxe1 in developing thyroid. The transcription factor and the functions controlled by it are indicated in different colours. Each factor regulates other transcription factors controlling the onset (arrow) or the maintenance (square) of their expression. (From Parlato et al., 2004)

The present knowledge on the molecular genetics of thyroid development mainly as deduced from the phenotype of knock-out animals, is summarized below. It is worth noting that while we have sufficient information on genes expressed in the thyroid primordium after its specification and indispensability for the maintenance of the thyroid morphogenesis, the factors required for the initiation of anlage formation are still unknown.

Embryonic Day	**Morphology**	**Functional (terminal) differentiation**		**Thyroid hormones**	**Controller genes**	
		Tg*, *TPO*, *Tshr	***NIS***		***Titf1*,*Foxe1*, *Pax8*,*Hhex***	***Fgfr2***
E8	Undifferentiated endoderm	-	-	-	-	-
E8.5	Thyroid anlage	-	-	-	+	-
E9.5	Thyroid bud	-	-	-	+	-

E11.5-13.5	Expansion of thyroid primordium	-	-	-	+	+
E14-15	Definitive bilobed shape	+	-	-	+	+
E16	Folliculogenesis	+	+	-	+	+
E16.5	Completion of organogenesis	+	+	+	+	+

Table I Summary of the different phases of thyroid development, indicating the morphological features, the expression of relevant genes and the capacity to produce thyroid hormones. +, Present; -, Absent.

Relevant genes in thyroid development

Titf1

Titf1 (formerly called TTF-1 for Thyroid Transcription Factor–1 or Nkx2-1 or T/EBP) is a transcription factor that recognizes and binds to specific DNA sequences via a 61 amino acid-long DNA binding domain called homeodomain, whose sequence is conserved from the fruitfly to humans with very few changes. Titf1 was initially identified in a rat thyroid cell line (*Civitareale et al., 1989*) as a nuclear protein able to bind to specific sequences in the Tg promoter. The corresponding cDNA was subsequently cloned and a comparative sequence analysis demonstrates that *Titf1* has a considerable degree of homology to the Drosophila NK-2 class of homeodomain proteins (*Kim and Niremberg, 1989*).

Titf1 is a member of the Nkx2 class of transcription factors and is encoded by a single gene whose official name is *Titf1* in mice and *TITF1* in humans, located on chromosome 12 and on chromosome 14q13 (*Guazzi et al., 1990*) respectively. The gene splits into at least 3 exons that express multiple transcripts (*Lonigro et al., 1996; Hamdan et al., 1998*). The most abundant is 2.3 Kb mRNA which encodes a phosphorylated (*Francis-Lang et al., 1992b; Zannini et al., 1996*) 42 kDa protein 371 amino acids long in humans (*Ikeda et al., 1996*). Functional studies have addressed to the question of whether the homeodomain is responsible only for the binding to the DNA (*Damante and Di Lauro, 1991*), while the transactivating property resides in the two apparently redundant domains localized at two ends of the protein (*De Felice et al., 1995*).

The expression pattern of Titf1 has been exhaustively studied in rodents. Titf1 is expressed in both differentiated follicular cells and in their precursors. It is detected in

the thyroid primordium as soon as the thyroid anlage is visible. In the thyroid gland, Titf1 expression is not restricted to the follicular cells; it is also present in parafollicular C-cells (*Suzuki et al., 1998*) and in the epithelial cells of the ultimobranchial body (*Mansouri et al., 1998*). In addition, Titf1 is expressed in selected areas of the forebrain, including the developing posterior pituitary, in the trachea and in the lung epithelium (*Lazzaro et al., 1991*).

Gene targeting experiments have allowed the study of the role of this transcription factor during embryonic life. Mice, in which both *Titf1* alleles have been disrupted, show a complex phenotype according to the wide expression domain of this gene.

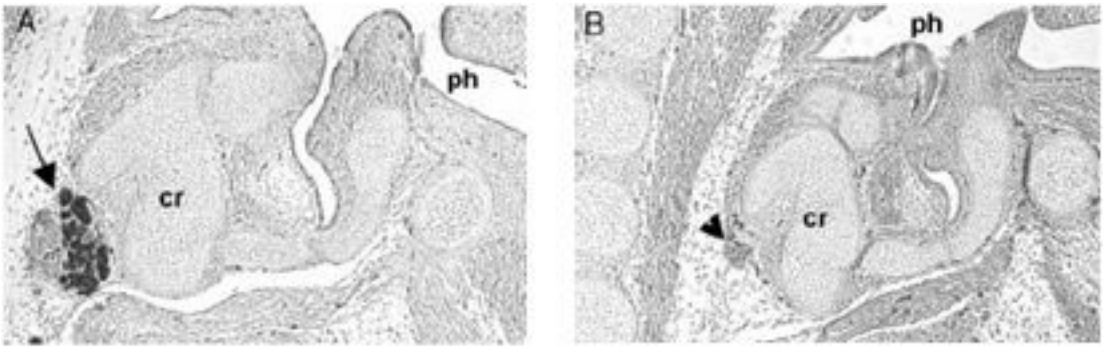

Figure 6 Sagittal sections of wild-type (A) and Titf1-/- (B) E15.5 embryos stained with Pax8 antibody. In wild-type embryo, the developing thyroid (arrow) is positioned dorsal to the cricoid cartilage. In the mutated embryo the thyroid tissue is undetectable (arrowhead). Cr: Cricoid cartilage; ph: pharynx (Di Felice and De Lauro, 2004).

In absence of Titf1, the newborn mice immediately die at birth and are characterized by impaired lung morphogenesis, lack of thyroid and pituitary and severe alterations in the ventral region of the forebrain (*Kimura et al., 1996*) (*Figure 6*).

Analyses during development demonstrate that the thyroid anlage forms in its correct position but at an early stage, morphogenesis of the gland is impaired. The thyroid primordium by E10.5 appears much smaller in size in comparison to wild type and subsequently undergoes degeneration probably in consequence of an apoptotic process (*Kimura et al., 1996*). Hence, Titf1 is dispensable for the initial commitment of thyroid cells, but is required for the survival and subsequent differentiation of the cells. However we do not know which genes are controlled by this transcription factor in the thyroid primordium. A detailed analysis of the phenotype of the affected tissue reveals that in absence of Titf1, the expression of *BMP4* (*Minoo et al., 1999*) and that of *Fgf8*

(*Takuma et al., 1998*) is abolished in the developing lung and in the posterior pituitary respectively. These data indicate that signalling molecules relevant for the morphogenesis of embryonic structures are controlled by this transcription factor. The finding that *Fgfr2* is expressed in the thyroid bud (*Parlato et al., 1999*), suggests that Titf1 could regulate the survival of TFCs through a Fgf-dependent mechanism.

In adult thyroid cells Titf1 plays a different role and controls the expression of thyroid specific genes. In transient transfection assay, Titf1 is able to activate both *Tg* and *TPO* promoters in non-thyroid cells (*De Felice et al., 1995*). However the function of Titf1 in differentiated thyroid cells has been studied only *in vitro* at present. A conditional knock-out of the gene encoding Titf1 is necessary to extend these data to a whole organism.

Pax8

Pax8 (Paired Box gene 8) is a member of a family of transcription factors characterized by the presence of a 128 amino acid-long domain that can recognize and bind to specific DNA sequences (*Frigerio et al., 1986*). This DNA binding domain is called paired domain as it was identified for the first time in the *Drosophila* segmentation gene as *paired*. Pax8 was identified (*Plachov et al., 1990*) in the mouse as a protein expressed in the developing thyroid gland. It was successively demonstrated (*Zannini et al., 1992*) that Pax8 paired domain recognizes and binds to a single site present in *Tg* and in *TPO* promoters.

The gene encoding Pax8 (called *Pax8* in mice and *PAX8* in humans) is located on chromosome 2 in both species (*Plachov et al., 1990; Stapleton et al., 1993*) and consists of 12 exons (*Okladnova et al., 1997*) that encode different alternative spliced transcripts (*Poleev et al., 1995; Kozmik et al., 1993*). All the isoforms generated, contain the paired domain located near the amino terminus and differ in their carboxy-terminal regions. Pax8a, the most abundant isoform, is a protein, 457 amino acids long in mice (*Plachov et al., 1990)* and 450 in humans (*Poleev et al., 1992*).

Pax8 is expressed in adult and developing thyroid since the early stages of gland morphogenesis. In addition, Pax8 is initially but transiently expressed in the myelencephalon and through the entire length of the neural tube during embryonic life (*Plachov et al., 1990*) and is present in the developing kidneys, where it is maintained throughout adult life.

Analysis of *Pax8* $^{-/-}$ mice (*Mansouri et al., 1998*) offers the possibility of studying the role of this transcription factor in embryonic life. Pax8 null pups show growth retardation and die within 2-3 weeks of their birth. The animals are affected by a severe hypothyroidism and present a rudimental gland composed almost completely of calcitonin-producing C cells while the TFCs are absent. In Pax8 null embryos the thyroid anlage forms, evaginates from the endoderm and begins to migrate into the mesenchyme. However by E11 the thyroid bud is smaller in comparison to that of wild type. In addition, other transcription factors, such as Foxe1 and Hhex, are down-regulated in the precursors of thyroid cells in the absence of Pax8 (*Parlato et al., 2000*). Finally, by E12.5 TFC is not detectable (*Mansouri et al., 1998*) (*Figure 7*).

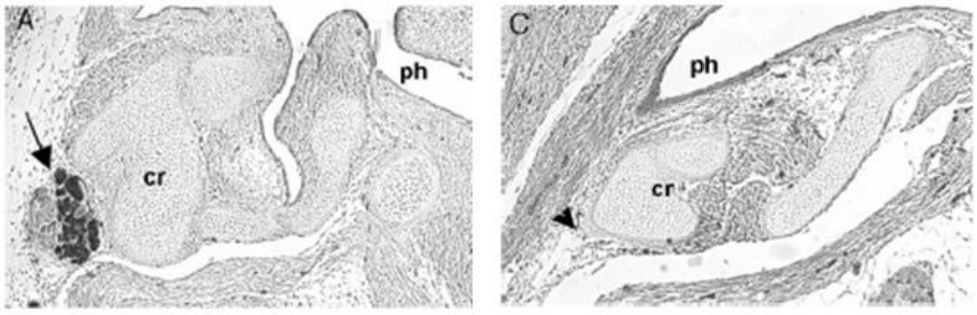

Figure 7 Sagittal sections of wild-type (A) and Pax8 -/- (C) E15.5 embryos stained with Titf1 antibody. In wild-type embryo, the developing thyroid (arrow) is positioned dorsal to the cricoid cartilage. In the mutated embryo the thyroid tissue is undetectable (arrowhead). Cr: Cricoid cartilage; ph: pharynx (Di Felice and De Lauro, 2004).

Thus, during morphogenesis, Pax8 holds a specific upper role in the genetic regulatory cascade that controls thyroid development and it is required for the survival of the TFCs and to maintain the tissue-specific gene expression program.

Like Titf1, Pax8 shows other functions in adult thyroid cells, which have been studied in cell culture systems. Transfection assays have demonstrated that Pax8 drives transcription from the *TPO* promoter and to a less extent, from the *Tg* promoter in non-thyroid cells (*Zannini et al., 1992*). In addition, Pax8 was shown to activate transcription of thyroid-specific genes at their chromosomal locus (*Pasca diMagliano et al., 2000*). The co-expression of Pax8 and Titf1 only in thyroid cells has suggested that these factors can cooperate in the stimulation of thyroid genes (*Miccadei et al., 2002*).

This hypothesis is supported by the data demonstrating that Pax8 and Titf1 directly interact *in vivo* in thyroid cells (*Di Palma et al., 2003*).

Foxe1

Foxe1 (formerly called TTF-2 for Thyroid Transcription Factor–2) was originally identified as a thyroid specific nuclear protein that can bind to a sequence present on both Tg and TPO promoters under insulin, IGF-1 or TSH stimulation (*Santisteban et al., 1992*). Later on, rat Foxe1 cDNA was cloned and the features of this protein have been characterized (*Zannini et al., 1997*).

Foxe1 belongs to a winged helix/forkhead family of transcription factors characterized by a 100 amino acid long DNA binding domain whose structure has a winged-helix motif (*Kaestner et al., 1993*) homologous to that of Drosophila *fork head* gene (*Lai et al., 1991*). Foxe1 contains two nuclear localization sequences flanking the DNA binding domain (*Romanelli et al., 2003*). The official name for the genetic locus encoding this transcription factor is *Foxe1* in mice {located on chromosome 4 (*Zannini et al., 1997*)} and *FOXE1* in humans {located on chromosome 9q22 (*De Felice et al., 1998; Chadwick et al 1997*)}.

Foxe1 is an intronless gene coding for a 42 kDa phosphorylated protein (*Dathan et al., 2002*). The protein contains an alanine stretch of variable length. In humans, the most frequent *FOXE1* allele, with 14 residues, is 371 amino acids long (*Macchia et al., 1999*).

Like Titf1 and Pax8, Foxe1 is detected in the thyroid primordium and its expression is maintained in TFCs during all stages of development and in adulthood. However, during embryonic life, Foxe1 has a wide domain of expression. Indeed, at early stages of development, Foxe1 is detected in the endodermal epithelium lining the primitive pharynx, the arches and the foregut and transiently in the Rathke's pouch. Subsequently Foxe1 is expressed also in the tongue, in the secondary palate, in the definitive choanae, and in the whiskers and hair follicles (*Dathan et al., 2002*). In humans, in addition to thyroid and foregut, FOXE1 expression is also found in embryonic thymus (*Trueba et al., 2004*), outer follicular hair sheath and the seminiferous tubules of prepubertal testis (*Sequeira et al., 2003*).

Analysis of *Foxe1* null mice (*Figure 8*) revealed the role of this transcription factor in thyroid development. Targeting inactivation of Foxe1 shows that homozygous Foxe1$^{-/-}$ mice are born at the expected Mendelian ratio but die within 48 hours. These mice display a severe cleft palate, probably responsible for the prenatal death, no thyroid in its normal location, absence of thyroid hormones and elevated TSH levels in the bloodstream (*De Felice et al., 1998*). In *Foxe1*$^{-/-}$ embryos, at early stages of thyroid morphogenesis, the formation of the thyroid anlage is not affected. However, at E10 in *Foxe1* null embryos, TFCs are still on the floor of the pharynx whereas in wild type embryos the thyroid primordium begins to descend towards its final location.

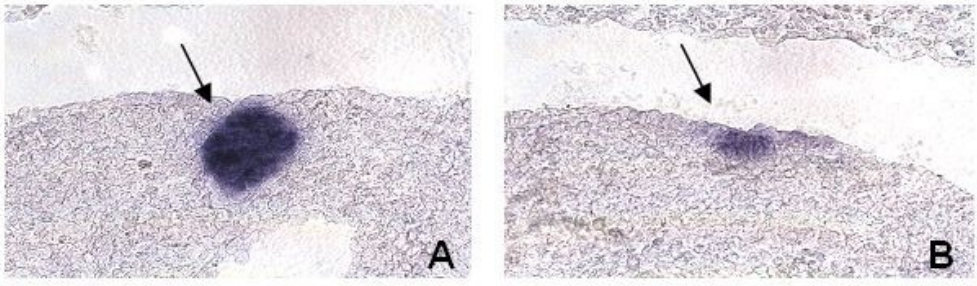

Figure 8 Sagittal sections of wild-type (A) and Foxe1-/- (B) E 10 embryos hybridized with Titf1 probe. Arrows point to the thyroid bud. In the mutated embryo, the thyroid bud is still on the floor of the primitive pharynx.

At later stages of development, in the absence of Foxe1, TFCs either disappear or form a small thyroid remnant still attached to the pharyngeal floor. In this case, the cells are able to go on their differentiative program as tested by the synthesis of thyroglobulin. These data indicate that in embryonic life Foxe1 has a specific role in controlling the migration of TFC precursors, but is not relevant for the specification and differentiation of the thyroid anlage. In addition, Foxe1 could be involved in the survival of TFCs since in many Foxe1 null embryos the thyroid primordium disappears (*De Felice et al., 1998; Parlato et al., 2004*). Analysis of *Foxe1* null embryos indicates that the migration of TFC precursors is a process promoted by Foxe1. However we do not know through which genes these programs are executed. In the adult gland the role of Foxe1 is not yet clear. *Foxe1* null newborns die at birth, thus conditional knock-out mice are necessary to study the functions of this gene in the physiology of the gland. Functional studies in rat thyroid cells in culture have shown that Foxe1 can act as a promoter-specific transcriptional repressor via a repression domain located at the

carboxy terminus of the protein (*Perrone et al., 2000*). Foxe1 represses the activities of *Tg* and *TPO* promoters induced by Titf1 and Pax8 respectively. This repression requires neither a direct binding between Foxe1 and Titf1 or Pax8 nor the binding between Foxe1 and DNA (*Perrone et al., 2000*). It is possible that this domain interacts with some specific cofactor required for the transcriptional activities of Titf1 and Pax8. Furthermore, it has been demonstrated that in differentiated thyroid cell lines the transcription of *Foxe1* mRNA is under TSH and insulin or IGF-1 control, which could suggest that Foxe1 plays a key role in the hormonal control of gene expression in thyroid cells (*Ortiz et al., 1997, Zannini et al., 1997*). However these controls do not seem to be effective in developing thyroid since in Pit-1 null mice, which lack TSH, GH and IGF-1, the expression of Foxe1 is not affected (*Postiglione et al., 2002*). Studies in mutant mice have shown that in absence of Pax8, the expression of Foxe1 is not detected in TFC precursors (*Parlato et al., 2004*) and Pax8 binding sites have recently been found in the 5' UTR of Foxe1 (*D'Andrea et al., 2006*).

Hhex

Hhex (formerly called Hex for Hematopoietically Expressed Homeobox or Prh for Proline-rich Homeobox) is a homeodomain-containing transcription factor that was first identified in a range of multipotent hematopoietic cells (*Crompton et al., 1992; Bedford et al, 1993*). It was successively demonstrated that Hhex is expressed in other tissues including the thyroid (*Thomas et al., 1998*).

Hhex is encoded by a gene called *Hhex* in mice and *HHEX* in humans located on chromosome 19 (*Ghosh et al., 1999*) and chromosome 10q23.32 (*Hromas et al., 1993*) respectively. The gene is split into 4 exons and codes for a protein 270 amino acids long. Hhex is considered an orphan homeobox-containing gene because the sequence of its homeodomain, responsible for the binding to the DNA, shows some differences with with respect to other homeodomains. Outside the homeodomain, Hhex contains an N-terminal proline rich region and a C-terminal acid region. These two regions are probably involved in repressing the transcription of the target genes (*Tanaka et al., 1999*).

During development Hhex is also expressed in the primordium of several organs derived from the foregut. Hhex is an early marker of thyroid cells, since it is present in the thyroid anlage at E 8-8.5 (*Thomas et al., 1998*) at the same stage in which Titf1,

Foxe1 and Pax8 are detected. In the adult, in addition to the thyroid, only liver and lungs maintain Hhex expression (*Bogue et al., 2000*).

The analysis of *Hhex* $^{-/-}$ embryos has revealed that this factor is absolutely necessary for thyroid morphogenesis (*Martinez-Barbera et al., 2000*).

In *Hhex* null embryos at E8.5 TFCs are present in the anterior wall of the pharynx (*Parlato et al., 2000*). One day later the thyroid primordium is absent or hypoplastic and the thyroid precursor cells do not express Titf1, Foxe1 and Pax8 (*Martinez-Barbera et al., 2000*) (*Figure 9*). Hence at early stages of development, the presence of Hhex could be required to maintain the expression of Titf1, Foxe1 and Pax8 in the thyroid primordium. As in the case of Titf1, Pax8 and Foxe1, a conditional knock-out mouse will be a useful tool in elucidating the role of Hhex in the adult thyroid gland.

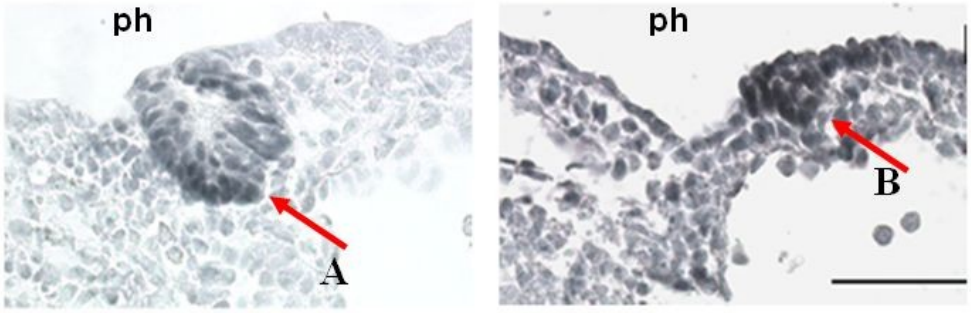

Figure 9 Development of the thyroid in E10 Hex$^{-/-}$ embryos. The thyroid primordium is evident in a wild-type embryo (A, arrow) ventral to the pharynx (ph). It is hypoplastic and remains connected to the floor of the pharyngeal endoderm in a Hex$^{-/-}$ embryo (B, arrow) (Parlato et al., 2004).

Studies on differentiated thyroid cells suggest that the network between Hhex and the other thyroid specific transcription factors, seems to be rather complex. Indeed, it has been reported that Hhex is regulated by Titf1 (*Puppin et al., 2003*); furthermore the overexpression of Hhex partly inhibits *Tg* promoter activity (*Pellizzari et al., 2000*). These data are consistent with the hypothesis that Hhex could be a transcriptional repressor as reported in other systems (*Tanaka et al., 1999; Brickman et al., 2000; Ho et al., 1999*).

Congenital hypothyroidism

Congenital hypothyroidism (CH) is the most common neonatal endocrine disorder, found to occur once in 3000-4000 live births (*Klett et al., 1997*) and results in severe neurodevelopmental impairment, if treatment is delayed. CH is characterized by

elevated levels of TSH in response to the reduced thyroid hormone levels produced by the follicular cells of the thyroid gland.

In 85% of the cases, CH is a consequence of thyroid dysgenesis (TD), which includes thyroid gland insufficiency due to abnormalities in gland development (*Grant et al., 1992*). In the remaining 15% of the cases, CH is caused by the thyroid hormone dyshormonogenesis (TDH) due to the defects in the biochemical mechanisms responsible for thyroid hormone biosynthesis. Mutations in the genes encoding for thyroglobulin and thyroperoxidase have been classically implicated in TDH (*Medeiros-Neto et al., 1994; de Vijderet al., 1997*). The study on the molecular genetics of TD seems to be a useful tool in the elucidation of the mechanisms underlying thyroid development, since in many cases it has confirmed data obtained in animal models and has provided new insights into thyroid morphogenesis and differentiation (*Van Vliet, 2003; De Felice and Di Lauro, 2004; Grueters et al., 2004; Polak et al., 2004; Park and Chatterjee, 2005*).

Thyroid dysgenesis

According to the definition by Fisher (*Fisher and Klein, 1981*), "… the term thyroid dysgenesis describes the abnormality in infants with ectopic or hypoplastic thyroid gland (or both) as well as those with total thyroid agenesis…". The term "thyroid dysgenesis" brings together a heterogeneous group of thyroid malformations, due to defects occurring at different stages of development resulting from different pathogenic mechanisms.

Ectopic thyroid, probably as a consequence of defects in thyroid bud migration, is the most common type (40-70% of cases) of TD. Ectopic thyroids can also be hypoplastic. The second most common variant, accounting for 20-40% of cases, is thyroid agenesis. Finally, hypoplastic eutopic thyroids account for 5-10% of cases. In addition, to these three types, thyroid hemiagenesis has also been included in the group of TD (*Maiorana et al., 2003*). However, the reported frequency of the different types of dysgenesis is influenced by the methods used for the diagnosis (*Grueters et al., 2002*).

Ectopic thyroid

Any disturbances during the process of thyroid bud translocation can lead to an ectopic thyroid that can be found at any point of the path followed by the developing gland towards its final location in front of the trachea. Though lingual thyroid is most frequent type, presence of thyroid tissue has been described in unexpected regions such as the submandibular region, heart or gut. Since it has been hypothesized that the cells of ultimobranchial bodies can differentiate into TFCs, thyroid tissue in submandibular region could be derived from cells of the utimobranchial bodies that did not merge with the thyroid bud (*Feller et al., 2000*). The aberrant presence of TFCs in other organs such as the gut could be explained by the differentiation of uncommitted endodermal cells.

More than 50% of patients affected by CH show an ectopic thyroid, but up to now, no mutation in known genes has been associated to this dysgenesis. In some familial cases of CH, the affected members show either athyreosis or ectopy. This observation raises the possibility that athyreosis and thyroid ectopy have a common underlying mechanism. This hypothesis is consistent with the data that mice deprived of *Foxe1* gene products show either ectopy with a very small thyroid or no thyroid at all (*De Felice et al., 1998*).

Athyreosis and agenesis

Both athyreosis and agenesis indicate the absence of thyroid tissue. However, agenesis should be used to define the absence of the gland as a consequence of a defective initiation of thyroid morphogenesis. The term athyreosis indicates a dysgenesis characterized by the disappearance of the thyroid due to alterations in any step following the specification of the thyroid anlage.

At the moment, no animal model for *bona fide* agenesis has been identified. Indeed, gene targeting experiments have demonstrated that neither Titf1, nor Foxe1, nor Pax8 nor Hhex seems to play a role in the thyroid anlage specification that correctly forms in embryos deprived of any of these proteins. Agenesis could be due to defects in genes relevant in the early regionalization of the endoderm, including the genes controlling the onset of *TITF1*, *FOXE1*, *PAX8* and *HHEX*. However the relevance of these genes in congenital hypothyroidism could be hard to demonstrate, since mutations in genes widely expressed in the endoderm at early stages of development could cause in embryo, lethal phenotypes and/or many additional defects. On the contrary, all knock

out mice described in the previous section seem good models of athyreosis. In these mutants morphogenesis of the gland begins but the thyroid bud disappears probably for a defective survival or proliferation of the precursors of the follicular cells. However, absence of the thyroid has been described in patients with CH associated with *FOXE1* defects and in one subject carrying a mutation in *PAX8*.

Hypoplasia

The term hypoplasia describes a gland containing a reduced number of TFCs. Thyroid hypoplasia is probably a genetically heterogeneous disorder since it could be a consequence of alterations in any of the steps controlling the expansion and/or survival of thyroid cells during organogenesis.

Genes involved in early stages of development (*TITF1*, *FOXE1*, *PAX8* and *HHEX*) as well as in the late steps (*TSHR*) could be reliable candidates for this dysgenesis. Indeed, mutations in either *TITF1 or PAX8* or *TSHR* genes have been found in patients with CH associated with hypoplasia.

Hemiagenesis

In thyroid hemiagenesis only one lobe of thyroid is present. It is usually the left lobe that fails to develop. This malformation is very rarely associated wih CH and has a prevalence of 0.2-0.05% in normal population (*Maiorana et al., 2003*). Thyroid hemiageneis occurs more frequently among members of the same family (*Castanet et al., 2000*) and in the relatives of patients with classic forms of TD (*Castanet et al., 2005*).

Genes involved in the pathogenesis of TD

Hence, the study of thyroid dysgenesis in humans can provide insights into the molecular mechanisms involved in thyroid development. Many evidences indicate that genetic factors are involved in the pathogenesis of TD. Here we will focus on genes known (or candidate) to be responsible for this affection (*Table II*).

It is worth noting that the mutations thus far identified in patients with congenital hypothyroidism associated with TD account only for a very small number of cases. However, these cases could be much more frequent than hitherto identified ones because mutations which might arise in specific regulatory elements were not searched for, in the studies published so far.

Organogenesis	Expected phenotype(a)	Genetic lesion in human diseases	Genetic lesion in mouse models	Other candidate genes
Budding	agenesis	unknown	Unknown	unknown genes responsible for budding (might include genes that induce the expression of TTITF1/NKX2-1, FOXE1/TITF2 , PAX8 and HHEX)
Migration	ectopic thyroid	unknown	FoxE1/Titf2 knock-out	FOXE1/TITF2 target genes expressed exclusively in thyroid precursors
Survival of precursor cells	athyreosis	FOXE1/TITF2 mutations	Foxe1/Titf2 knock-out Titf1 /Nkx2-1 knock-out Pax8 knock-out Fgf10 knock-out Fgfr2 knock-out HHex knock-out	TTITF1/NKX2-1, FOXE1/TITF2, PAX8 and HHEX target genes and cofactors expressed exclusively in thyroid precursors
Expansion of cell population	hypoplasia	PAX8 mutations (b) TITF1/NKX2-1 mutations(b) TSHR mutations TSH induced genes	Tshr knock-out Tshr hyt/hyt mouse Tshr dw/dw mouse	TSH induced genes
Interactions with neural crest-derived cells	hypoplasia		ET-1 knock out Hoxa3 knock-out Eya 1 knock-out Pax3 knock-out(splotch)	Other Hox genes

Table II Summary of known and potential genes involved in the pathogenesis of TD.

TITF1 *disease*

No *TITF1* mutations are found in patients with non syndromic CH (*Lapi et al., 1997; Perna et al., 1997*). On the contrary, inspired by the phenotype of *Titf1* null mice, studies focussed on subjects with thyroid affections associated with respiratory distress and neurological problems, have reported heterozygous point mutations of chromosomal deletions in the *TITF1* locus have been reported (*Devriendt et al., 1998; Iwatani et al., 2000; Krude et al., 2002; Pohlenz et al., 2002; Moeller et al., 2003; Doyle et al., 2004; Moya et al., 2006*). In these patients choreoatheosis is the most frequent neurological defect. In accordance with these findings numerous data point to the identification of *TITF1* as the candidate gene in benign hereditary chorea, an autosomal dominant movement disorder (*Breedveld et al., 2002*).

Homozygous *TITF1* mutations have not been reported in humans. While in humans loss of function of a single allele produces an overt phenotype, the *Titf1*$^{+/-}$ mice show mild neurological defects and a very slight hyperthyrotropinaemia (*Moeller et al., 2003*). The mechanisms explaining the dominant effect of the TITF1 have not yet been addressed. Only in one case, *in vitro* assays demonstrated that the mutated gene is translated into an altered protein which acts as dominant negative and interferes with the activity of thyroid specific promoters (*Moya et al., 2006*). In other cases dominant negative effects have been excluded. Thus, the reduction of levels of functional TITF1 protein remains the most likely mechanism that causes the disease. *TITF1*$^{+/-}$ subjects display a highly variable thyroid phenotype. In many patients thyroid alterations have not been reported; in other cases patients show elevated TSH levels with mild or severe hypoplasia of the gland. There is no clear correlation between the phenotype severity and the type of mutations; in addition a variable phenotype has been reported also in the same familial cluster. Other modifier genes probably contribute to the phenotype, but at the moment this is only a working hypothesis.

PAX8 *disease*

In human, heterozygous mutations in *PAX8* have been reported in both sporadic and familial cases of CH with TD. Eight different mutations, seven located in the paired domain (*Macchia et al., 1998; Congdon et al., 2001; Komatsu et al., 2001; Vilain et al., 2001; Meeus et al., 2004*) and one in the C domain (*de Sanctis et al., 2004*) have been described. Patients show a variable phenotype ranging from mild to severe hypoplasia of the thyroid in presence of elevated levels of TSH in the bloodstream. The variable expression of the phenotype could be due to the influence of other modifier genes. Assays *in vitro* demonstrate that the ability of the mutated PAX8 proteins to bind to specific DNA target is either strongly reduced or absent; consistently, the transcriptional activity of proteins is lost. All affected individuals are heterozygous for the mutations and the familial cases show an autosomal dominant transmission of the disease. This data indicate that in humans, loss of function of a single allele is sufficient to produce the disease and a reduced dosage of the gene product (haploinsufficiency) causes dysgenesis; in contrast, in the murine model *Pax8*$^{+/-}$ mice display a normal phenotype (*Mansouri et al., 1998*). This discrepancy could be related to the genetic background of the mouse line used in generating the corresponding animal models.

FOXE1 disease

Homozygous loss of function mutation in conserved residues within the FOXE1 forkhead domain was first reported (*Clifton-Bligh et al., 1998*) in two siblings affected by syndromic congenital hypothyroidism, characterized by athyreosis, cleft palate, bilateral choanal atresia and spiky hair {Bamforth-Lazarus syndrome (*Bamforth et al., 1989*)}. The phenotype is consistent with the expression domain of FOXE1 and partially overlaps that displayed by *Foxe1* null mice. After this report, a different mutation in *FOXE1* has been recorded in two siblings with athyreosis and a less severe extrathyroidal phenotype (*Castanet et al., 2002*). In this case the incomplete phenotype could be due to the residual functional activity of the mutant protein assessed *in vitro*. Recently a third loss of function mutation within the *FOXE1* forkhead domain has been described (*Baris et al., 2006*) in a child displaying extrathyroidal defects (cleft palate, bilateral choanal atresia and spiky hair) and CH but not athyreosis; actually the patient presented eutopic thyroid tissue. The variable thyroid phenotype displayed by patients carrying *FOXE1* mutations could be due to different effects of the various mutations. Another possibility is the rule of modifier genes in making the phenotype manifest.

TSHR *disease*

Several years ago Stanbury (*Stanbury et al., 1968*) reported a case of hypothyroidism in the absence of goiter and suggested that the phenotype could be due to an impaired response to TSH. This phenotype was described also in mutated mice ($Tshr^{hyt/hyt}$) (*Stein et al., 1989*), in which hypoplasia of thyroid was associated with high levels of TSH and reduced thyroid hormone. The cloning of cDNA for the TSH receptor (TSHR) has made it possible to study the molecular bases of the phenotype described by Stanbury. Indeed loss of function mutations in *TSHR* gene are responsible for a syndrome (resistance to TSH) (*Refetoff, 2003*) characterized by elevated thyroid hormone. *TSHR* mutations have been identified in a number of families. Individuals carrying heterozygous loss of function mutations are euthyroid even though most of them present borderline elevation of TSH. Subjects homozygous or compound heterozygous for mutations in *TSHR* are affected and the disease is transmitted as an autosomal recessive trait displaying variable expressivity. A group of patients are euthyroid and show hyperthyrotropinaemia associated with normal or hypoplastic (fully compensated resistance to TSH). In another group, patients manifest mild hypothyroidism (partially compensated resistance to TSH). The patients of the third group (severe uncompensated resistance to TSH) are affected by severe hypothyroidism

with hypoplastic gland (*Park and Chatterjee, 2005*). The amount of residual functional activity of the mutant TSHR could be responsible for the variable expressivity of the phenotype.

3. Aim of the present project

The overall aim of the thesis was the discovery of novel genes that could be relevant to thyroid development and differentiation by an unbiased search for transcripts enriched in the early organ primordium.

To address this issue, I compared the microarray transcriptome profiles of E10.5 thyroid progenitor cells isolated by Laser Capture Microdissection (LCM) with that of the entire embryo of the corresponding stage.

I could identify in this way a large number of transcripts in the embryonic thyroid bud which function in this organ development has never been described and thus providing insights to explain some of the cases of CH still unresolved.

4. Materials and methods

Embryo dissection and embedding

C57BL/6 mice (Jackson) were crossed to generate wild-type embryos. Embryonic age (E) was estimated by considering the morning when a vaginal plug was detected as 0.5 dpc. After cervical dislocation embryos were dissected on ice under aseptic conditions in cold DEPC treated PBS (PBS-DEPC).

For cryoprotection, embryos were immediately transferred to 30% sucrose in PBS-DEPC and incubated overnight at 4°C. After embedding in Tissue-Tec (Sakura) specimens were quick-frozen over dry-ice/ethanol slurry and stored at -80°C.

Preparation of frozen sections

For laser capture microdissection (LCM) and downstream applications the thyroid buds of 6 E10.5 embryos from different litters were isolated and pooled together as one sample. In this way three biological replicates were generated. During sectioning and the LCM procedure care was taken to minimize the time sections were exposed to room temperature. Blocks were mounted in a cryostat (Leica) and allowed to equilibrate for 5 min. Sagittal sections were cut and immediately inspected under a light microscope.

Once the central part of the lung-bud was visualized, indicating that the plane of sectioning was close to the midline, consecutive sections (8 sections per slide) were collected on Polylysine slides (Mentzel-Gläser). Each slide was immediately put on dry-ice and stored for a maximum of 90 min. Staining and LCM was performed once sections covering the thyroid rudiment of 3 embryos had been collected (see below).

Staining and LCM

For staining slides were sequentially incubated at room temperature for 30 s in 70% ethanol (EtOH), 20 s in DEPC-treated water, 20 s in 70% EtOH, 20 s in 95% EtOH, 2 s in Eosin Y staining solution (Sigma), twice for 10 s in 95% EtOH, twice for 45 s in 100% EtOH, 5 min in xylene and thereafter allowed to dry for 5 min.

LCM of thyroid buds was performed immediately thereafter using the PixCell II system (Arcturus) under 20 x magnification with a laser spot size of 7.5 µm, laser

output power of 100 mW and pulse duration of 1.5 ms. Thyroid primordial tissue from three embryos was captured on one CapSure HS thermoplastic cap (Arcturus).

RNA isolation and amplification

Total RNA from thyroid precursor cells dissected by LCM was isolated using the PicoPure RNA isolation kit (Arcturus) according to instructions from the manufacturer. Whole-embryo RNA from E10.5 littermates was isolated in triplicate using Trizol reagent (Invitrogen).

RNA quality and quantity was analyzed by an Agilent 2100 Bioanalyzer (Agilent Technologies) on RNA Pico Chips (Agilent). RNA integrity numbers (RIN) were generally >8 indicating good quality of RNA. Each biological replicate was diluted to a starting amount of 7.5 ng total RNA and labeled cRNA for chip hybridization was produced by two rounds of in vitro transcription-based linear amplification using the Two-Cycle Target Amplification and Labeling kit (Affymetrix) according to instructions from the manufacturer. cRNA quality, size-distribution and quantity was analyzed by an Agilent 2100 Bioanalyzer.

Gene chip analysis

The biotinylated cRNA, obtained as above described, was fragmented and hybridized overnight at 45°C to the GeneChip® Mouse Genome 430 2.0 Arrays, which includes over 39000 expressed sequences selected from mouse databases. Each experiment was performed in triplicate.

For each microarray, internal standards were established by hybridizing predetermined amounts of biotinylated cRNA to the microarray as recommended by the microarray manufacturer together with the target cRNA. Chips were washed and scanned with the Affymetrix Complete GeneChip Instrument System, generating digitized image data (DAT) files.

DAT files were analyzed with AGCC (Affymetrix, Santa Clara, CA) producing CEL files. RMA (Benjamini Y, 1995) normalization and data analysis was performed using GeneSpring 10.0.2 (Agilent Technologies, Santa Clara, CA).

The expression values obtained were filtered for fold change greater than 1.5, only up-regulated probe sets in thyroid buds were selected and the resulting gene list

was subjected to *t*-test (*p*-value cutoff of 0.05) with Benjamini-Hochberg (B-H) FDR (Benjamini Y, 1995) correction.

Probe Generation and Labeling

For probe synthesis, clones were purchased from OPEN BIOSYSTEM or GENE SERVICE Company when available, or templates were synthesized by PCR using specific primers and cDNA from E10.5 mouse embryo.

Probes were generated by PCR-based method and the addition of sequence of the T7 or Sp6 universal promoters (T7: GAA TTT AAT ACG ACT CAC TAT AGG GAG A; Sp6: CGA TTT AGG TGA CAC TAT AGA) to the gene specific primers. For practical purposes, I always attached T7 primer to the reverse and the Sp6 to the forward primer. The annealing temperature at this point depends only on the sequence of the gene specific part of the primer. The T7 or Sp6 sequences play no role because there are no binding sequences for them in the cDNA. I determined the best annealing temperature by running an analytical (gradient) PCR with 4 tubes in a range of 52°C - 68°C. Designing primers in this manner avoids having to subclone (i.e. ligation, transformation, mini prep, digest, gel, etc.).

The first PCR product was loaded onto an agarose gel, the desired band was excised and DNA was purified with a gel extraction kit (QIAquick Gel Extraction Kit, QIAGEN). In order to prepare sufficient template to obtain the probe, a new PCR is prepared using the purified gel extract (from first PCR) as template. A part of the PCR product was thus analyzed on an agarose gel. If no unspecific bands are present and the desired band is of the correct size, the PCR product was purified using the Qiagen PCR purification kit (QIAquick PCR Purification Kit, QIAGEN).

0.5 µg of above purified DNA template was used in the *in vitro* transcription reaction for the synthesis of antisense RNA labelled with DIG using a DIG RNA labeling kit (Roche Diagnostics) according to the manufacturer's specifications. Briefly, 20 µl of master mix for each probe template was prepared to contain final concentrations of 1 mM ATP, CTP, GTP, 0.65 mM UTP, 1X transcription buffer, 1 unit/µl RNase inhibitor, and 20 unit/µl T7 or Sp6 RNA polymerase, after which each of the samples was incubated at 37°C (2 h). Two microliters of 10 unit/Al RNase-free DNase I were added to each sample, which was then incubated at 37 °C (15 min) to remove the DNA template. RNA denaturing gel electrophoresis was used to confirm

that each product consisted of a single band and to check that a good quality of synthesized RNA was obtained.

The RNA was purified by G-25 Sephadex MiniQuick Spin Columns (Roche) and the yield estimation was performed by comparing the provided DIG-labelled control, following essentially the instructions of the labelling kits. Dilutions series of both are prepared and spotted on a piece of nylon membrane. Subsequently, the membrane is colorimetrically detected.

Direct comparison of the intensities of samples and control allow the estimation of labelling yield. RNA was diluted in RNA dilution buffer (DEPC-treated water, 20xSSC and formaldehyde, mixed in a volume ratio of 5/3/2) for greater stability. 1µl of the diluted control and samples is spotted on a piece of nylon membrane, fixed to the membrane by cross-linking with UV-light. The membrane is incubated in blocking solution (maleic acid 100 mM, NaCl 150 mM, 1% blocking reagent) for 30 min at room temperature (RT). Anti-DIG alkaline phosphatase is diluted (1: 5000) in blocking solution and incubated with the membrane for 30 min at RT. The membrane is washed twice, 15 min per wash, in washing buffer (maleic acid 100 mM, NaCl 150 mM) at RT and incubated in colour substrate solution (i.e. BM Purple, Roche).

The colour is developed in the dark without shaking. The colour precipitate starts to form within a few minutes and continues for approx. 16 h. When the spots appear in sufficient intensity, the reaction is arrested by washing the membrane with sterile water for 5 min.

DIG-ISH

The DIG-ISH was based on the work of *Jing Yu Andy McMahon Laboratory*, with some modification.

Briefly, sections were fixed in 4% PFA, washed in PBS and treated with 1 µg/ml of PK in PBS for 10 minutes at room temperature. After pre-treatment with PK, sections were subsequently washed in PBS and subjected to acetylation step using 0.25% Acetic Anhydride in 1M Triethanolamine-HCL for 10 minutes.

Thereafter, slides were hybridized over night (50% Formamide; 5X SSC, Ph 4.5; 50 µg/ml yeast tRNA; 1% SDS; 50 µg/ml Heparin) using a probe concentration of 0.5-1 µg/ml at 68°C.

For the detection of hybridization, sections were incubated with anti-digoxigenin alkaline phosphatase-conjugated Fab fragments (ROCHE) at 1:4000 dilution. Staining was developed for 24/48 h, according to probe-signal, with BM Purple AP Substrate (ROCHE). Finally, slides were fixed in 4% PFA- 0.2% gluteraldehyde and mounted in glycerol.

The *in situ* reaction was controlled under an AXIOPLAN 2 microscope equipped with Axiocam digital camera (Zeiss), the images were processed using Axion Vision software and edited by Image J software.

Genotyping

Mutant embryos were identified by PCR analysis of embryonic DNA extracted from yolk sacs. The yolk sacs were incubated overnight at 60°C with lysis buffer (50 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ ml proteinase K) and genomic DNA was extracted adding 0.3 volumes of 6 M NaCl and precipitated with isopropanol. Samples were amplified for 33 cycles (94°C for 30 s, 57°C for 60 s, 72°C for 30 s). $Pax8^{cre/cre}$ embryos were genotyped as described (*Maxime Bouchard et al., 2004*).

Immunofluorescence

Immediately after dissection in ice-cool PBS, embryos were immersion-fixed in 4% paraformaldehyde in PBS. Embryos were immersed after washing 2 x 15 min in PBS in 30% sucrose/PBS at 4°C with gentle rocking until they sink. (usually O/N). Embedding was done in O.C.T. compound (Sakura, Zoeterwoude, the Netherlands) and care was taken to obtain proper orientation of the embryos in the molds.

Freezing of embryos was done over an EtOH/Dry ice slurry. 10 µm thick sections were cut on a cryostat microtome (Microm HM 500M) and collected on polylysine glass slides (Menzel-Gläser, Braunschweig, Germany). Air-dried sections were permeabilized by incubation with 0.1% Triton X-100 for 20 min, incubated in PBS with 2% normal donkey serum (Jackson ImmunoResearch) for 1 hour at room temperature, and then incubated overnight at 4˚C with primary antibody diluted in blocking buffer.

Immunolabelled sections were incubated with secondary antibodies diluted in blocking buffer for 1 hour and thereafter with Streptavidin-FITC for 30 minutes at

room temperature. All incubation steps were followed by washing in 0.1% Triton X-100 for 3 x 5 minutes. Microscopy and imaging were performed in a Zeiss AttoArc II epifluorescence microscope or a Bio Radiance 2000 Laser Scanning Microscope.

5. Results

Analysis of critical gene expression in developing tissue progression, in a normal as well as in disease status, requires the microdissection and extraction of a microscopic homogeneous cellular subpopulation from its complex tissue environment. This subpopulation can then be compared with adjacent interacting, but distinct, subpopulations of cells in the same tissue. The method of harvesting pure cell populations from heterogeneous tissues should entirely preserve the state of the cell macromolecules, thus being suitable for analysis of their gene expression. Laser capture microdissection (LCM) (*Figure 10*) has been developed to provide a fast and dependable method of capturing and preserving specific cells from tissue, under direct microscopic visualization (*Bonner R.F. et al, 1997*). The use of an inverted microscope on slides without coverslips in LCM makes tissue recognition difficult; therefore, good tissue morphology is essential. However, while preserving tissue morphology, histochemical fixatives function by altering the structure of macromolecules (*Auerbach C et al, 1977; Stanta G et al, 1991*), which may affect the integrity of nucleic acids. Fixation can also affect the ability of the instrument to capture cells efficiently. For these reasons, the quality of nucleic acid and proteins from intact cells can be problematic, and it depends on the methods used to preserve the tissue specimen (*Goldsworthy S.M. et al., 1999*). LCM method is central to my purpose and therefore needs to be carefully optimized with respect to the E10.5 thyroid bud. The method has previously been established in the laboratory after initial optimization of conditions for embryo fixation (*Parlato R. et al., 2002*). This protocol has been applied in preliminary experiments to isolate the thyroid bud of E10.5 mouse embryos.

In the following study, I attempted to address the problems of RNA degradation and tissue preservation as discussed above.

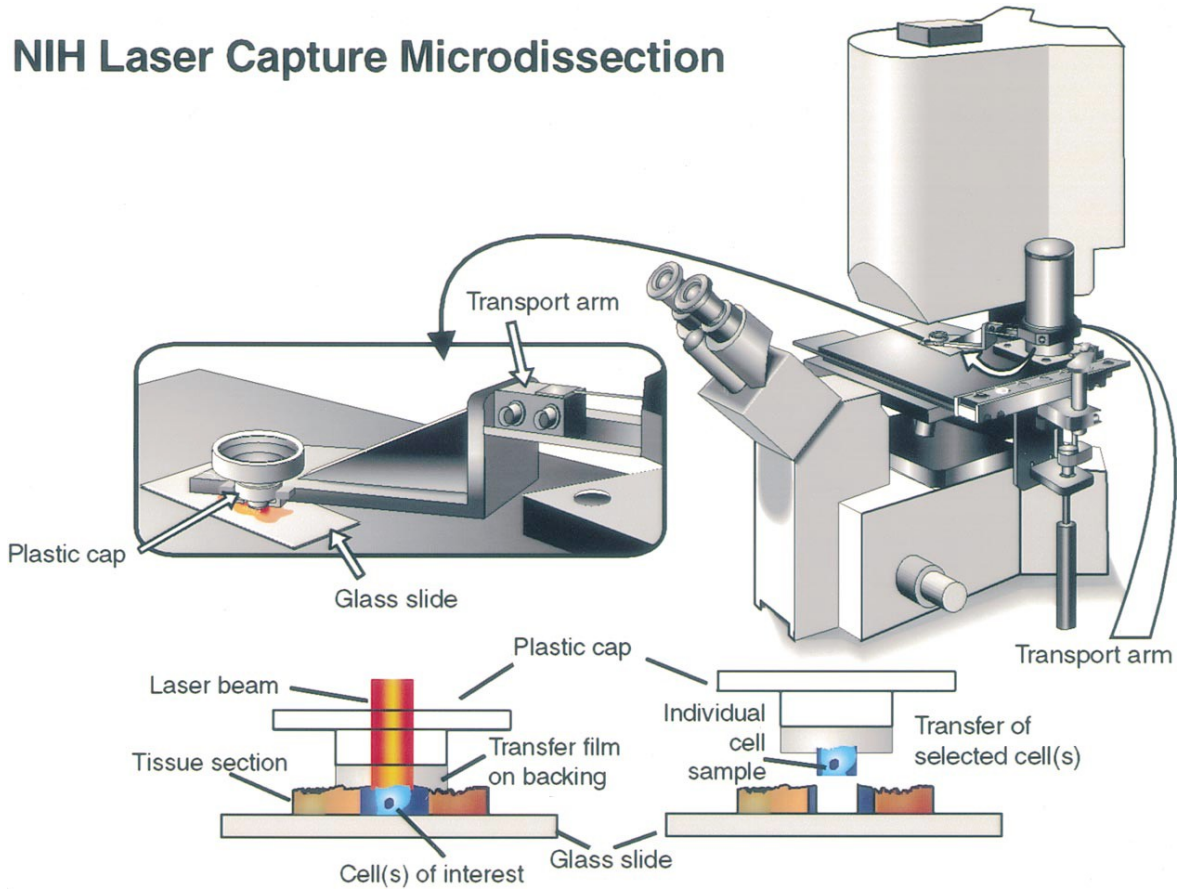

Figure 10 Laser Capture Micriodissection system. A thermolable polymer is placed on a tissue section on a slide. An infrared laser melts the polymer in the vicinity of the laser pulse. The resulting polymer-cell composite is removed from the tissue.

5.1 Effects of Fixation on Recovery of intact RNA

I first re-evaluated the effects of different fixation procedures on the quality of RNA extracted from embryonic E10.5 mouse embryos. Ideal fixation must provide acceptable morphology, allow proper laser capture of selected cells, and preserve the integrity of the RNA.

Embryos were treated for 12 h with methanol/30% sucrose, acetone/30% sucrose, Zinc Fix/30% sucrose or with 30% sucrose without any previous fixation. RNA was extracted using TRizol reagent (Sigma). Each RNA sample was evaluated for quality by inspection of 18S and 28S rRNA integrity. The absence of the rRNA bands observed after fixation of embryos with alcoholic solvents (*Figure 11, lanes 5-8*) indicates that these preparations have deleterious effects on the quality of RNA recovery. On the other hand, RNAs extracted from mouse embryos treated with 30% sucrose or Zinc Fix procedure show clear 18S and 28S bands (*Figure 11, lanes 1-4*).

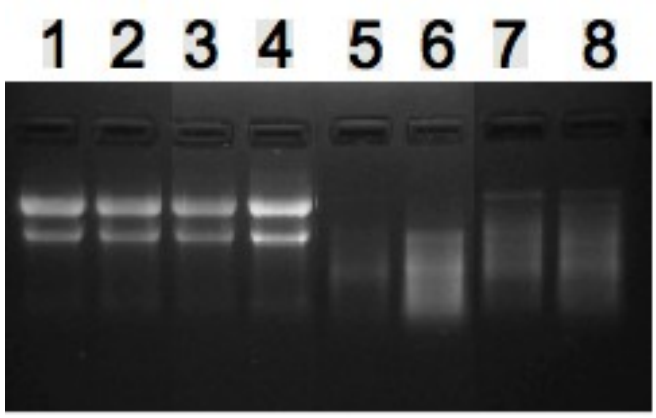

Figure 11 Influence of different fixatives on RNA recovery. Ethidium bromide-stained agarose gel showing RNA extracted from E10.5 frozen embryos after incubation in 30% sucrose (lanes 1-2), Zinc Fix/30% sucrose (lanes 3-4), acetone/30% sucrose (lanes 5-6), and methanol/30% sucrose (lanes 7-8).

5.2 Tissue Morphology

In order to check whether our procedure was able to preserve tissue morphology, I analyzed histology quality from embryos treated in different ways. A number of E10.5 embryos were placed over-night in 30% sucrose, embedded in OCT compound, without any fixation, and cryosectioned. Other embryos were incubated over night in Zinc Fix solution (and/or in Zinc Fix-30% sucrose) and cryosectioned. Figure 3 shows 10 µm frozen sections handled as above. Sections treated with 30% sucrose (*Figure 12A*) display a well preserved tissue structure thus allowing the identification of the cells of interest; in fact the developing thyroid can be seen in the midst of surrounding embryonic tissues (red circle in the picture). On the contrary, Zinc Fix procedure (*Figure 12B*) yields a poor retention of tissue morphology and makes it not suitable to identify the embryonic thyroid bud at E10.5, without contamination from surrounding tissues.

Therefore, the use of unfixed embryos, cryopreserved in sucrose provided the most satisfactory protection for RNA integrity and gave rise to good preservation of morphology, thus it was chosen for all subsequent experiments.

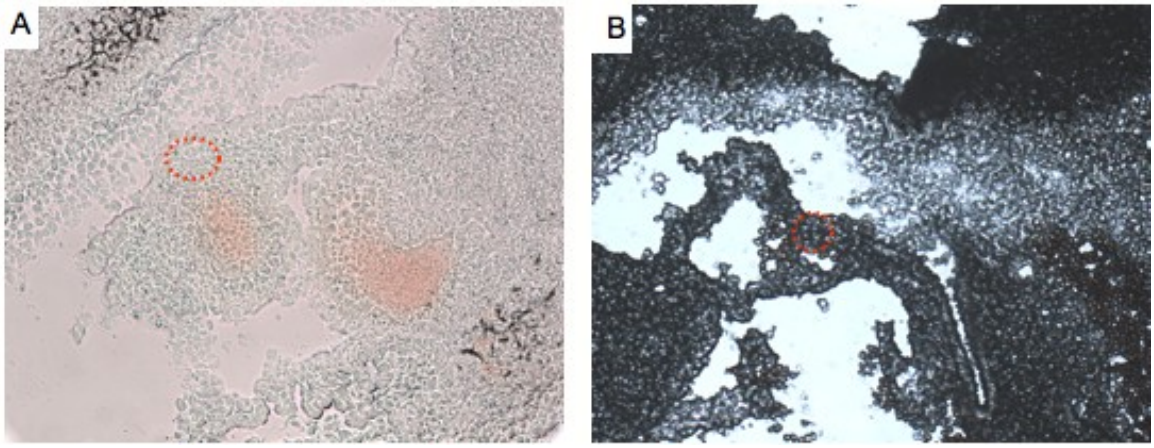

Figure 12 Histology quality from embryos treated with two different fixation protocols. 10 µm section from unfixed E10.5 mouse embryo incubated in 30% sucrose before freezing (A) or treated with Zinc Fixative solution (B). Red circle indicates the developing thyroid.

5.3 Sensitivity of sections to different pre-LCM treatments

The LCM procedure could require a long time to be completed, especially if more than one section has to be collected from the same slide. It means that sections will be exposed to room temperature during LCM. In order to establish how long I can keep the slides at room temperature without destroying RNA, I checked RNA quality in samples kept at room temperature during sectioning or after sectioning. The experiment was done by scrape-off of sections from slides. RNA was extracted with the Arcturus Pico Pure RNA kit following manufacturer's protocol. RNAs were quality-checked with the Agilent Bioanalyzer.

These tests showed that slides can be kept at room temperature for at least 90 min, still obtaining a good quality RNA as assessed on Agilent Bioanalyzer, with a RIN (RNA Integrity Number) between 7 and 9.5 (data not shown) . A RNA integrity number of at least 7 will be considered sufficient for downstream applications.

Frozen sections are highly recommended to maximize quantity and quality of RNA recovery (*Krizman DB et al., 1996; Hiller T et al., 1996*). However, the staining steps used for histological identification of cells of interest could damage mRNA in the cells, with the consequence of loss of representation of certain genes in microarray hybridization analysis. Thus, the influence of the staining method on RNA was assessed by comparing the quality of total RNA extracted from tissue scrapes after using different stains. No RNA degradation was observed when staining solutions containing hematoxylin or Eosin Y were used in comparison with not stained sections.

Moreover, satisfying results were obtained both for fresh embryo sections prepared and stained immediately and for embryo sections procured one day before and conserved at -80°C before staining steps (*Figure 13*). Finally, I chose the staining protocol in which only Eosin Y was included as stain, since this method gave more reproducible staining quality.

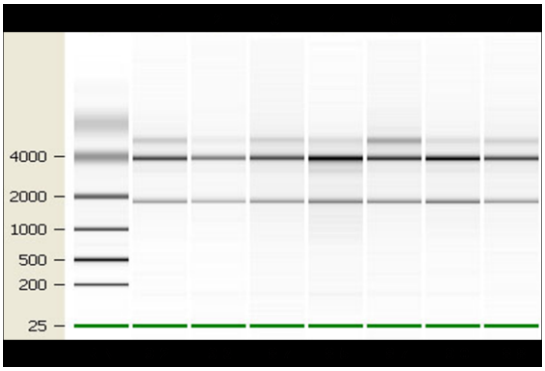

Figure 13 Analisys of RNA quality on Agilent Bioanalyzer system. Lanes 1 to 4 show fresh embryo sections prepared and immediately stained (1:not stained; 2:stained with Eosin Y; 3: stained with hematoxylin; 4:stained with hematoxylin). Lanes 5 to 7 swhow embryo sections prepared 1 day before and conserved at -80°C (5: not stained; 6: Eosin Y; 7: hematoxylin).

5.4 Optimization of RNA Amplification

Microarray analysis coupled with LCM is a good way to analyze the gene expression profiles of a given cell population isolated on the basis of its morphology in heterogeneous tissues (*Luzzi et al., 2001*). However, intrinsic characteristics of LCM allow isolation of only small amounts of total RNA (generally a few nanograms for each sample), thus making an RNA amplification step necessary prior to microarray analysis (*Upson et al.,2004; Luzzi et al., 2005; McClain et al., 2005;Schindler et al., 2005*). In fact, higher quantities are generally required to perform hybridization on arrays, starting from a few micrograms to many micrograms, depending on protocols and arrays.

The fidelity of the RNA amplification step is critical to the extraction of meaningful information from microarray experiments. To optimize the choice of the RNA amplification step, the performance of three different systems for RNA amplification has been evaluated by microarray analysis: the Genechip® Expression 3 Amplification Two-Cycle Target Labeling and Control Reagents Kit from Affymetrix, the RiboAMP® and the RiboAMP HS® Kits from Arcturus.

Table III shows that the Affymetrix Kit gives a best range of amplified RNA and provides enough amount of labeled RNA for microarray analysis. Moreover, to enhance kit efficiency I introduced the following modification to the protocol: I added the T4 Gp32 gene protein (*Rapley R. et al., 1994; Villalva C. Et al., 2001)*, a RNA-binding protein from bacteriophage T4 that provides better accessibility of the mRNA template to the reverse transcriptase and helps in reducing higher order structures of RNA

molecules, thus enhancing overall processivity of cDNA synthesis. This protein was added in both first strand cDNA synthesis reactions.

With this modification, 5, 10, and 15 ng of total RNA, subjected to double linear amplification using the Affymetrix Two-cycle amplification and labelling kit yield large amount of labelled amplified cRNA to hybridize to the array (data not shown).

Table III Evaluation of different system for RNA amplification based on the amount and the size range of labelled amplified RNA.

	Affymetrix	RiboAmp	RiboAmpHS
Total RNA input	20 ng (triplicate)	20 ng (triplicate)	? ng (triplicate)
Obtained labelled aRNA	87±8 µg	39±8 µg	21±3 µg
RNA product bases	200/800bp	200/600bp	200/700bp

5.5 Effect of input RNA amount on the amplification

To investigate whether a different amount of RNA input could give different or ambiguous results after amplification and array hybridization, I performed microarray experiments comparing the array data generated from unamplified and amplified RNAs. I compared several parameters of hybridization quality among our samples. The ratios of the hybridization intensity from the 3□ and 5□ ends of the GADPH and Beta-actin transcripts were generally low, although higher in amplified samples compared with standard. This is consistent with the shorter aRNAs from amplified samples seen on the agarose gel, and is caused by the amplification creating targets that are skewed to the 3□ end of the transcript. I examined overall signal and percent present call (% present) to evaluate whether the amplification samples procedure produces high-quality of array results.

As seen in Table IV, these measures were similar among not amplified and amplified samples, and all indicated good hybridizations, even using 5 ng of total RNA as a starting material.

TableE IV The quality of the amplified samples was assessed by performing microarray experiments comparing the array data generated from unamplified (NA) and amplified RNA samples (5, 10 and 15 ng).

RNA input:	5 ng	10 ng	15 ng	NA
Average signal (P)	4250.3	4526.6	3591.6	4223.1
Average signal (All)	1065.3	1147	1069.6	1036.7
Number present	8811 19.50%	9435 20.90%	11712 26.00%	8551 19.00%
3'/5' ratio Beta-actin	12.81	9.96	7.86	2.74
3'/5' ratio GPDH	6.88	7.88	7.7	2.59

Then, a comparison between the three low amount RNA input samples and the Not Amplified sample showed that the linear amplification technique will anyway differ from the standard one in that I will have some underrepresented genes (possibly the ones with a very low expression level) and some overrepresented genes (possibly the ones with a very high expression level) (*Figure 14*).

The evidence that comparing the low amount RNA input samples between themselves I obtain a very good correlation suggests that these differences are irrelevant with respect to the overall experiment results, because we should avoid false positive values, obtaining a good quality information (*Figure 15*).

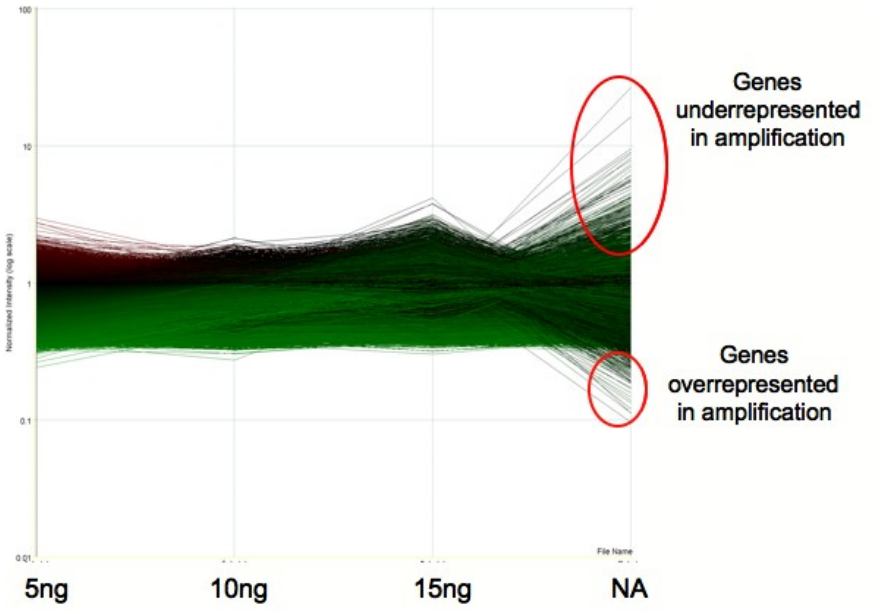

Figure 14 Line graph showing the gene expression between amplified and and Not Amplified samples.

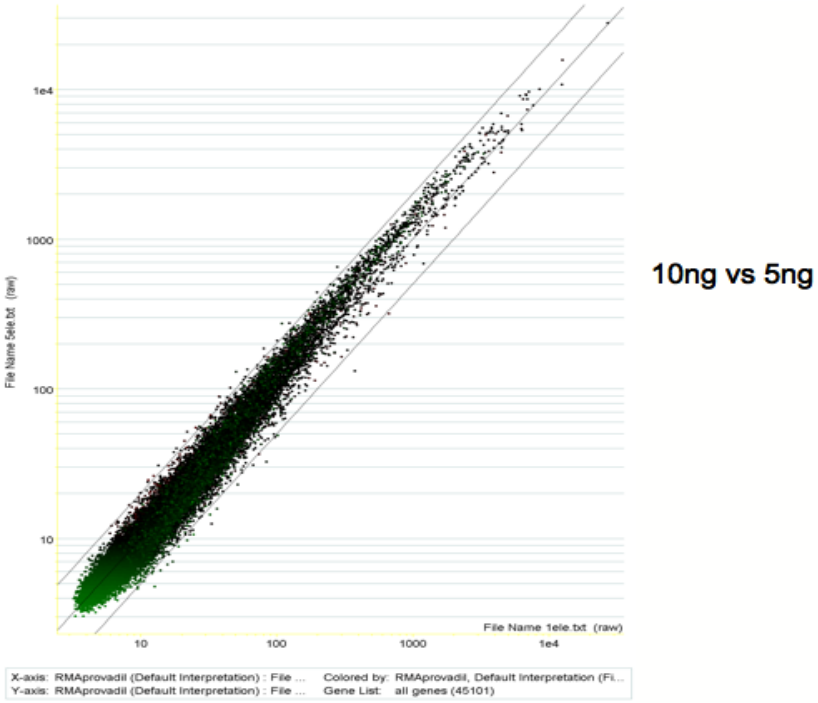

Figure 15 An example of scatter plot generated from data of microarray hybridization with two RNA amplified samples (10 and 5 ng) showing a very good correlation.

Preliminary conclusion

I have described a very good protocol that can be used to prepare tissues for LCM that preserves excellent tissue morphology and still permits extraction of high-quality RNA. Additionally, gene profiling in mice using the Affymetrix system gives the opportunity to measure and compare gene expression levels using down to 5 ng of total RNA as starting material.

There is quite a good correlation comparing gene profiling expression using amplified RNA and not amplified RNA. Satisfactory results were observed on the chips when comparing two different amplified samples.

Encouraged by the high degree of reproducibility from the tests abovementioned, I established that the use of LCM technology is optimal for my purpose, making thus insights in identifying genes involved in the early stages of organogenesis of developing thyroid.

5.6 Identification of genes enriched in the early thyroid primordium

To elucidate the genetic networks that govern thyroid development and differentiation, it is important to identify genes abundantly expressed in the early organ primordium. To obtain this information, I took advantage of the LCM and Affymetrix microarray techniques. Following this approach, I compared the transcriptome profile of E10.5 thyroid progenitor cells with that of the entire embryo of the corresponding age.

Using LCM, we dissected thyroid precursor cells from sagittal mouse criosections, stained with Eosin Y, independent of surrounding embryo structures, thereby generating a pure population of only developing thyroid cells (*Figure 16*).

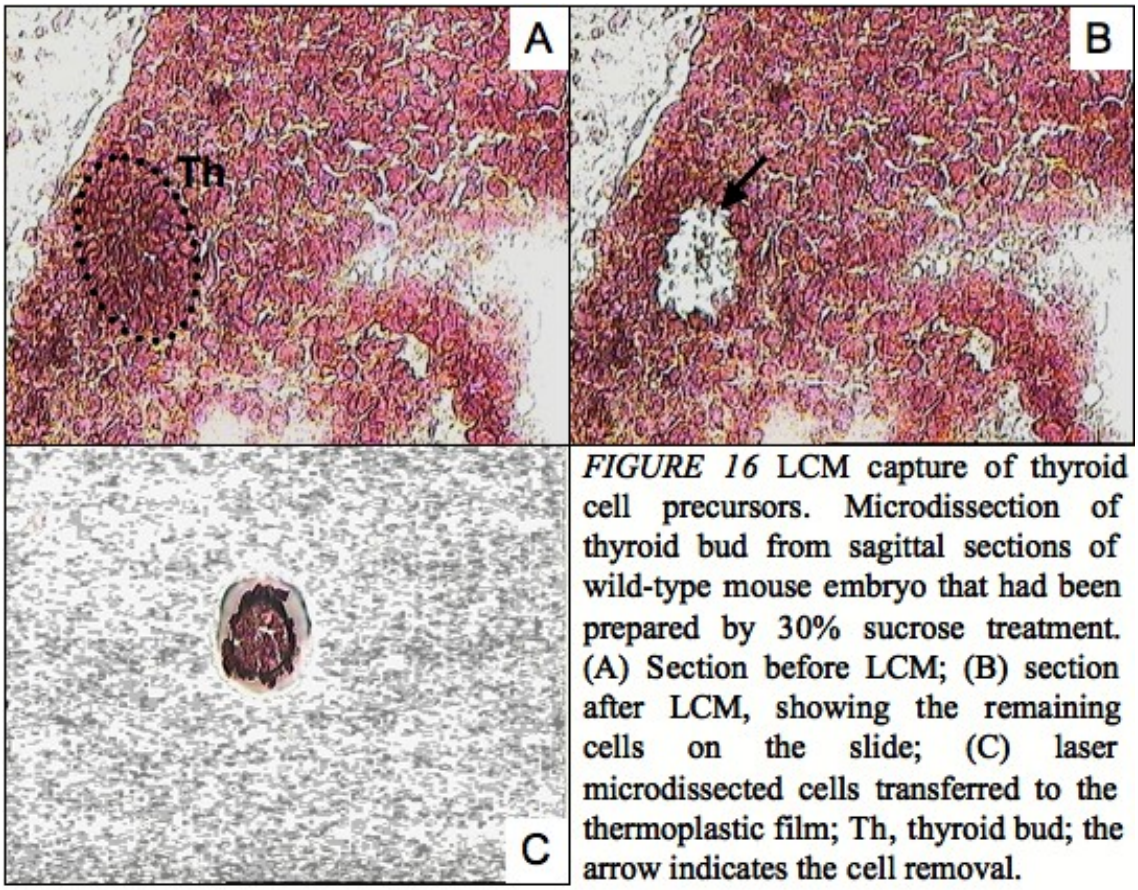

FIGURE 16 LCM capture of thyroid cell precursors. Microdissection of thyroid bud from sagittal sections of wild-type mouse embryo that had been prepared by 30% sucrose treatment. (A) Section before LCM; (B) section after LCM, showing the remaining cells on the slide; (C) laser microdissected cells transferred to the thermoplastic film; Th, thyroid bud; the arrow indicates the cell removal.

At this embryonic stage (E10.5), the thyroid bud of wild-type mice is composed of around 100-150 cells and it is, usually, present in 5 to 6 sections of 10 μm thickness.

In order to obtain enough RNA for downstream applications, I pooled cells microdissected from several wild-type embryos. Three independent pools were generated for wild type embryos. Total RNAs were extracted from E10.5 wild-type whole embryos or only from the captured sample cells, and were quality-checked with the Agilent Bioanalyzer 2100, for in vitro amplification and labelling.

Linear RNA amplification was done based on the method described by *Eberwine J (1996)*, using the GeneChip® Expression 3□ Amplification Two-Cycle cDNA synthesis kit from Affimetrix®. Total RNA obtained from thyroid buds was subjected to double linear amplification using the Affymetrix kit, whereas whole embryo RNAs were processed once with a standard labelling protocol (Affymetrix One-Cycle kit) and another time with the Affymetrix Two-cycle amplification and labelling kit. In each case, around 20 μg of labelled amplified cRNA was obtained for each sample.

Labelled cRNAs were hybridized onto the Affymetrix MOE 430 2.0 arrays, containing over 39000 expressed sequences selected from mouse databases.

Each experiment was performed in triplicate. A flow chart of the approach is contained in Figure 17.

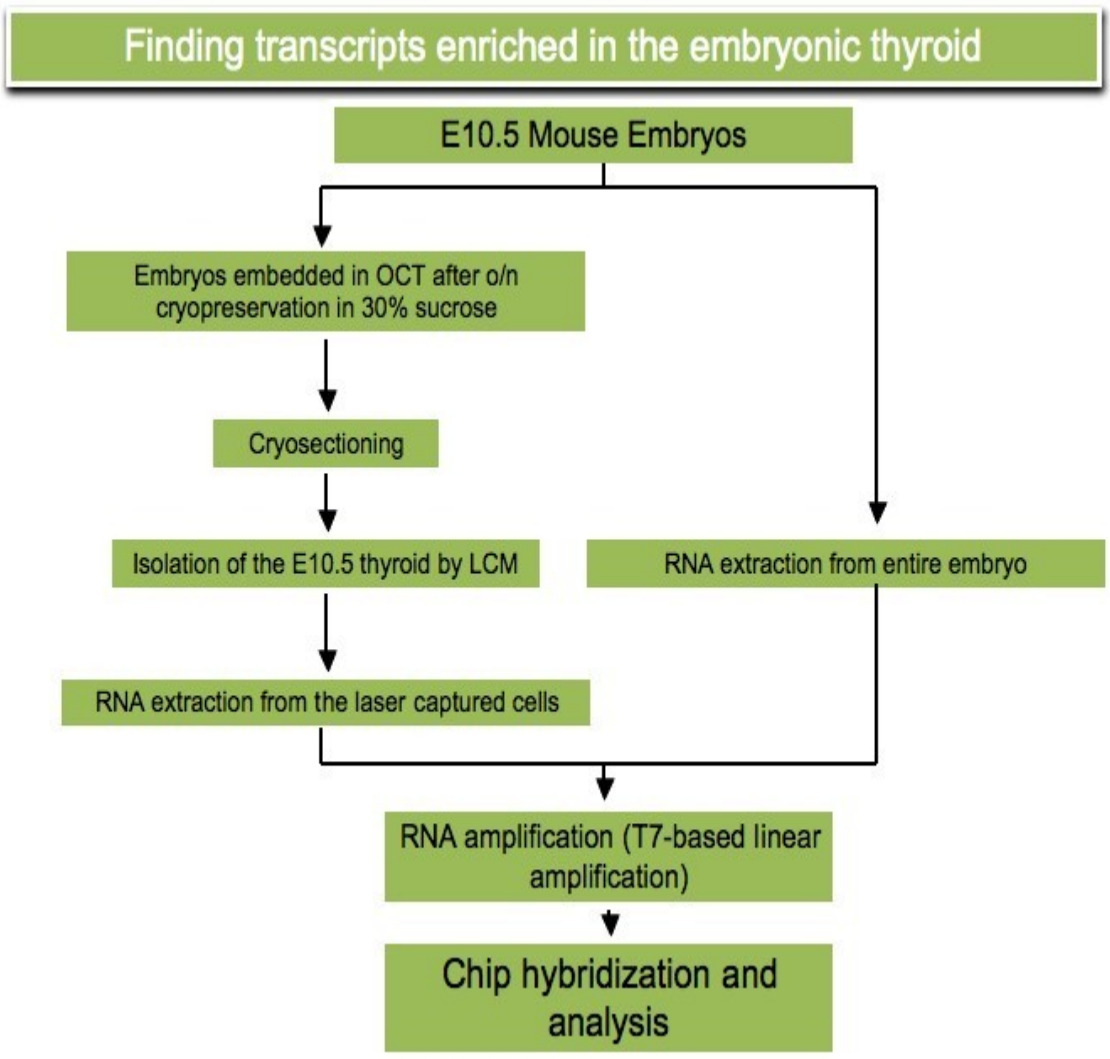

Figure 17 Flow chart of the experimental design and the procedure used.

Expression Profiling experiments were performed by the Gene Expression Core Facility at BioGeM, Ariano Irpino, Italy.

Data were collected and extracted with the Affymetrix GeneChip Command Console software. Data were subsequently normalized using the RMA algorithm, and then filtered for FC greater than 1.5 (using the Whole embryo data as a baseline for the thyroid data).

I compared thyroid buds with whole embryo, both unamplified and amplified, obtaining different gene lists. I applied an Unpaired T-test with variances assumed equal; Table V shows the number of up-regulated genes with a FC greater than *50*, *20*, *10, 5* and *1.5*.

p>0.05	>50	>20	>10	>5	>1.5
Thyroid vs Whole embryo 2-cycle	10	39	141	484	3589
Thyroid vs Whole embryo 1-cycle	17	80	270	916	5671

Table V Number of up-regulated genes in thyroid bud with a FC greater than *50*, *20*, *10* and *1.5*, identified by bioinformatic analysis comparing the thyroid bud transcriptome with that of the entire embryo, both unamplified and amplified.

Thus, bioinformatic analysis identified several transcripts as significantly enriched in the thyroid bud as compared to expression in the whole embryo. Such an enrichment is expressed as Fold Change (FC), a value that indicates how many folds each transcript is more abundant in the RNA of thyroid bud versus the RNA of the whole embryo. The fact that several of the genes enriched on array analysis of LCM material had been previously identified to be highly expressed in the thyroid bud, such as Hhex and Pax8, supports the reliability of my experimental approach.

Furthermore, I verified thyroid bud-specific expression of candidate genes by *in situ* hybridization (ISH).

5.7 Target validation

To confirm a sampling of array data and to search for genes having an effective (or unique) expression pattern in the developing thyroid primordium, I performed *in situ hybridization* (ISH) for a number of candidate genes, which were present in the Thyroid vs Whole embryo 2 cycle list, selected on the basis of their potential relevance in relation to known biological mechanism and relative enrichment. A list of selected genes is presented in Table VI.

List of genes selected to verify their expression in the embryonic thyroid

Gene name	Symbol	Function	FC
RIKEN cDNA 4930426D05 gene	4930426D05Rik	Unknown	108
Interferon inducibile GTPase 1	Iigp1	Cytokine-mediated Signaling pathway	96
Prolactin receptor	Prlr	receptor activity	79
B-cell leukemia/lymphoma 2	Bcl2	Regulation of apoptosis	45
Stanniocalcin 2	Stc2	hormone activity	28
Glucagon receptor	Gcgr	signal transducer activity receptor activity	26
Sorbin and SH3 domain containing 2	Sorbs2	zinc ion binding	24
Solute carrier family 16 (monocarboxylic acid transporters) member 2	Slc16a2	transport symporter activity	19
Transducin-like enhancer of split 2 homolog of Drosophila E(spl)	Tle2	transcription regulation of transcription	16.8
T-box 3	Tbx3	DNA binding transcription factor activity	16.2
Claudin 3	Cldn3	structural molecule activity protein binding	15
Solute carrier family 4 (anion exchanger), member 4	Slc4a4	transport symporter activity	13
Transforming, acidic coiled-coil containing protein 3	Tacc3	microtubule cytoskeleton organization structural	12
Nephronectin	Npnt	cell adhesion cell-matrix adhesion ureteric bud development	10.3

Table VI Candidate genes are listed by the difference in their relative enrichment expressed as Fold Change (*Thyroid bud vs two cycle Whole Embryo genes list, FC≥10*). Common gene names and functions are shown in this list.

I directed our studies toward the identification of thyroid bud enriched genes using ISH. Thus, even though *in situ hybridization* may not be the most sensitive method of transcript detection, it shows a detailed picture of expression patterns which is typically lost when using other methods of detection, such as RT-PCR, that do not allow for the anatomical preservation of tissue sections of interest.

Cryostat sections of frozen tissue and paraffin embedded tissue sections have both been effectively used for ISH. In general, paraffin-embedded tissues show better morphology than frozen tissue. However, several works present in literature demonstrated that ISH using cryosections of freshly frozen material results in less artefactual labelling and in higher overall sensitivity, since paraffin embedding requires more tissue processing and can result in RNA loss and low ISH signal (*Pintar and Lugo et al., 1985*). The major advantages of cryosectioning over traditional paraffin wax sectioning are that tissue embedding and sectioning are carried out under frozen conditions, thus minimizing RNase activities, and allowing a better preservation of mRNA target in the tissue, which is essential to ISH (*Didier Dècimo et al., 1990*).

For these reasons, I established to perform validation of expression in mouse embryos by non-radioactive ISH using frozen sections. Digoxigenin-labeled RNA probes were generated by PCR amplification and the addition of RNA polymerase promoters to the specific primers (see materials and methods) and hybridization was performed essentially as previously described by *Jing Yu and A. McMahon Laboratory*, with some modifications.

In situ hybridization analysis confirmed that eleven of the selected genes displayed a strong expression in the E10.5 embryonic thyroid, whereas three have produced negative or ambiguous results at that time (*Figure 18*). Thus, eleven of the selected fourteen probes, gave a hybridization pattern in complete agreement with microarray readings (11/14 = 79%), the remaining three probes gave no hybridization signal or not explicable (or understandable) result (3/14 = 21%).

These results suggest that the overall rate of agreement between microarray readings and *in situ hybridization* is remarkably high. Moreover, it is worth noting that the number of false positives may be actually lower, since in the last cases the abundance level of the transcripts may be too low for the detection limit of the non-isotopic method used or, simply, the in situ needed an optimization step.

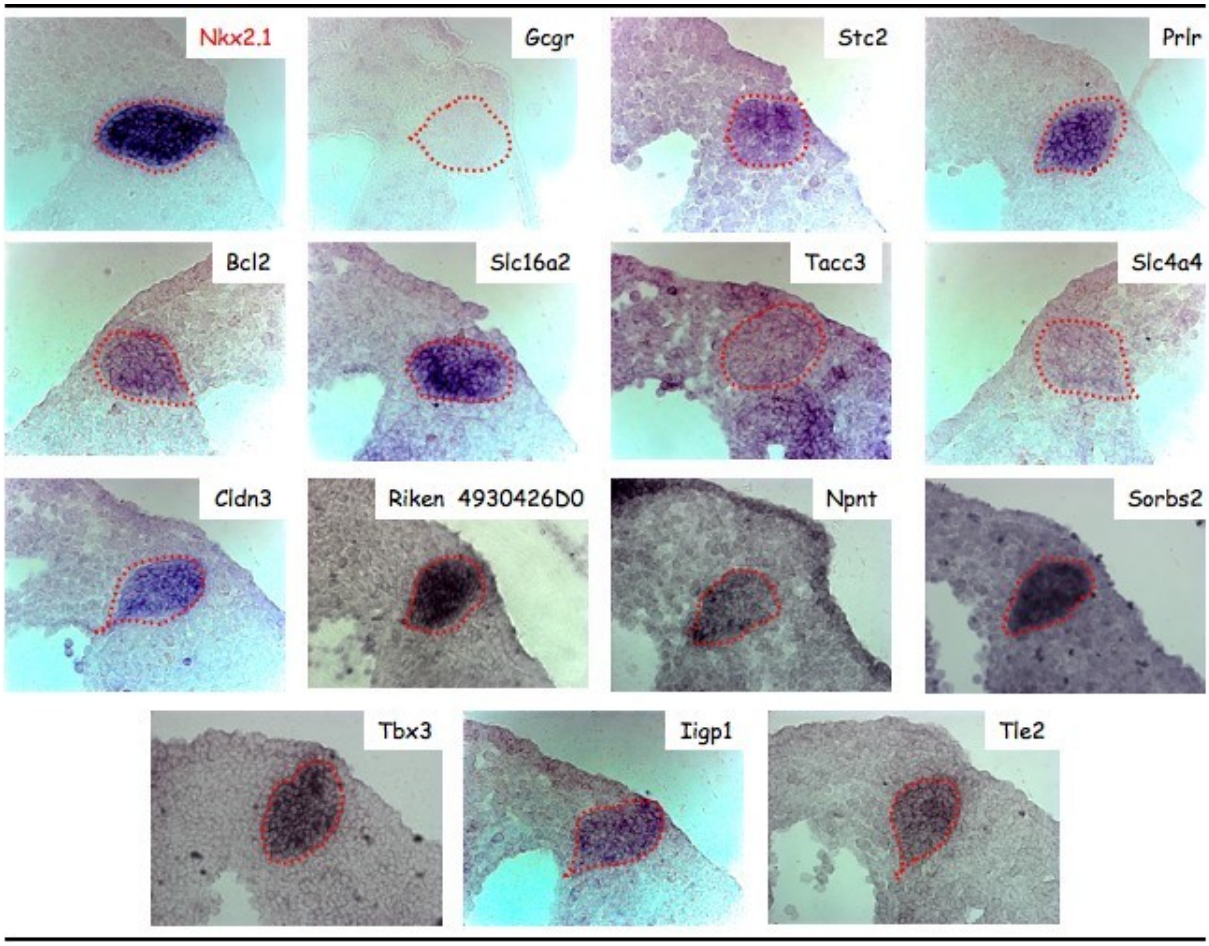

Figure 18 *In situ* hybridization confirms clear expression of 11 of the selected 14 transcripts in the thyroid bud (outlined in red). Sagittal sections of E10.5 mouse embryo were hybridized with probes corresponding to the list shown in TABLE VI. Nkx2.1 (in red) is shown as positive control.

Then, I used the *GenePaint* **http://www.genepaint.org** database to assess the staining patterns of these genes in the E14.5 thyroid. Interestingly, many of the genes I confirmed as enriched in E10.5 thyroid bud showed agreeable correlation in the data present in the abovementioned database (*Figure 19*).

Thus, in addition to my microarray data, there is independent evidence from literature demonstrating the expression in developing thyroid of some genes I detected and suggesting that the LCM technique clearly holds the potential to correctly identify transcripts enriched in a given tissue.

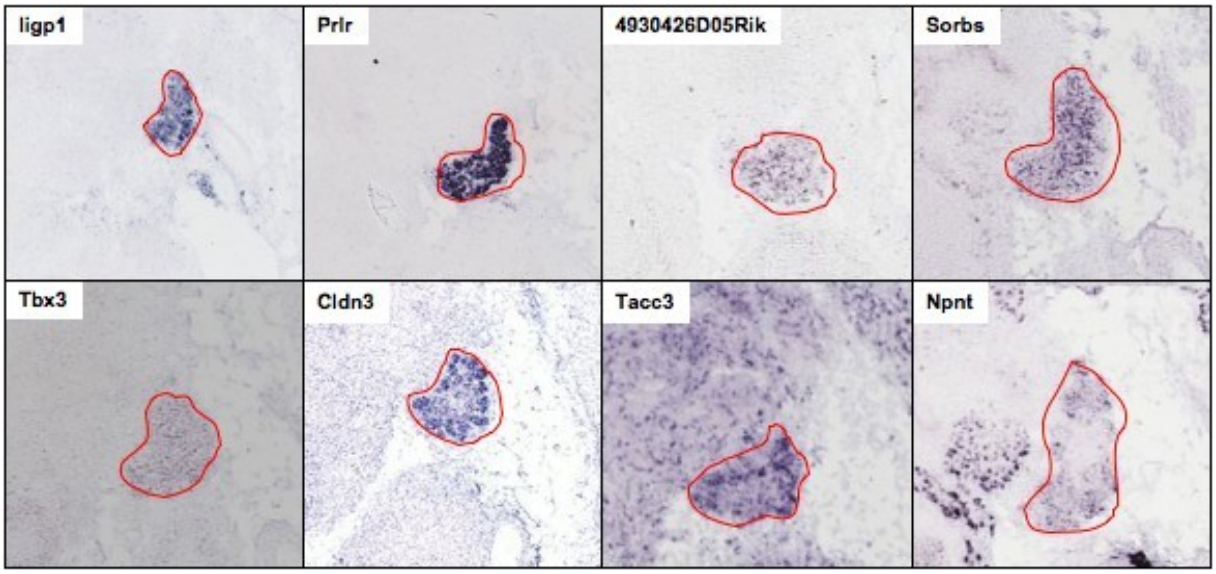

Figure 19 *In situ* hybridization staining patterns for 8 genes present in TABLE VI on E14.5 whole embryo sagittal sections. Images were obtained from the *GenePaint* website and magnified to show the developing thyroid (encircled in red).

5.8 Regulation of an enriched gene in a model of thyroid dysgenesis

The Bcl2 proto-oncogene suppresses apoptosis in a variety of cell types and is expressed in many tissues (*Lu Q-L et al., 1993; LeBrun DP et al., 1993; Allsopp TE et al., 1993; Garcia I et al., 1992*). Expression of Bcl2 is essential for normal renal development. Mice that are homozygously deficient in Bcl2 develop polycystic kidneys and die shortly after birth (*Veis DJ et al., 1993; Nakayama K et al., 1994*).

Pax8 is a member of the paired box class of transcription factors and is developmentally regulated (*Gruss P and Walther C, 1992; Noll M, 1993*). Pax8 is expressed in the developing kidney, thyroid gland and other tissues (*Plachow D et al., 1990*). While the function of Pax8 has been well established in thyroid gland (*Mansouri et al., 1998*), its role in other structures expressing Pax8, such as the spinal cord, the midbrain/hindbrain boundary or the kidney, is less clear probably due to partially redundant functions provided by Pax2 and Pax5 in this organ (*Dressler GR et al., 1990; Asano M & Gruss P, 1992; Urbank P et al., 1994*). Interestingly, Bcl2 and Pax8 are both expressed in the kidney during development, demonstrating an identical temporal and spatial expression patterns and expression of both genes is reduced in cells undergoing apoptotic cell death during renal development (*Chandler D et al., 1994;*

Poleev A et al., 1992). In addition, Pax8 has been shown to transcriptionally activate Bcl2 expression (*Hewitt et al., 1997*). Thus, these data suggest that Pax8 may drive Bcl2 expression in the kidney and other tissues in which they are co-expressed.

I have studied the possible functional relevance of the anti-apoptotic regulator Bcl2 that is among the transcripts I identified as enriched in the E10.5 thyroid primordium in a model of thyroid dysgenesis. Pax8 knockout mice display an impaired thyroid development with a complete degeneration of follicular cells (*Mansouri et al., 1998*). Consequently, Pax8 is an established key regulator of thyroid differentiation. However, little is known about Pax8 target genes that might be relevant in thyroid development. It is noteworthy that as the thyroid bud forms but later regresses in Pax8 null embryos, possibly by an apoptotic process, the finding of the enrichment of the anti-apoptotic regulator Bcl2 is of potential interest.

Thus, the expression of Bcl2 has been investigated by ISH and immunofluorescence (IF) in Pax8 null embryos. Interestingly, Bcl2 seems to be down regulated in Pax8 deficient thyroid precursor, at mRNA and protein levels (*Figure 20 and 21*).

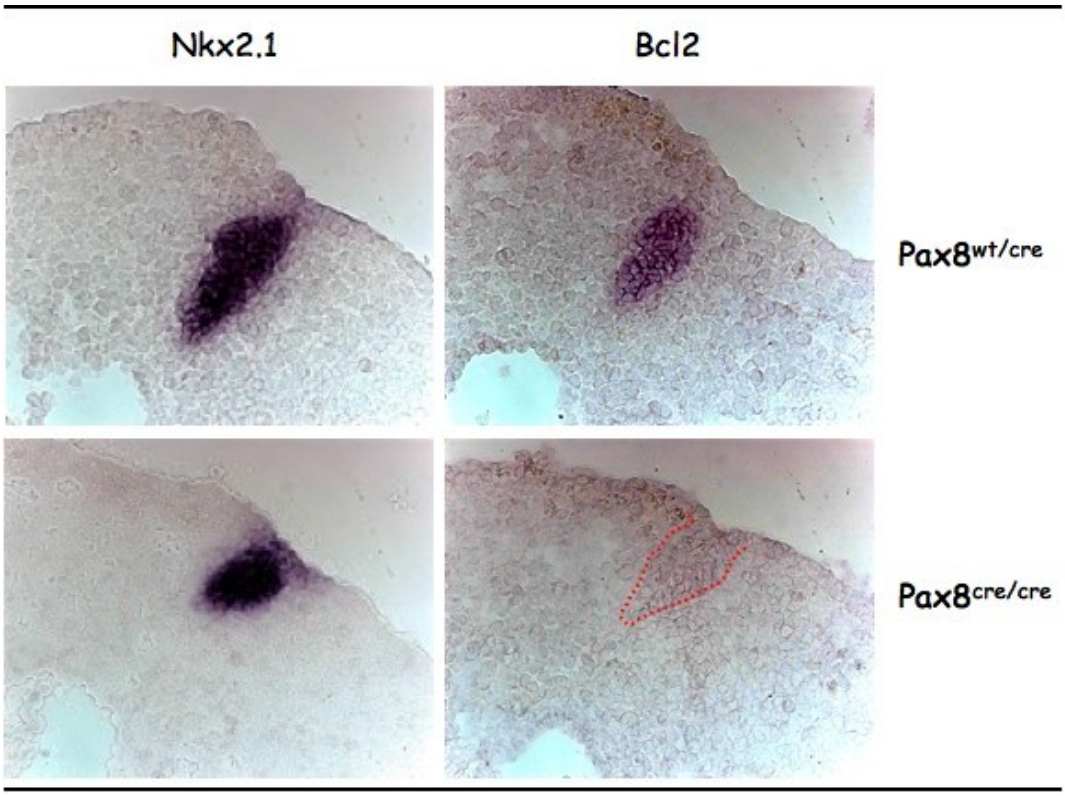

Figure 20 DIG-ISH on E10.5 embryo frozen sections of Bcl2 transcript in the presence or absence of Pax8. Thyroid precursors lack the expression of Bcl2 in the absence of Pax8 (*Pax8*$^{cre/cre}$), as shown.

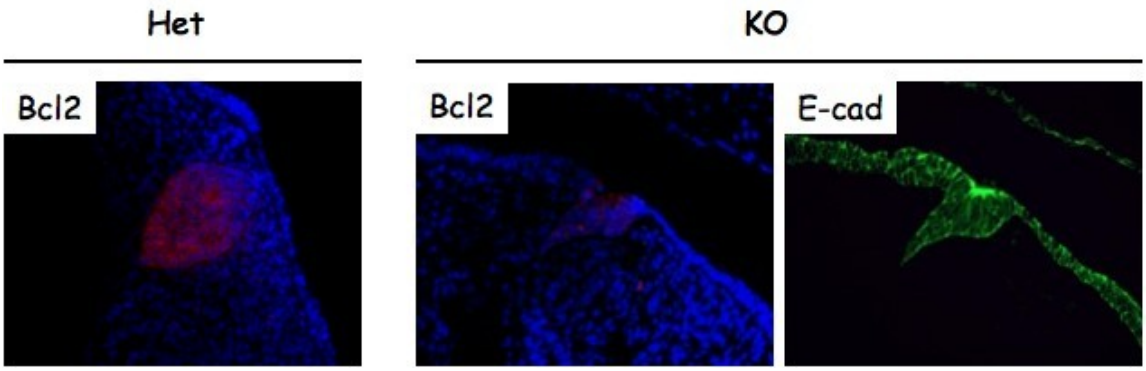

Figure 21 Distribution of Bcl2 protein in the thyroid precursor cells from control (Het) and Pax8 Knockout mice. E10.5 whole embryo sagittal sections were stained by immunofluorescence with antibodies anti-Bcl2, anti-E-cad and DAPI. The staining intensity of Bcl2 in the thyroid bud is reduced in Pax8 null mice with respect to control (Het), as shown. Anti-E-cad antibody is shown as marker of epithelial cells forming thyroid primordium, reduced in size, in adjacent section of Pax8 KO mice embryo.

6. Discussion and Conclusions

During the last decade, gene expression profiling has had a vast impact on biological research. Microarray hybridization has been largely used to study the transcriptome of many different tissues and cell lines (*Duggan et al., 1999*).

However, when the cells to be studied are located in heterogeneous tissue, and therefore, are surrounded by purposeless cells, gene expression of adjacent tissue structures can strongly affect or even conceal the specific signature of the particular cell type of interest. Laser-assisted microdissection could be a useful tool to overcome such obstacles. LCM makes possible the isolation of unique cells or group of cells from heterogeneous tissues and subsequent nucleic acids extraction and analysis (*Emmert-Buck et al., 1996; Suarez-Quian et al., 1999*).

LCM, therefore, makes cell specific expression profiling possible, which may be decisive in addressing the function and the specificity of each cell in a in vivo physiological or physiopathological setting (*Bonner et al., 1997; Luo et al., 1999; Sgroi et al., 1999*). The elegance of this technique is that no tissue is destroyed in the process. LCM operates by positive rather than negative selection. Direct visualization of the transferred tissue, with its histology intact, is also possible to ensure that the correct population of cells is obtained. However, RNA degradation constitutes the main drawback of this technique, considering that RNA quality has a major impact on the subsequent gene expression profiling results (*Goldsworthy et al., 1999; Fleige and Plaffl, 2006; Copios et al., 2007)*.

Carrying out LCM experiment for gene expression profiling requires (1) acceptable tissue morphology allowing histological selection of the desirable cell type, (2) preserved integrity, and (3) preserved biological accessibility to the RNA. Besides maintaining tissue morphology and high-quality RNA, another challenge to be dealt with is the low amount of RNA obtained from microdissected material. Standard protocols for microarray analysis are based on starting amounts of 1–5µg total RNA. Yet, LCM yields at best nanograms of RNA. To overcome this obstacle, it is necessary to employ RNA amplification methods to generate the required microgram amounts of RNA.

In this work, I described a very good method to obtain frozen tissue sections that combine excellent tissue morphology with good preservation of RNA, suitable for

downstream applications. This makes these sections optimal substrates for LCM in order to obtain the harvesting of specific cell population for gene expression analysis.

To optimize the fixation method required to preserve RNA molecular integrity, I have compared the effect of different histological fixatives. Although several authors have examined the use of various fixation procedures to preserve both molecular and morphological information in tissue specimens (*Goldsworthy et al., 1999; Shibutani et al., 2000*), I have found that only the treatment with 30% sucrose preserves both morphological features and RNA integrity. The brief Eosin Y stain provides essential cellular discrimination without significantly affecting the ability to perform subsequent molecular analysis. This represents an important advantage, particularly when tissue availability is limiting, because it makes possible the use of serial sections and molecular diagnosis. These findings, however, stand in contrast to previously published data by *Ellis et al. 2002* where RNAlater-fixed and PBS-washed pancreas samples provided reasonably good tissue morphology.

In addition to maintenance of RNA quality, standardization of RNA amplification and chip hybridization is of extreme importance. The generation of high-quality amplified RNA that is representative of the original mRNA population is crucial for the interpretation of differential gene expression in quantitative transcriptome approaches. In principle, amplification of mRNA can be performed either exponentially using PCR-based methods, as described previously (*G. M. Makrigiorgos et al., 2002; N. N. Iscove et al., 2002; Zhumabayeva B. et al., 2001*) or linearly using T7 RNA polymerase-strategies. T7-based linear amplification of small amounts of total RNA is widely used to obtain complementary RNA (cRNA) quantities sufficient for microarray analysis (*L. Luo et al., 1999; S.R. Lakhani et al., 2001; C. King et al., 2005; JJ Upson et al., 2004; O. Kabbarah et al., 2003*). To avoid biases in amplification, PCR-based methods have not been used here for global transcriptome analysis of small amount of total RNA, although amplification from even traces of total RNA would be possible. Recently, optimized PCR methods that claim to result in a minimally biased amplified RNA population have been published (*G. M. Makrigiorgos et al., 2002; N. N. Iscove et al., 2002*). Due to sampling errors and reaction-intrinsic properties when amplifying over 30-35 cycles, however, faithful amplification of low-copy-number RNAs and saturation effects may still represent important issues. These issues can potentially be minimized by linear amplification strategies.

To improve the accuracy of RNA amplification, I tested different commercial kits based on the T7 amplification strategy by microarray analysis. For our purpose, I have established the Affymetrix kit to be optimal in terms of amplification efficiency and reproducibility. Additionally, understanding that the reverse transcription (RT) is a crucial step in the procedure encouraged the development of different RT strategies aimed at increasing RT efficiency and minimizing reaction-intrinsic biases. I and others (*M. Kenzelmann et al., 2004; L.R. Baugh et al., 2001*) have shown that T4 Gp32 protein may essentially contribute to the quality and quantitative efficiency of the RT reaction. In order to enhance the reaction efficiency, this protein was added in both first strand cDNA synthesis reactions. With this modification, starting with as little as 5 nanograms of total RNA and using two rounds of linear amplification, a sufficient quantity of labeled target is generated for microarray hybridization. In addition, I obtained quite a good correlation comparing gene profiling expression using amplified RNA and not amplified RNA samples. Satisfactory results were observed on the chips when comparing two different amplified samples.

Considering all the parameters analyzed in this study, a protocol for RNA isolation from laser microdissected samples with subsequent Affymetrix chip hybridization was established that was also successfully applied to thyroid precursor cells microdissected from E10.5 mouse embryos to study genes involved in the early stages of thyroid organogenesis.

I compared the transcriptome profile of E10.5 thyroid progenitor cells with that of the entire embryo of the corresponding age. Mouse thyroid primordia at the bud stage (E10.5) were isolated by laser capture microdissection (LCM). Three biological replicates of ~10 nanograms RNA were acquired by pooling several buds. In parallel, RNA from whole embryos was obtained. RNA integrity was confirmed and after two rounds of IVT-based linear amplification and labelling, samples were hybridized to Affymetrix microarrays. Bioinformatic tools identified several transcripts as significantly enriched in the thyroid bud as compared to expression in the whole embryo. Among the transcripts thus identified, genes already known to be highly expressed in the thyroid bud, such as Hhex and Pax8, were found supporting the reliability of our experimental approach.

From this list a number of genes have been selected for validation by *in situ* hybridization based on the abovementioned criteria. Of these, eleven have been confirmed as enriched in the thyroid bud whereas three have produced negative or

ambiguous results. This *in situ* hybridization analysis indicates that the list has a high degree of validity, and confirms that the LCM approach clearly holds the potential of correctly identify putatively enriched genes. Interestingly, some genes I detected as enriched in the thyroid primordium were in agreement with the data present in the *GenePaint* database, showing a similar staining pattern in E14.5 developing thyroid (see Results).

Indeed, literature shows a strong involvement in thyroid development and functionality for many of the genes I identified with this approach, as discussed below.

Among the genes validated by *in situ*, Bcl2 displayed effective expression in the thyroid bud, as previously described (*Q-L Lu et al., 1993*). The main biological function of Bcl2 is to inhibit apoptosis or, conversely, to promote cell survival. Mice homozygously deficient for Bcl2 display polycystic kidney with dilated renal tubules as a part of phenotype (*Veis DJ et al., 1993; Nakayama K et al., 1994*) Other related biological functions concern the control of cell cycle. At the molecular level, inhibition of apoptosis as well as control of cell cycle and differentiation occurs through a complex process of protein-protein interaction. Bcl2 is expressed in all tissues that express Pax8, which is an established key regulator of thyroid gland differentiation (*Mansouri et al., 1998*), suggesting a direct interaction of both genes in the tissues where they are co-expressed. Indeed, it has been demonstrated that Bcl2 is transcriptionally activated by Pax8 (*Hewitt et al., 1997*). I demonstrated in vivo that Bcl2 is strongly down regulated in Pax8 null mice, at mRNA and protein level, indicating that the athyreosis occurring in these mice may be due to an aberrantly activated apoptotic mechanism that normally is prevented by Pax8 acting via Bcl-2.

These findings suggest that suppression of apoptosis is an important event in the development and maintenance of thyroid epithelial structures. To functionally test the relevance of this hypothesis the generation of a murine model where Bcl-2 is over-expressed in Pax8 deficient thyroid progenitors has been initiated.

The Tle2 transcript, according to my data, was strongly expressed in the thyroid bud at E10.5. Tle2 belongs to the Gro/TLE family proteins that are required for many developmental processes, including lateral inhibition, segmentation, sex determination, dorsal/ventral pattern formation, terminal pattern formation, and eye development. It has been shown that Tle2 acts as transcriptional repressor and is expressed during neuronal development (*D. Grbavec et al,. 1998*). Additionally, it has been observed that Tle2 is expressed throughout pancreas development suggesting that it may act at

multiple stages of pancreas development. Interestingly, data from literature demonstrate that Tle2 is able to interact with Nkx2.2, Nkx2.6, Hes1 and Arx, which are key controllers of endocrine cell specification and maturation (*Brad G Hoffman et al., 2008*). These interactions are thought to play roles in the transcriptional functions of these proteins, possibly by causing local chromatin remodeling effects that may interfere with the functions of transcriptional activators (*Edmondson, D. G. et al., 1996*). These data allow us to hypothesize about the possible ability of Tle2 to interact with transcription factors important in the development of the thyroid gland that shares with pancreas the origin from gut tube. In this direction, the possible influence of Tle2 on thyroid-specific transcription may be investigated.

Prolactin receptor (Prlr) gene showed a robust expression in the thyroid primordium. Its expression was also detected in the thyroid gland at E15.5 (data not shown). At that stage, the developing thyroid is featured by the expression of a number of genes required for thyroid function such as thyroglobulin (Tg), TSH receptor (Tshr), and the onset of thyroid peroxidase (TPO), small thyroid follicles begin to appear and a day later the gland displays an evident follicular organization.

Prlr belongs to a class I cytokine receptor superfamily (*Boutin JM et al., 1988; Bazan JF et al., 1989; Kelly PA et al., 1991*). Expression of Prlr mRNA in thyroid of mice, in both follicular cells and "C" cells, has been documented by *Kedzia et al.* 2005. The same authors reported mild hypothyroidism in Prlr knockout mice. Surprisingly, in Prlr null animals, the authors described medullary thyroid carcinoma (MTC) arising from parafollicular C cells producing calcitonin. The incidence of these carcinomas attained 41% in Prlr null mice, whereas this malignant tumor occurs sporadically or as a component of the familial cancer syndrome in humans. Additionally, *P. Costa et al*. 2006 demonstrated an overexpression of Prlr in human medullary thyroid carcinomas by tissue microarray. These observations suggest a possible link between Prolactin signaling via its receptor and thyroid function.

Another gene I detected as enriched in the developing thyroid was Tbx3. Its expression in the epithelium of the pharynx at the thyroid diverticulum, and in the mesenchyme of the pharyngeal arches has been previously reported, confirming the reliability of our data (*Deborah L. et al,. 1996*). Tbx3 is a T-box transcription factor involved in developmental patterning, regulation of proliferation, senescence, cell cycle exit, and apoptosis (*Naiche LA et al., 2005; Hoogaars WMH et al., 2007).* Mutations in Tbx3 cause ulnar-mammary syndrome (UMS) in humans (*Bamshad M et al., 1997)*.

Tbx3 deficient mice developed outflow tract malformations and ventricular septal defects (*Martijn L. Bakker et al., 2008*). Furthermore, Tbx3 physically interact with Nkx2.5 to activate or repress target genes (*P. Barrett, unpublished data, 2007*). Nkx2.5 null embryos have cardiac malformations (*Lyons et al., 1995*), and it is noteworthy that in Nkx2.5 mutant embryos the thyroid bud is smaller than in wild type embryos (*De Felice and Di Lauro 2004).* Interestingly, structural aberrations in other organs are overrepresented among children with thyroid dysgenesis, indicating that CH should potentially be considered as a manifestation of a different syndrome. Among these associated defects, vascular anomalies are the most frequent (*Devos et al., 1999*). Additionally, it has been observed an altered *Shh* expression in Tbx3 null mice (*K Mesbah et al., 2008*). Importantly, genetic deletion of Shh causes hemiagenesis and ectopic development of the thyroid in mouse (*H Fagman et al., 2004*). These data suggest that endodermal signaling is also impaired in Tbx3 mutant embryos and the loss of *Tbx3* thus alters the balance of signaling molecules in the caudal pharynx, revealing a pleiotropic role for *Tbx3* in the control of pharyngeal development.

Overall, these findings allow me to argue that Tbx3 may have an important role in the thyroid morphogenesis, but further researches in this direction are mandatory.

Together with the genes discussed above, my study provides other novel targets which function in thyroid development has never been described, although in many cases their function was studied in other tissues. In vivo analysis on the possible influence of validated target genes on thyroid morphogenesis will be investigated by the generation of murine models of targeted inactivation.

Importantly, I have specifically shown that specimen obtained by LCM and subjected to microarray analysis can uncover regionally enriched genes which are otherwise not identified, for example, by hand-dissection. Thus, I demonstrated that LCM not only permits the isolation of tissue that may be hard to dissect with more rudimentary tools, but also allows for the relative enrichment of specific transcripts that may be diluted out in larger, non homogeneous samples. Here, I have demonstrated that LCM permits sensitive recovery of high-quality RNA from a given cell population. mRNA obtained by LCM can be further analyzed for function-specific gene expression by microarray technology. Additionally, these observations on genes expressed in the thyroid bud provide an infrastructure for further studies aimed at the elucidation of the specific functional roles and interactions of these factors during thyroid morphogenesis. Moreover, to confirm the relevance of the obtained results, selected targets will be

investigated by analyzing orthologous genes in human patients with thyroid dysgenesis. In fact, this list will be an important resource in further efforts to elucidate the genetic networks that control thyroid morphogenesis and might underlie CH.

While my energies were specifically aimed at understanding the molecular aspects of thyroid gland development, I strongly believe that this method represents a useful tool for the unbiased analysis og global gene expression in a wide range of specific cell types enmeshed in a complex tissue background.

7. References

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