EMBRYONIC ORIGIN AND DEVELOPMENT OF THYROID PROGENITOR CELLS

An experimental study focused on endoderm, EphA4 and Foxa2

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Cover picture: EphA4 (green) expression in thyroid progenitor cells. The picture is captured at the developmental stage when the ultimobranchial body (ub) fuse with the midline thyroid anlage (th) at E13.5. Note that the EGFP (green) signal is completely absent in the ultimobranchial body remnant.

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ABSTRACT

EMBRYONIC ORIGIN AND DEVELOPMENT OF THYROID PROGENITOR CELLS

An experimental study focused on endoderm, EphA4 and FoxA2

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The thyroid gland consists of two endocrine cell types, the follicular cells that produce the thyroid hormones T3 and T4 and the parafollicular C-cells that synthesize calcitonin. It is well-known that these cells have different embryonic origin, although details of their specification and development during organogenesis are still largely lacking. Tumors arising from the two cell types are distinct entities with different treatment and prognosis.

In paper I mouse thyroid morphogenesis was investigated to provide a detailed map of the different stages: specification and placode formation (Embryonic day 8.5-9.5), budding (E10.5), migration (E11.5), fusion and bilobation (E13.5), and eventually differentiation (E15.5 and onwards). Special interest was paid to patterns of proliferation of progenitor cells and relationship to embryonic vessels in the neck. Results of this study formed a platform for further investigation in papers II-IV.

Paper II was designed to investigate the germ layer origin of C-cell precursors by genetic and biochemical tracing of cells expressing T-box (Tbx1) and forkhead (Foxa1 and Foxa2) transcription factors. This showed that mouse C-cells in all probability arise from the pharyngeal endoderm, at difference with the prevailing concept of a neural crest origin originally identified for birds. Microarray analysis indicates that Foxa2 is a novel marker of human medullary thyroid carcinoma cells.

Paper III and IV identified the EphA4 receptor as a novel modulator of follicular and C-cell proliferation in post-natal life. The effect on the C-cell lineage was obviously cell non-autonomous as EphA4 was expressed only in the follicular cells. EphA4 was further found to be expressed in the embryonic thyroid in a distinct spatiotemporal pattern, although no thyroid malformation was detected in EphA4 null embryos presumably due to redundant functions of other Eph receptors. The cognate ephrin ligands interacting with EphA4 in the thyroid awaits to be identified.

Key words: thyroid, C-cells, Tbx1, Foxa1, Foxa2, EphA4, neural crest, endoderm

LIST OF PUBLICATIONS

The thesis is based on the following papers, referred to in the text by their roman numerals.

Fagman H, Andersson L and Nilsson M.
The developing mouse thyroid: embryonic vessel contacts and parenchymal growth pattern during specification, budding, migration, and lobulation.
Dev Dyn. 2006 Feb;235(2):444-55

Andersson L*, Westerlund J*, Carlsson T, Lania G, Baldini A, Fagman H and Nilsson M.
Foxa2 expressing mouse embryonic C-cells originate from progenitors in the foregut endoderm.
Manuscript
* contributed equally to this work

Andersson L, Westerlund J, Carlsson T, Amendola E, Fagman H and Nilsson M.
Role of EphA4 forward signaling in thyroid development: Embryonic expression pattern and regulation of folliculogenesis and C-cell lineage expansion.
Endocrinology (*Accepted*)

Andersson L, Liang S, Carlsson T, Liao X, Weiss R.E and Nilsson M.
Impaired thyroid growth in *EphA4* deficient mice in an experimental goitrogenesis model.
Manuscript

TABLE OF CONTENTS

ABBREVIATIONS

ADAM	A-Disintegrin-And-Metalloprotease
AIP	Anterior intestinal portal
cAMP	Cyclic AMP
CIP	Caudal intestinal portal
$ClO4^-$	Perchlorate
E	Embryonic day
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EMT	Epithelial to mesenchymal transition
Ephrin	Eph family receptor interacting proteins
FTP	Follicular thyroid carcinomas
IGF-1	Insulin-like growth factor-1
MAPK	Mitogen activated protein kinase
MTC	Medullary thyroid carcinomas
NCC	Neural crest cell
NIS	$Na+/I^-$ symporter
PAA	Pharyngeal arch artery
PKA	Protein kinase A
PTC	Papillary thyroid carcinomas
SH2	SRC-Homology-2
T3	Tri- and tetraiodotyronine
T4	Thyroxine
TBG	Thyroid-binding globulin
TG	Thyroglobulin
TPO	Thyroperoxidase
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
UB	Ultimobranchial bodies

INTRODUCTION

This thesis is focused mainly on the embryonic development of the mouse thyroid gland. The reason is, much is yet unknown of the molecular mechanisms that determines its formation, from the earliest onset of specification of progenitors cells destined to a thyroid cell fate, to the gradual process of morphogenesis leading to the final anatomy, histoarchotecture and cellular content of two endocrine cell types. Paper I provides a complete map of the morphogenetic stages as the mouse thyroid develops, previously lacking in the literature. The findings in paper I form the basis of the microscopic evaluation of data in the next papers, in which novel aspects on the embryonic origin of thyroid C-cells (paper II), regulation of progenitor cell interactions (paper III), and modulation of post-natal thyroid growth (paper IV) by the Eph receptor EphA4 are presented. To introduce the work I will here summarize the basic concepts of thyroid structure and function, and the developmental process from two originally separated primordia in the foregut endoderm. In addition, the genes and molecules of interest will be highlighted.

The thyroid gland: general

Anatomy and cell types

The butterfly-shaped thyroid gland is located in the anterior neck, on the trachea just inferior to the larynx (Figure 1). A median tissue called isthmus connects the two lateral thyroid lobes. The functional units of the gland are the follicles in which thyroid hormone is synthesized and stored. Each follicle consists of a single layer of epithelial cells (follicular cells or thyrocytes) resting on a basement membrane and secluding an inner follicular cavity, the lumen, which serves as the actual production place of thyroid hormones and storage after their synthesis. Calcitonin producing C-cells located between the follicles in a so-called parafollicular position are the second endocrine cell type of the thyroid. Although C-cells are scattered throughout the gland, they are mostly distributed medially in the centre of each lobe reminiscent of the embryonic development (Figure 1).

Hormone synthesis

Thyroid hormones are key regulators of metabolism and development in all vertebrates and they apply their effects on almost every cell in the body. Thyroid hormones have multiple actions which include: somatic growth, homeostasis of the cardiovascular system, development of the nervous system and regulation of the metabolic rate (Boelaert and Franklyn 2005). Iodine is an essential component of thyroid hormone. From an evolutionary point of view, when a variety of species moved from an iodine-rich environment for example the sea to the relatively iodine-deficient environment, it is thought that this change forced the development of both the follicular structure and novel molecular mechanisms that are more efficient in capturing inorganic iodide (I^-) required for the formation of thyroid hormones (Venturi, et al. 2000). The cells thus actively take up iodide from the circulation. Iodide is transported into the thyrocytes by the sodium iodide

(Na^+/I^-) symporter (NIS), which is expressed only at the basolateral cell surface and functionally coupled to the sodium ion gradient generated by Na^+/K^+-ATPase (Dai, et al. 1996). The negatively charged iodide therefore enters the follicular lumen against its concentration gradient (De La Vieja, et al. 2000). Iodine accumulated in the follicle lumen is covalently bound to a large glycoprotein, thyroglobulin (TG), that constitutes the prohormone in which the thyroid hormones are integrated. This occurs by iodination and coupling of tyrosyl residues in TG after it is secreted by the thyrocytes into the follicle lumen. Hormongenesis normally takes place

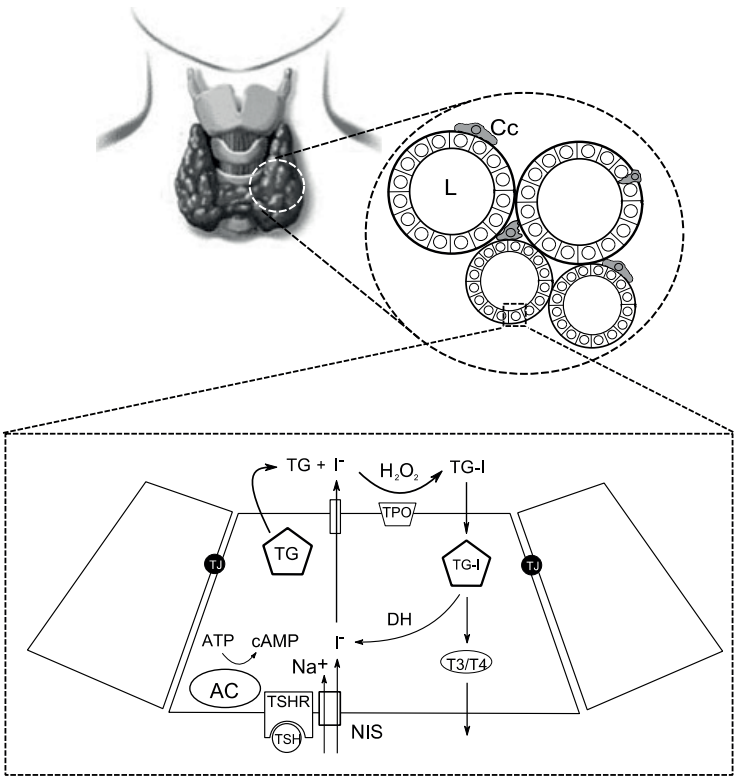

Figure1. Structure of the thyroid gland and overview of thyroid hormone synthesis. Note that C-cells (Cc, grey) can either have a parafollicular position or be integrated in the follicular wall, but always outside the epithelial layer of thyrocytes facing the follicle lumen (L). See text in thesis for further explanation of abbrivations. AC, adenylate cyclase; ATP, adenosine triphosphate; c, capillary; cAMP, cyclic adenosine monophosphate; Cc, C-cell; DH, dehalogenase.

extracellularly at the apical cell surface. Iodine becomes oxidised once it enters the follicular lumen in a process that requires hydrogen peroxide (H_2O_2) produced by the thyrocytes themselves and thyroperoxidase (TPO) that catalyzes the reaction. Effective means to inhibit thyroid hormone synthesis is withdrawal of iodide by competitive inhibition of NIS-mediated iodide uptake by perchlorate (ClO_4^-), and prevention of iodination by antithyroid drugs (e.g. methimazole) that blocks TPO activity. Both compounds may be used clinically for treatment of hyperthyroidism and experimentally in investigations of thyroid functions. Secretion of thyroid

hormones into the bloodstream begins with endocytosis of iodinated TG from the follicle lumen, followed by protolytic cleavage of the prohormone by lysosomal enzymes which result in the release of T_3 and T_4 (tri- and tetraiodotyronine, the latter also known as thyroxine) for transport out of the cell and into the capillaries. The majority of circulating hormones is bound to plasma proteins (thyroid-binding globulin (TBG), transthyretin and albumin); only the free pool of T4 and T3 is metabolically active in target cells (Figure 1).

Regulation of function

The synthesis and release of thyroid hormone into the circulation is regulated by thyroid stimulating hormone (TSH) from the pituitary gland by a negative feedback mechanism (Shupnik, et al. 1989). The release of TSH from cells in the anterior pituitary is modulated by thyrotropin-releasing hormone (TRH) produced by the hypothalamus. TSH production is suppressed when T4 levels are high, leading to decreased activity of the thyroid gland. Contrary to this, TSH is elevated in response to reduced T4, which stimulates the thyroid to increase both the TG production and the iodide uptake and also to accelerate thyroid hormone synthesis and release. Disturbances of hormone supply at any level eventually lead to impaired thyroid function, which can be diagnosed by monitoring the circulating levels of TSH, T3 and T4. At the cellular level TSH exerts its effects by activation of the TSH receptor (TSHR). Several intracellular signalling pathways act downstream of TSHR, the most important being generation of cyclic AMP (cAMP) that triggers or enhances the activity of protein kinase A (PKA).

Regulation of growth

The thyroid size in adults is not much changed during the lifetime unless exposed to goitrogenic stimulation. However, on demand thyroid growth and enlargement can be outstanding, e.g. in severe iodine deficiency. In this situation it is the elevated TSH concentration in blood that triggers the thyroid cell to proliferate. Once a matter of debate, it is now established that TSH is a mitogen that stimulates thyroid cell cycle progression and mitosis through the TSHR-cAMP-PKA pathway (Roger, et al.). In addition, thyrocytes are susceptible to various peptide growth factors, e.g. epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1), which modulate thyroid growth both normally and in pathological conditions.

Growth regulation of the fetal thyroid is very much an unexplored field. Of interest, as evidenced by the mild structural phenotype in TSHR null mice (Postiglione, et al. 2002), TSH has no role in the expansion of the follicular cell lineage during thyroid organogenesis in the embryo. Several embryonically active factors that if genetically deleted lead to agenesis or hypoplasia of the gland have been identified (some of these will be mentioned and discussed in coming paragraphs). However, in most cases this appears to be secondary to defective morphogenetic signals. Mitogens required for embryonic thyroid growth are yet to be discovered.

Papers I and III of the thesis are in part focused on embryonic thyroid growth and the expansion of thyroid progenitors to their differentiated descendants. Post-natal growth of the thyroid and growth responsiveness in adult gland is investigated in papers III and IV.

Thyroid development

Progenitors of different origin

The thyroid gland is in mammals formed by fusion of one midline and two lateral anlagen, all developed from the anterior foregut. This developmental process follows a sequence of morphogenetic steps that are similar in humans and mice (Figure 2). The progenitor origins of the two endocrine cell types populating the thyroid are essentially different and will be focused on in detail in several of the papers included in the thesis. A short summary of the main background information is therefore warranted.

The follicular cells

Thyroid specification giving rise to progenitors of the follicular cell lineage takes place in the anterior endoderm. The cells assemble to form the midline thyroid primordium, which is first observed as a thickening of the foregut endoderm at the base of the prospective tongue. This thickening is termed the thyroid placode and can be distinguished molecularly from the rest of the endoderm by the co-expression of four transcription factors: Nkx2.1, Foxe1, Hhex and Pax8 (Civitareale, et al. 1989; Guazzi, et al. 1990) (Plachov, et al. 1990) (Zannini et al. 1997) (Bedford, et al. 1993; Crompton, et al. 1992) (Figure 3). These transcription factors also have central functions in other embryonic tissues, but it is only in the endoderm cells committed to a thyroid fate the combination of all four can be found. Several of these transcription factors not only regulate thyroid development but also thyroid differentiation, e.g. the expression of NIS and TG in both embryonic and adult thyrocytes. There are no controversies regarding the endoderm origin of the follicular cells. However, it is still not known whether the placode is the actual site of specification or whether progenitors not yet expressing the characteristic set of biomarkers arise in other parts of the endoderm and migrate into the placode by lateral movements.

The C-cells

The ultimobranchial bodies (UB) serve as vehicles for the transport of C-cells into the thyroid prior to the fusion with the midline primodium. Even though the mechanisms by which C-cells enter the embryonic thyroid have been known for almost half a century, the embryonic origin of the C-cell precursor is still being discussed. In 1974, a neural crest origin of avian C-cells became evident in the classical quail-chick grafting experiments, in which the cranial neural crest division that comes to populate the pharyngeal apparatus was found to differentiate into C-cells in the ultimobranchial gland (Le Douarin, et al. 1974; Polak, et al. 1974). Although not formally proven, it is generally assumed that mammalian C-cells are also descendants of neural crest (Adams and Bronner-Fraser 2009). However, this concept of C-cell origin was recently challenged by results achieved from fate mapping experiments in mice. Kameda et al. used Wnt1-Cre/Rosa26R double heterozygous mutant embryos to map neural crest-derived cells as they migrate into the pharyngeal arches, but failed to identify any Wnt1 expressing cells in the ultimobranchial bodies or in the thyroid co-localizing with the C-cells. From this, the authors proposed that C-cells might originate from endoderm rather than the neuroectoderm (Kameda, et al. 2007).

The embryonic origin of various cell types has been significantly modified in recent years due to the adoption of new techniques. Of particular interest, this notion accounts for gut endocrine cells (Thompson, et al. 1990). As early as 1924 Danisch suggested a neural origin for enteroendocrine cells that were believed to migrate from the solar plexus into the intestinal epithelium (Thompson et al. 1990). In the late 1960´s Pearse introduced the APUD (amino precursor uptake and decarboxylation) model, in which both C-cells and neuroendocrine cells of the gut together with a great number of other cell types were all considered as neural crest derivatives (Pearse 1968, 1977). Some years later in 1990, Thompson et al. showed that gut endocrine cells share a common stem cell with other epithelial cell lineages and therefore are specified within the endoderm (Thompson et al. 1990).

The embryonic origin of an organ-specific cell is of central interest when it comes to tumours, because certain embryonic features related to growth and migration traits established during development may be recapitulated in malignantly transformed cells arising from it (Dubeau 2008). In a broader sense, the embryonic origin is also recognized in thyroid cancer. Malignant thyroid tumours are divided in two groups reflected by the ancestor cell: follicular and papillary thyroid carcinomas (FTP, PTC) from the follicular epithelium and medullary thyroid carcinomas (MTC) from the C-cells. MTC belongs to the neuroendocrine family of tumours mainly designated by the expression of neuronal biomarkers but also the assumed embryonic origin of human C-cells. Whether embryonic programmes putatively involving oncogenic effects of developmental growth signals are reactivated in thyroid carcinoma cells is, however, unknown.

Morphogenetic stages

A central theme in this thesis is thyroid morphogenesis. In particular paper I, where we tried to pinpoint the key morphogenetic events in mouse, as the mechanisms to these sequence of events are largely unknown. To be able to discover organ malformation and phenotypic changes in for example mouse models of disease, it is important to have a detailed overview of normal development. It is therefore appropriate to shortly summarize the various stages of the developmental process leading to the final anatomy and composition of the gland (Figure 2).

As already introduced, the thyroid consists of cells from fused anlagen, one median and two laterals, which emerge from different regions of the pharyngeal endoderm. In the following overview, the two anlagen will be described separately until they fuse. The main steps that can be identified and addressed are; specification and placode formation, budding and detachment of bud, downward migration of midline anlage, symmetric bilobation, fusion of primordia and eventually lobe growth and differentiation of progenitors. Mouse and human thyroid developments follow essentially the same pattern although timing is different (Trueba, et al. 2005); estimated embryonic ages for each developmental stage in mice and humans are highlighted. However, most details are derived from mouse studies (Figure 2).

As mentioned, the cells forming the thyroid placode (Mouse: E8.5-9.5, Human: 4^{th} week) in the anterior foregut endoderm co-express *Nkx2.1*, *Foxe1*, *Pax8* and *Hhex*. These transcription factors are all obligatory for normal thyroid development. The first noticeable specified thyroid

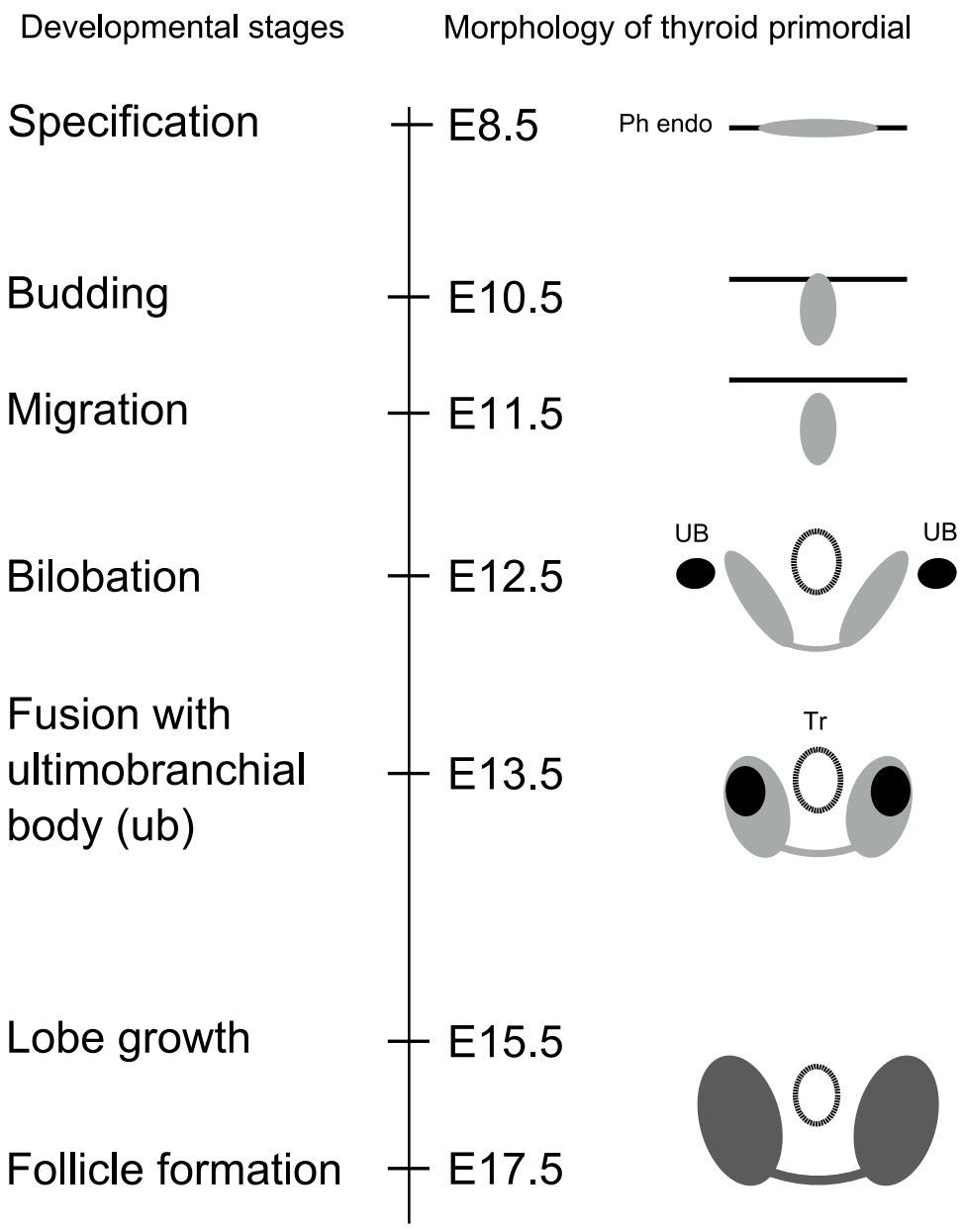

Figure 2. Overview of thyroid development. Tr, trachea; UB, ultimobranchial bodies; Ph.endo, pharygeal endoderm. See text in thesis for further explanation.

progenitors can be recognized as a subpopulation of cells that become crowded, resulting in the thickening of the ventral wall of the primitive pharynx, in the midline at the level of the first and second pharyngeal arches. The thyroid placode gradually becomes pseudostratified before the actual budding process takes place.

The ultimobranchial bodies (UB), which constitute the lateral thyroid anlage, can be recognized in the mouse embryo approximately one day later than the thyroid placode. The paired UB originates from the fourth pharyngeal pouches. However, a morphologically disceranble placode of gathered UB progenitors cannot be distinguished before budding takes place. The thymus and parathyroid glands develop from the third pouch by a similar mechanism. All three organs that bud from these locations require the transcriptional activity of Tbx1, expressed in the pharyngeal endoderm.

The increase in number of thyroid progenitor cells enlarges the placode which forms a cup shaped evagination or diverticulum of the midline pharyngeal floor (Mouse: E9.5-11.5, Human: 4-5th week). Budding occurs caudally into the ventral mesenchyme, and as the bud grows the connection to the pharyngeal endoderm, the thyroglossal duct, becomes thinner and eventually disappears. The mechanisms of thyroid budding and regression of the thyroglossal duct have not been elucidated. In other organs budding from the endoderm i.e. the liver, lung and pancreas the outgrowing bud is formed due to proliferation of progenitor cells (Zaret and Grompe 2008).

The budding UB is formed by a single layer of tall epithelial cells surrounding a lumen that initially is continuous with the pharyngeal cavity. The lumen is retained for a considerable time after budding is completed. Similar to the midline primordium, the budding mechanism is not known.

Around day E11.5-12.5 (Human: 5-7th week), the developing thyroid has moved in a caudal direction away from the endoderm, but it still holds the midline position. There are different theories about embryonic thyroid migration, if it is an active or passive process or both. How this migration of the thyroid progenitors is regulated will be discussed further in paper I. The detached UB now move freely from their site of origin in the pharyngeal endoderm approaching the expanding midline thyroid.

One day later in the mouse embryo (Mouse: E13-13.5, Human: 7-8th week), the two thyroid anlagen forming three migrating primordial tissues starts to fuse. The UB are enclosed in the parenchyma of the midline thyroid so that both progenitor tissues contribute to the formation of the prospective thyroid lobes. By E15.5, the first confirmation of follicular organization appears with small follicles distributed within the gland. At this stage, calcitonin producing C-cells derived from the UB can also be distinguished in the follicles (Mouse: E13.5-17.5, Human: 10-12th week).

Thyroid transcription factors

As already mentioned, the thyroid progenitors of the midline primordium express four transcription factors*: Nkx2.1, Pax8, Foxe1 and Hhex* (Figure 3). As these transcription factors are absolutely indispensable in normal thyroid development, their expression pattern and developmental roles will be summarized shortly.

Nkx2.1

Nkx2.1 (formerly known as *TTF-1*, for thyroid transcription factor 1) is a homeodomain-containing transcription factor that first was identified because of its binding to specific DNA

sequences present in the TG and TPO promoter (Civitareale et al. 1989; Guazzi et al. 1990). During embryogenesis, *Nkx2.1* is expressed in the midline thyroid anlage, the trachea, lungs, pituitary and the forebrain (Lazzaro, et al. 1991). Furthermore, *Nkx2.1* transcripts have been described to be present in C-cells (Suzuki, et al. 1998) and in the ultimobranchial bodies (UB) (Mansouri, et al. 1998). However, the pharyngeal endoderm adjacent to the thyroid placode and developing UB does not express *Nkx2.1*, thus indicating its specificity as biomarker of thyroid progenitor cells.

Mice deficient of Nkx2.1 are stillborn, due to respiratory failure (Kimura, et al. 1996). The thyroid bud is hypoplastic and regresses completely by E12-E13 (Kimura, et al. 1999; Parlato, et al. 2004). In these animals, the early thyroid development is not disturbed. As the formation of the thyroid placode and subsequent pseudostratification of the early bud in the pharyngeal endoderm take place apparently normally (Parlato et al. 2004). Thus, *Nkx2.1* seems to be important in completing the budding process. Later on, thyroid rudiment regresses presumably through apoptosis of progenitor cells. In addition, the UB are correctly shaped at first but degenerate after budding (Kusakabe, et al. 2006). As a result, both UB and C-cells are absent in *Nkx2.1* knockout mice. The experimental results indicate that *Nkx2.1* is expendable for the initial specification of both thyroid primordia, but is required for the survival of all known cell types involved in thyroid development.

Haploinsufficiency in humans give rise to a syndrome characterised by thyroid hypoplasia, neurological disturbance and pulmonary disease (Krude, et al. 2002; Pohlenz, et al. 2002). Screening of patients with CH indicate that only a very few cases of thyroid dysgenesis are due to mutations in *Nkx2.1*. In humans, homozygote *Nkx2.1* mutations have not been found and are therefore probable not compatible with survival of the foetus. Mice heterozygous for *Nkx2.1* exhibit mild hypothyroidism with elevated serum levels of TSH.

Pax8

Pax8 (paired box gene 8) is a member of a family of transcriptions factors characterised by the present of a 128-amino acid DNA binding domain (paired domain) (Plachov et al. 1990). It is involved in thyroid development and gene expression after follicular differentiation. Comparable to *Nkx2.1, Pax8* recognizes specific sequences in the promoter region regulating TPO and TG *Pax8* is detected in the developing thyroid from E8.5 onwards. Moreover, expression of *Pax8* is maintained in the thyroid follicular cells during all stages of development and in adulthood. Remarkably, *Pax8* directly interacts with *Nkx2.1* in differentiated thyroid follicular cells and is the only one of the four transcriptions factors that has an endoderm expression restricted to the median thyroid primodium (Figure 3).

Mice lacking the *Pax8* gene are born at an expected Mendelian frequency, but with low body weight already at birth. Growth retardation becomes more and more prominent and only 20% of the pups survive until three weeks of age (Friedrichsen, et al. 2003; Mansouri et al. 1998). These animals suffer from severe congenital hypothyroidism (CH) as indicated by undetectable T3 and T4 (Friedrichsen et al. 2003) and high TSH level (Friedrichsen, et al. 2004). Hypothyroidism is an obvious cause of death since *Pax8* mutants can be rescued by supplementary thyroxin (T_4) treatment (Friedrichsen et al. 2004). In *Pax8* null mice embryos the midline thyroid anlage is properly specified, and the bud forms, detaches and migrates normally. However, just like in the

Nkx2.1 knockouts, the thyroid regresses totally soon afterwards (Mansouri et al. 1998). In contrast, the UB develop normally and C-cells can differentiate in these animals. In fact, newborn *Pax8* null mice have an undeveloped gland that more or less completely consists of C-cells. Thus indicating that the gland is devoid of thyroid follicular cells, consisting only of cells derived from UB.

In humans haploinsufficiency of the *Pax8* gene causes CH, with variable thyroid phenotypes, either hypoplasia or ectopic thyroid tissue (Macchia, et al. 1998).

Foxe1

The forkhead/winged-helix domain transcription factor *Foxe1* (Zannini, et al. 1997) (formerly called TTF-2 for thyroid transcription factor-2), is detected in the entire foregut endoderm at E8.5. Expression of *Foxe1* in the thyroid cell precursors is continued during development and persists in adult thyroid follicular cells. Foxe1 deficient mice are born at an expected ratio but die a few days after birth, almost certainly due failure to thrive because of the severe cleft palate (De Felice, et al. 1998). The thyroid gland cannot be detected in its normal position and hormone analysis reveals severe CH. In early thyroid morphogenesis, the thyroid develops normally in the *Foxe1* knockout animals. The placode is formed and a rudimentary bud is shaped. However, at E9.5, thyroid precursor cells either retains a position at the base of the prospective tongue, present also in late development (E15.5), or in some cases no thyroid gland can be found at all. These results have proposed that *Foxe1* is necessary during thyroid development in order to govern a normal migration of the mouse thyroid primodium, and supporting progenitor cell survival (De Felice et al. 1998). Even though the thyroid have changed position and size the progenitors are able to differentiate and start to synthesise TG (De Felice et al. 1998).

In humans, homozygote *Foxe1* mutations, give rise to a rare disease characteristic of cleft palate, athyreosis and spiky hair (Clifton-Bligh, et al. 1998).

Hhex

The *Hhex* was first identified in hematopoietic cells and was therefore named Hhex (hematopoietically expressed homeobox) (Bedford et al. 1993; Crompton et al. 1992). *Hhex* is extensively expressed in the endoderm and later becomes concentrated in organ primordial derived from the foregut, such as the thyroid, liver, lungs, thymus and pancreas (Bogue, et al. 2000; Thomas, et al. 1998). *Hhex* is an early marker for thyroid morphogenesis. At E9.5 the thyroid primodium is absent or hypoplastic (Martinez Barbera, et al. 2000), only a few non-migrating cells compose the thyroid primodium. Before the regression, the remaining thyroid progenitors do not express *Nkx2.1*, *Foxe1* or *Pax8*. *Hhex* deficient mice die at E15.5 due to multiple malformations, most notably, the liver is absent and a major reduction of forebrain tissue is observed.

No *Hhex* mutations have been found in humans. The severity of the phenotype found in mice suggests that this is not compatible with life.

Dysgenesis

Defective development of the thyroid gland is the most frequent cause of congenital hypothyroidism (CH), a condition that affects infants from birth (congenital) and results from a partial or complete loss of thyroid function (hypothyroidism). CH is the most common endocrine disorder in newborns, with an incidence of about 1 of 3500 newborns (Toublanc 1992). Dwarfism and mental retardation, also known as cretinism, are the predominate features of children with untreated CH. Early detection of CH with neonatal screening programs together with substitution

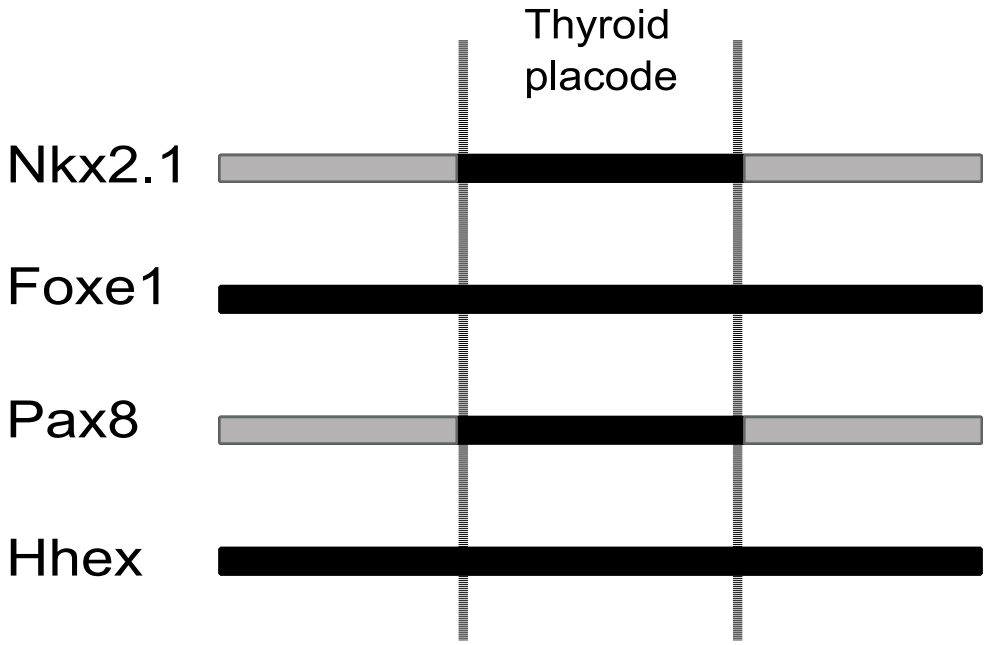

Figure 3. Expression pattern of Nkx2.1, Foxe1, Pax8 and Hhex in the pharyngeal endoderm. Nkx2.1 and Pax8 are restricted to thyroid progenitors forming the thyroid placode. Foxe1 and Hhex are ubiquitously expressed in foregut endoderm cells. Black marks protein expression in the pharyngeal endoderm.

therapy using thyroxine is therefore mandatory to prevent irreversible brain damage. Thanks to early treatment, CH patients will reach full intellectual capacity as indicated in comparison studies with healthy control siblings (1981; Fisher and Klein 1981). In about 85 percent of cases with CH the thyroid gland is either absent (thyroid agenesis or athyreosis), abnormally located (thyroid ectopy), or severely reduced in size (thyroid hypoplasia). All of these conditions are collectively named thyroid dysgenesis (Fisher and Klein 1981) and are in most instances sporadic and of unknown pathogenesis; mutations in known developmental genes have been described for only a handful of CH patients in which the cause is dysgenesis (Heinrichs, et al. 2000; Van Vliet 1999). In the remaining cases, a normal-sized or enlarged thyroid gland (goiter) is present but the production of thyroid hormones is decreased or absent. The cause can be traced to a defect in one of the many genes implicated in thyroid hormone synthesis (i.e. *TG, TPO, NIS, pendrin, Thox2, iodotyrosine dehalogenase*). Dyshormonogenesis are transmitted as classical autosomal recessive mendelian traits.

Foregut development

Some issues investigated and discussed in this thesis concern the endoderm and in particular its anterior part forming the foregut. Key morphogenetic events and molecules implicated in endoderm development will therefore be highlighted in the following paragraphs. The presentation is by no means complete, thus for comprehensive reviews on this topic, see following articles; (Kaestner 2005; van den Brink 2007; Zaret and Grompe 2008).

During gastrulation of vertebrate embryos, the epiblast will form three germ layers; endoderm, mesoderm and ectoderm. The endoderm later differentiates into the epithelial lining of the pharynx, lungs, oesophagus, stomach and intestines. The endoderm also contributes significantly to the formation of glandular organs like the thyroid, parathyroid, thymus, pancreas and liver. The mesoderm will form the cardiovascular system, kidneys, muscle, blood and bone. The ectoderm gives rise to the epithelium of the skin (epidermis) and the central nervous system.

The endoderm and gut tube

In the mouse embryo gastrulation starts at E6.25 with the ingression of epiblast cells through the primitive streak. In this process the epiblast cells undergo epithelial to mesenchymal transition (EMT), a phenotypic change that supports cell migration. From earlier fate mapping studies, it is proposed that the endodermal sheet can be subdivided in four distinct regions (Lawson, et al. 1986; Lawson and Pedersen 1987). Region I, the anterior-most region, forms the ventral foregut that later on during development differentiate into the lungs, part of the stomach, the ventral primordium of the pancreas and the liver.

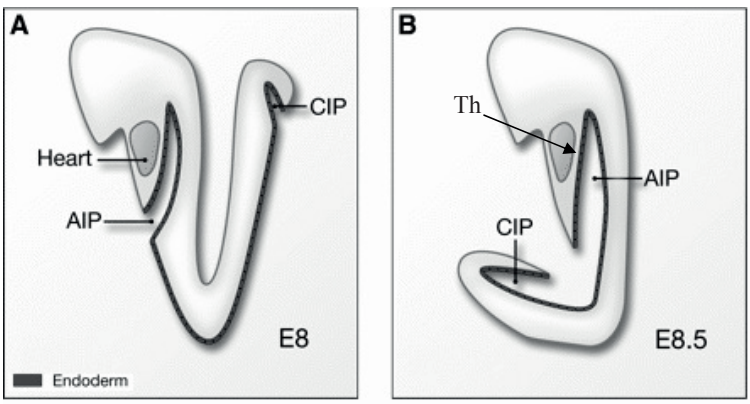

Figure 4. Folding of the embryo. A: The ventral side is located towards the outside of the embryo at E8. B: After a series of complex turning movements (for more details see the thesis text), the ventral side ends up at the inside of the embryo forming the anterior intestinal portal (AIP) and caudal intestinal portal (CIP). Arrow pointing at the presumptive location of the thyroid anlage. Adapted from Van den Brink 2007.

The dorsal foregut is formed from region II and will form the oesophagus, part of the stomach, and the dorsal pancreas and duodenum. Finally, region III and IV become the small and large intestine, respectively. Even though it has not been addressed experimentally, thyroid progenitors are most likely to be specified within region I.

The gut is formed by a complex series of folding events that convert the flat endoderm sheet into a tubular structure (Grapin-Botton and Melton 2000). The folding begins in the anterior part of the embryo by a ventral folding of the anterior-most endoderm. This fold generates a pocket, the so-called anterior intestinal portal (AIP). A similar fold, the caudal intestinal portal (CIP), arises later at the posterior end of the embryo and moves anteriorly. The two folds meet at the stalk of the yolk sac were the forming gut closes ventrally. Concurrently, the lateral endoderm folds ventrally, by which closure of the gut tube is completed. The folding of the endoderm sheet alters the anterior-posterior positioning of cells as compared to the site they where originally incorporated in the definitive endoderm (Grapin-Botton and Melton 2000). Progenitors of budding organs may thus move considerable distances before they reach the spot where organogenesis starts (Zaret and Grompe 2008).

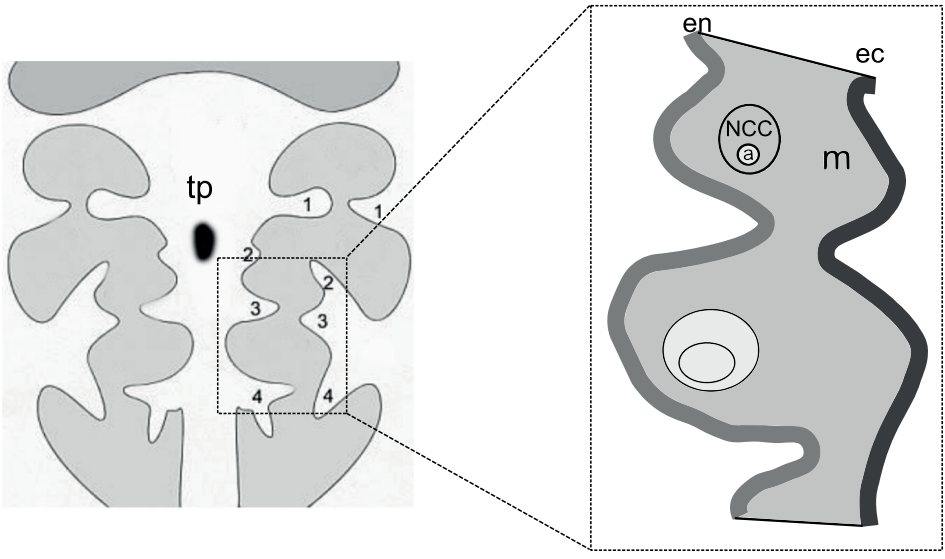

Figure 5. Coronal section through the pharyngeal arch region at E11 in mouse. Numbers specify the pharyngeal archers and pouches. Thyroid primodium (tp) is located between arches I (1) and II (2). The ultimobranchial bodies (UB) are formed from the 4th pharyngeal pouches. The right figure shows a magnification of two pharyngeal arches. Each arch has an ectodermal layer (ec) on the outside and an endodermal layer (en) on the inner side. Internally, the arches have a concentric structure throw which a centrally located pharyngeal arch artery (a) passes, surrounded by neural crest cells (NCC) and mesoderm (m). Adapted from Manly and Capecchi, 1995.

Pharyngeal arches and pouches

The pharyngeal apparatus is the most cranial part of the newly formed foregut. It consists of a series of paired bulges on the lateral surface of the embryonic head known as the pharyngeal arches. The arches are separated by clefts on the outside (skin surface) and pouches on inside facing the pharyngeal cavity. As already pointed out, the pharyngeal arches and pouches are transient embryonic structures that give rise to many of the tissues and organs in the head and neck region of vertebrates, notably craniofacial bone and muscles, the pharynx, and the thyroid, parathyroid and thymus. The arches and pouches are named by roman numerals (I-VI) as they

develop in a cranial to caudal direction, the most cranially positioned are numbered I. Each arch consists of cells from all three germ layers, ectoderm, mesoderm and endoderm. The external arch surface is covered by ectoderm, the internal by endoderm (Figure 5). The centre of each arch is traversed by a vessel, the so-called pharyngeal arch artery (PAA), which connects the heart to the dorsal aorta. Each artery is surrounded by mesoderm and neural crest cells (Figure 5), the latter of which have migrated into the arches from the rhombomeres (Krull 2001).

The neural crest cells of the upper arches will later differentiate into cranio-facial connective tissue and skeleton (Jiang, et al. 2000; Noden 1983). Crest cells of the lower arches are believed to contribute to the development of pharyngeal pouch derivatives. As thyroid relationships to embryonic vessels in the neck were investigated in paper I, the development of this vasculature need to be shortly described. The PAA develop bilaterally and sequentially as the pharynx segments and allows blood to flow from the heart tube to the dorsal aorta (DeRuiter, et al. 1993). The arteries form in a cranial to caudal direction accompanying the temporal development of the corresponding arches. Hence, the arch arteries are numbered I, II, III, IV, and VI (artery number V either does not exist or forms incompletely without any involvement in adult structures). In mouse embryos, at E10.5 both PAA number I and II have regressed into capillary beds whereas arteries number III, IV and VI persist in their respective arches. Starting from E11.5, the caudal PAAs undergo extensive asymmetrical remodelling and eventually convert into the definitive great vessels arising from the aortic arch (Srivastava and Olson 2000).

Role of Sonic Hedgehog (*Shh*) gene in pharyngeal apparatus

Hedgehog proteins are major morphogens in embryonic development. Three mammalian hedgehog genes, Sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*), and Desert hedgehog (*Dhh*) have been identified, each of them implicated in organ morphogenesis in the gut (Chiang, et al. 1996; Hebrok, et al. 1998; McMahon, et al. 2003; van den Brink 2007). *Shh* is of particular interest since it is expressed in the foregut endoderm and was recently shown to regulate mouse thyroid development (Fagman, et al. 2004). In the mouse embryo *Shh* expression is first detected at E7.25 in the midline mesoderm. However, already at E7.75 *Shh* is expressed in the node, the notochord, and the definitive endoderm in which it is initiated in the anterior endoderm and thereafter is expanding into the posterior endoderm (Echelard, et al. 1993). Of interest, *Shh* is excluded from the thyroid and pancreatic anlagen indicating a non-cell autonomous mode of action in the development of these tissues (Apelqvist, et al. 1997; Fagman et al. 2004). In contrast, *Shh* is ubiquitously expressed in the pharyngeal arches and pouches, both in the ectoderm and the endoderm. Consequently, *Shh* null embryos develops a severely malformed pharyngeal apparatus (Chiang et al. 1996).

Shh acts as a key regulators of embryogenesis and has been found to be crucial to foregut development (Litingtung, et al. 1998). Specifically, separation of the oesophagus and trachea and also branching of the distal airways fails in *Shh* deficient mice (Litingtung et al. 1998) Shh binds to the receptor Patched (Ptc) on target cells (Marigo and Tabin 1996) eventually leading to relieved inhibition of the interacting co-receptor Smoothened (Smo) (Murone, et al. 1999). *Shh* downstream signalling involves both *Foxa2* and *Tbx1* signalling. *Shh* is not investigated in this

thesis work, but as *Foxa2* and *Tbx1* expression are studied in paper II the signalling pathway will be considered.

Role of the T-box transcription factor (*Tbx1*) in pharyngeal apparatus

Tbx1 belongs to the T-box family of transcription factors that is characterized by homology in a DNA-binding domain known as the T-box (Bollag, 1994; Chieffo *et al.,* 1997). The members are expressed in a tissue specific fashion during embryogenesis and adulthood (Papaioannou and Silver, 1998; Smith, 1999). *Tbx1* is required for the development of the pharyngeal apparatus and therefore of potential interest in thyroid morphogenesis. Indeed, in our lab, we recently documented a developmental defect in thyroid in mice embryos deficient for Tbx1 (Fagman et al. 2004). *Tbx1* expression is restricted to the pharyngeal endoderm and the mesodermal core of the pharyngeal arches; no *Tbx1* expression can be found in the neural crest-derived mesenchyme of the pharyngeal arches (Garg, et al. 2001). It is also not expressed in the midline thyroid primordium (Fagman et al. 2004).

For almost 10 years ago, *Tbx1* was discovered as the gene responsible for the phenotype observed in patients with DiGeorge syndrome. Typical features of DiGeorge syndrome also recognized in *Tbx1* deficient mice are cardiovascular malformations in the head and neck region. Hence, in *Tbx1*$^{-/-}$ embryos the first pharyngeal arch is abnormally patterned, the second is hypoplastic, and the third, fourth and sixth are not possible to distinguish (Baldini 2005). In addition, thymus and parathyroids are rudimentary or lacking (Liao, et al. 2004), making this a mouse model of the complete spectrum of anomalies characterizing DiGeorge syndrome. The thyroid phenotype can be separated in two defects. Firstly, lack of C-cells that probably depends on the absence of ultimobranchial bodies. Secondly, thyroid hypoplasia that may be related to impaired vessel interactions of the budding thyroid (Fagman et al. 2004). The basis of the latter hypothesis is presented in paper I. Moreover, *Tbx1* is used for tracing descendants of the pharyngeal endoderm into the developing thyroid (paper II).

Role of forkhead box (*Fox*) genes

The forkhead box A (*Foxa*) gene family was first named hepatocyte nuclear factor 3 on the basis of its regulatory role on liver-specific gene expression. The family has three members: *Foxa1, Foxa2* and *Foxa3* (previously termed HNF3-α, -β and –γ). The *Foxa2* gene is the first to be activated during mouse embryogenesis and its expression is detected in the anterior primitive streak and the node at E6.5 (Friedman and Kaestner 2006). Both *Foxa1* and *Foxa2* are expressed very early in the definitive endoderm and this persists in endoderm-derived organs such as lungs, liver, stomach and pancreas in which tissue specific genes are regulated (Bohinski, et al. 1994; Cereghini 1996; Wu, et al. 1997). Both genes are also expressed in adult organs, although the functions are probably different than during development. The effects of *Foxa1* and *Foxa2* are often redundant, but exceptions exist. For example, in the early embryo *Foxa2* is expressed in the node, the notochord and floorplate whereas *Foxa1* is detected only in the notochord and floorplate (Monaghan, et al. 1993; Sasaki and Hogan 1993).

The significance of *Foxa2* in early embryogenesis has been shown in embryos deficient for Foxa2. Embryos die by E11 due to severe malformations in structures that originate from the three germ layers, e.g. the neural tube, somites and gut tube (Ang and Rossant 1994; Weinstein, et al. 1994). Information about all the liver-specific *Foxa* target genes together with the observation that the *Foxa* genes are expressed very early during the formation of the definitive endoderm in the mouse, led to the theory that the *Foxa* proteins have important functions in mammalian liver development (Le Lay and Kaestner). In the liver, all three *Foxa* members are expressed, but neither of them is alone required for hepatogenesis indicating redundancy. However, using conditional knockouts it has been demonstrated that *Foxa1* and *Foxa2* together are necessary for the induction of liver specification (Lee, et al. 2005).

Foxa2 is in addition reported to be expressed in the developing thyroid (Monaghan et al. 1993; Sato and Di Lauro 1996), although the documentation (by *in situ* hybridization) was rather poor and could not be referred to a specific cell type. *Foxa2* have been shown to regulate the calcitonin gene (Viney, et al. 2004), suggesting that it is expressed in differentiated C-cells. Whether *Foxa2* (or *Foxa1*) is a biomarker of embryonic C-cells and their ancestors was investigated in paper II.

Eph receptors and ephrins

The Eph-ephrin receptor-ligand system is emerging as a novel principle of regulation in the embryonic development of possibly all organ systems. It has been most thoroughly investigated in the nervous system, from which many of the basic concepts of molecular interactions and cellular effects originate. Ephs and ephrins are implicated also in the development of different organs from the endoderm, e.g. intestine, pancreas and thymus (Islam, et al. ; Konstantinova, et al. 2007; Munoz, et al. 2009). No studies have so far investigated a putative role of Eph-ephrin signalling in the thyroid gland. In papers III and IV the expression pattern and function of the EphA4 receptor in the developing and adult thyroid were addressed. General and specific features of the Eph-ephrin system will be presented here to give the necessary background for the discussion of these papers.

History and classification

The first Eph gene was discovered in an erythropoietin-producing human hepatocellular carcinoma cell line (ETL-1) more than twenty years ago (Hirai, et al. 1987). Some years later, in 1994, the first report on an Eph family receptor interacting proteins (ephrins) was published (Beckmann, et al. 1994). The main features of Ephs and ephrins discussed at that time was the repulsive effects on developing axons guiding the growing neuronal processes toward their terminal destination. Since then, both the number and functions of Eph and ephrins identified in vertebrates have increased and diversified. Both the ligands and the receptors are membrane-bound molecules expressed on adjacent interacting cells.

There are 14 Ephs and 8 ephrins in mammals, which therefore makes them the largest family of receptor tyrosine kinases (RTK). Both Eph receptors and ephrin ligands are divided into two subclasses, A and B, based on structural homologies and preferable binding partners (1997; Murai and Pasquale 2003). There are nine EphA (EphA1-EphA8 and EphA10) and five EphB (EphB1-EphB4 and EphB6) receptors. All Eph receptors are monomeric transmembrane proteins consisting of a extracellular part that contains the N-terminal ephrin binding domain, a cystein-rich region and two fibronectin type-III repeats, and an intracellular/cytoplasmic part which includes four functional units, a juxtamembrane domain, a tyrosine kinase domain, a sterile α-motif (SAM) and the PDZ-domain binding motif (Figure 6). Functional aspects of the most important receptor domains will be discussed in the following paragraphs.

The A-class ligands (ephrinA1-A5) are membrane-anchored by a glycosylphosphatidylinositol (GPI) moiety and bind typically to EphA-class receptors. B-class ligands (ephrinB1-B3) are transmembrane molecules with an extracellular domain that binds to EphB-class receptors and an intracellular terminus that possesses signalling properties (Figure 6) (further discussed below). The Eph-ephrin interactions are promiscuous within each class (A or B), but the binding affinities vary significantly (Himanen, et al. 2004; Pasquale 2004). Exceptions to this rule are EphA4 and EphB2 that can bind both A- and B-type ephrins (Himanen et al. 2004; Pasquale 2004). In contrast to other RTKs subfamilies, Eph receptors often show redundant functions within a class as a result of overlapping expression pattern and similar binding affinities towards the ephrins. This makes functional analysis in e.g. genetically modified mice (Eph/ephrin knock-out/knock-in) difficult and complicated.

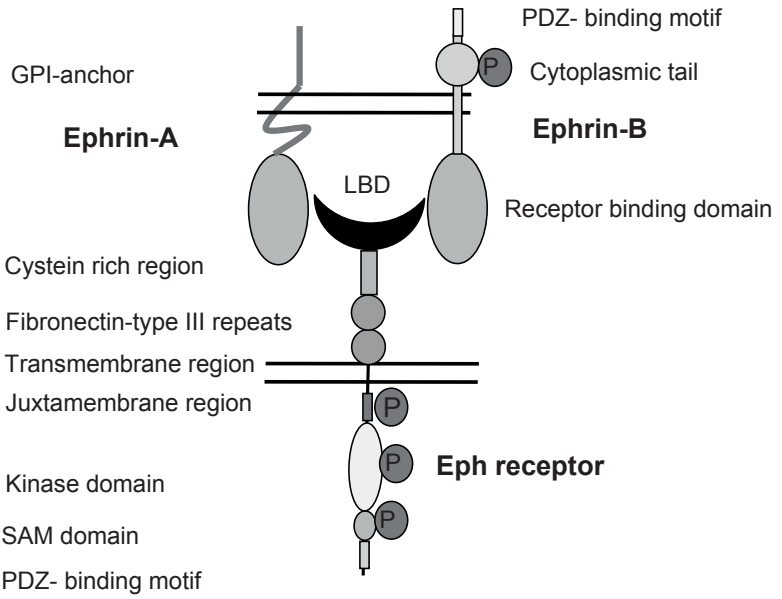

Figure 6. An illustration, which shows an ephrin-A and ephrin-B-expressing cell (top) and an Eph-expressing cell (bottom). GPI, glycosylphosphatidylinositol; SAM, sterile α-motif; P, phosphates; LBD,Ligand Binding global Domain.

Ligand-receptor binding

Because both Ephs and ephrins are membrane-bound intimate cell-cell contact is required for binding and signalling. Downstream effects of Eph receptor activation is referred to as forward signalling. However, during receptor-ligand binding Ephs often acts as a ligand that elicits a receptor response in the cognate ephrin, leading to reverse signalling in the ephrin-expressing cells. The dual ability of the receptor-ligand complex to function in both a forward and a reverse fashion, mimicked only by the semaphorin/semaphoring receptor system (Pasquale 1997) is known as bidirectional signalling.

A feature that distinguishes the Eph–ephrin system from other RTKs is that the classical activation of RTK by receptor dimerization is not sufficient, but requires formation of a multimeric Eph/ephrin complex that creates a big cluster for robust Eph autophosphorylation and transmission of a biological response (Vearing and Lackmann 2005). Monovalent interaction between Ephs and ephrins is the first step followed by the formation of a heterodimer complex. Tetramerization occurs by the binding of two Eph-ephrin heterodimers, which initiates autophosporylation of their respective cytoplasmic domains. Biochemical and X-ray crystallographic investigations have generated abundant structural information about the interacting domains of the EphB2 and ephrin-B2 forming a complex (Himanen and Nikolov 2003; Himanen, et al. 2001). The result shows that a large intermolecular interface is established during high-affinity ligand-receptor dimerization, whereas a second smaller interface is responsible for the assembly of the dimers into tetramers. More specifically, the ligand-receptor dimerization interface centres on the long G–H loop of ephrin-B2, which is inserted into a channel on the surface of EphB2. The EphB2 loops forming the sides of this channel are unstructured in the unbound receptor but are folded upon ligand binding to generate an extensive interaction surface that is complementary to the ligand G–H loop. Recently the crystal structures of EphA2/ephrin-A1 and EphA4/ephrin-B2 complex have been investigated (Himanen, et al. 2009; Himanen and Nikolov 2003; Qin, et al. 2010).

Forward signalling

The initial step after binding of ligand is autophosphorylation of tyrosine residues located in the intracellular part of the Eph receptor (Kalo and Pasquale 1999). For complete activation of the receptor tyrosine kinase autophosphorylation of the juxtamembrane tyrosine residues is required (Binns, et al. 2000; Kullander, et al. 2001). This allows the juxtamembrane domain to be released from the interaction with the kinase domain converting it into an active state (Wybenga-Groot, et al. 2001).

Once the receptor is activated, adaptor molecules associate with its cytoplasmic tail to transmit signals into the cell. Adaptor proteins are a growing class of proteins that contain functional protein-interaction domains, such as SRC-Homology-2 (SH2) and SH3 domains, but often lack intrinsic enzymatic activity. Downstream signalling from Eph receptors in which adaptor proteins participate involves e.g. cell shape changes by Rho activation, cell proliferation through the mitogen activated protein kinase (MAPK) pathway, cell-cell adhesion via cadherin complexes

and cell-matrix interactions by means of altered expression and binding properties of integrins (Noren and Pasquale 2007; Pasquale 2005).

Signalling connections that may apply only to a particular Eph class are the exchange factors Ephexin (Eph-interacting exchange factor) for EphA receptors and intersectin and kalirin for EphB receptors. Ephexin is a guanine nucleotide exchange factor for Rho-family GTPases that directly interacts with the EphA receptors. When Ephrin-A bind to EphA the activity of Ephexin leads to RhoA activation and Cdc42/Rac1 inhibition, which in turn reorganize the cytoskeleton and cell morphology changes (Shamah, et al. 2001). Other examples of selectivity are the lipid phosphatase Ship2 found to interact only with EphA2, and the GTPase-activating proteins SPAR/E6TP1 interacting only with EphA4 and EphA6 (Richter, et al. 2007; Zhuang, et al. 2007).

Reverse signalling

B-class ephrins mediate reverse signalling of importance for a number of morphogenetic events mostly notified in the central nervous system. Examples of this are axon guidance (Cowan, et al. 2004; Pasquale 2005), cell migration (Lu, et al. 2001), midline fusion (Dravis, et al. 2004), plasticity (Armstrong, et al. 2006) and synaptogenesis (Kayser, et al. 2006). Activation of ephrin-Bs through binding to a cognate Eph receptor leads to the recruitment of Src-family kinases that phosphorylate tyrosine residues on the cytoplasmic domain. This leads to a conformational change that is more favourable for signalling. Phosphorylated ephrin-B serves as a docking site for SH2-containing adaptor proteins, such as Grb4, which then activate signalling pathways ultimately leading to changes in the actin cytoskeleton and focal adhesions (Cowan and Henkemeyer 2001). B-type ephrins also have a carboxyl-terminal PDZ domain-binding site that recently was shown to be functionally important (Egea and Klein 2007).

Much less is known of the reverse signalling mechanism of Ephrin-As as these are anchored to the cell membrane by a GPI linkage and lack an intracellular domain. To signal ephrin-A must associate with other transmembrane proteins capable of activating intracellular signalling pathways. One example of this is the association of p75 with ephrin-A resulting in a functional complex in which p75 acts as co-receptor or signalling partner (Lim, et al. 2008).

Attraction (adhesion) versus repulsion

Ephs and ephrins control cell positioning during normal and oncogenic development by modulating cell-cell adhesion and cell-cell repulsion. A repulsive response involving the retraction of cellular processes was in fact the first discovered outcome of Eph forward signalling (Flanagan and Vanderhaeghen 1998). The physiological importance of Eph-ephrin mediated adhesive effects is highlighted by the defects in midline fusion of the neural tube observed in ephrin-A5 knockout mice (Holmberg, et al. 2000). These defects lead to severe congenital malformations including craniofacial malformations and anencephaly (Holmberg et al. 2000) Whether Eph-ephrin interactions result in adhesion or repulsion are dependent of the class of molecules and cell types involved and on the presence of modulating binding partners. As Eph and ephrin binding occurs through high-affinity interactions, the expected outcome would be a

close adhesion between the cells. However, the transient binding between an Eph receptor and the cognate ephrin tethered to the cell membrane usually results in contact-mediated repulsion. To accomplish this termination of receptor-ligand binding after initial activation and signalling is therefore a crucial event.

Recent findings indicate that the dissociation of complex-bound Ephs and ephrins is a highly regulated process involving endocytosis or ectodomain cleavage (Egea and Klein 2007). In cultured cells binding of EphB and ephrin-B results in the rapid formation of intracellular vesicles containing EphB-ephrin-B complexes. Interestingly, the endocytosed complex includes full-length proteins indicating that one of either the receptor or the ligand are trans-endocytosed from one cell to the other nearby (Mann, et al. 2003; Marston, et al. 2003; Zimmer, et al. 2003). Rac activity and actin polymerization in the Eph expressing cell have been shown to play an important role in the internalization of the receptor-ligand complex and repulsion of endothelial cells (Marston et al. 2003). However, putative signals elicited by or possible impacting on the endocytosis are poorly understood. So far no studies have confirmed that this mechanisms operates *in vivo* to regulate adhesion and repulsion.

Release of cell surface-bound ephrin ligands by A-Disintegrin-And-Metalloprotease (ADAM) transmembrane metalloproteases is another mechanism by which cell behaviors switches from adhesion to repulsion. ADAM10 is located on Eph expressing cells and cleaves the ephrin from its membrane tether thereby breaking the intercellular connection and allowing the ligand-receptor complex to internalize (Janes, et al. 2009). In general, ADAM function is enhanced when the Eph kinase is active and repressed when the kinase is inhibited. *In vitro* studies have showed that tyrosine phosphorylation of EphA3 generates a measurable movement of the kinase domain away from the plasma membrane. Only this positional change of the EphA3 kinase domain permits ADAM10 to come close enough to cleave the tightly bound ephrin-A5 from its plasma membrane anchor (Janes et al. 2009). The ephrin fragment retained in the membrane are further cleaved by γ-secretase followed by proteasomal degradation (Janes et al. 2009).

Eph and ephrins in early development

The expression of Ephs and ephrins is induced in a distinct spatiotemporal pattern during several important developmental stages including gastrulation, segmentation, somitogenesis and organogenesis. EphB3 is a good example of these dynamics. Already at the gastrulation stage *EphB3* is detected in the primitive streak in chick embryos, but the expression is extinguished once the cells have ingressed (Baker, et al. 2001). Somewhat later *EphB3* is strongly transcribed in the floor of the foregut, the anterior lateral endoderm and in the underlying cardiogenic mesoderm, and transiently expressed in the lateral ectoderm, neural tube, and neural crest. In the developing brain, *EphB3* expression is restricted to the mesencephalon. Similar changes are observed in mesoderm-derived tissues: at first *EphB3* is expressed in the sclerotome and in somitic mesoderm, but later on in development *EphB3* is predominantly found in the dermatome. In addition, *EphB3* is detected in the developing heart, liver, posterior ventral limb bud mesenchyme, pharyngeal arches, and head mesenchyme (Baker et al. 2001), illustrating the pluripotency of Ephs in vertebrate development.

Similar to *EphB3, ephrin-A1* transcript is first detected in the primitive streak and in addition in the developing allantois. At embryonic day 8-8.5 *ephrin-A1* is expressed in the anterior pharyngeal endoderm and in the presumptive endocardial cells, dorsal aorta and primary head veins. Later on *ephrin-A1* is detected in intersomitic vessels and the limb bud vasculature; each position of *ephrin-A1* expression correspond to regions of vasculogenesis or angiogenesis (McBride and Ruiz 1998). This explains why *ephrin-A1* null mice are early embryonic lethal due to impaired cardiovascular development.

Biological effects

The complexity of Eph-ephrin interactions is illustrated by the high diversity of biological outcomes, which can be distinguished at the single cell level, in tissue patterning, and in organogenesis in which the final anatomy such as organ size may be influenced. As many of these aspects are considered in the interpretation of data presented in papers III and IV, major achievements on the physiological roles of Ephs and ephrins will be briefly highlighted in the following order: cell migration, cell proliferation, tissue patterning, epithelial homeostasis, and organ-specific effects (nervous system, cardiovascular system, intestine and glands). The contribution of defective or aberrant Eph-ephrin signalling in cancer and other disease will also be summarized. Eventually, the current knowledge of EphA4-mediated effects in mice will be pointed out as a prelude to the discussion of thyroid phenotypes in *EphA4* mutants. In all instances, the presentation is to exemplify rather than provide a comprehensive review.

Cell migration

Locomotion is a fundamental cell behaviour that can be adopted by most cells in environments that promote changes in the cytoskeleton and adhesiveness characterizing the migrating phenotype. Regulation of the migration and adhesive interactions of cells is fundamental for the assembly and maintenance of organized tissues during animal development. Eph and ephrins are essential for these processes and have a number of interesting and characteristic properties that may underlie the mechanism by which they control cell adhesion and the assembly of the actin cytoskeleton. Repulsive Eph signals constrain the organization, and prevent the intermingling of subpopulations of cells that are marked by differential Eph or ephrin expression. This generates interfaces where Eph bidirectional signalling stabilizes patterns of cellular organization.

Neural crest cells (NCC) have been called the ‘explorers of the embryos’ because they migrate all over the embryo and differentiate into a diverse variety of cells. NCC arise by the delamination of cells from the dorsolateral edge of the neural epithelium, and migrate along a variety of pathways to specific destinations (Bronner-Fraser 1993). The NCC is one of the most pluripotent embryonic tissues as the progeny develop into such different cells and tissues as ganglionic neurons of the peripheral nervous system and craniofacial cartilage, bone and skeletal muscles cells. To reach the final destination streams of NCC cohorts migrate long distances in which Ephs and ephrins appears to play modulatory roles. For example, in *Xenopus* embryos, cranial NCC migrating throw the third arch express EphA4 and EphB1, whereas the neighbouring second arch and crest cells express *ephrin-B2* (Smith, et al. 1997). Ephrin-B2 is known to interact with both EphB1 and EphA4. Further experiments with truncated EphA4/EphB1 receptors and ephrin-B2

showed that these molecules are involved in restricting the intermingling of second and third arch neural crest and specifically in guiding third arch neural crest to the accurate destination (Smith et al. 1997).

Cell proliferation

In contrast to most RTKs that typically bind peptide growth factors, it is much more unusually for Eph receptors to have a direct role in mitogenesis. Whereas other RTKs activate the mitogen activated protein kinase (MAPK) pathway, Eph receptors can also work the other way around and have an inhibiting effect on the MAPK pathways (Miao, et al. 2001). However, it is clear that Ephs modulate cell proliferation impacting on organ size (Munoz, et al. 2006; North, et al. 2009). It is also evident that Eph signalling can regulate the balance of stem cell renewal versus differentiation and fate determination in a spatially regulated manner. In the epithelium of the small intestine opposing gradients of EphB2/EphB3 and ephrin-B1/ephrin-B2 along the crypt–villus axis influence stem cell renewal by controlling cell positions, which in turn determines the exposure of cells to growth factors that emanate from the bottom of the crypts (Holmberg, et al. 2006). These data establish that ephrins and Eph receptors are key players of migration and proliferation of the intestinal stem cell niche.

Tissue patterning

The expression pattern of Eph receptors and ephrins is known to restrict cell movements between hindbrain segments. The hindbrain is subdivided into rhombomers consisting of segmental units that organize nerves and streams of neural crest cells that migrate into the branchial arches. The rhombomers are established by the segmental expression of genes such as *Krox-20* required for the formation of segments, and by *Hox* genes that create the anterior-posterior axis in the embryo (Reviewed by (Lumsden and Krumlauf 1996; McGinnis and Krumlauf 1992; Sham, et al. 1993)). Specifically, *EphA4*, *EphB2* and *EphB3* are expressed at high levels in rhombomeres number r3/r5 (Henkemeyer, et al. 1994; Nieto, et al. 1992), whereas *ephrin-B1*, *ephrin-B2*, and *ephrin-B3* are expressed at high levels in r2/r4/r6 (Bergemann, et al. 1995; Flenniken, et al. 1996; Gale, et al. 1996a) se fig Wilkinson (Wilkinson 2000). The repulsive action of ephrin-B ligands thus makes cells expressing the ligand in even numbered rhombomeres to move away from cells expressing the Eph receptors in odd numbered rhombomeres. This mechanism of creating tissue boundaries is recognized also in other developmental processes, e.g. somitogenesis (Watanabe, et al. 2009) suggesting it is a general principle of tissue patterning.

Tissue homeostasis

Eph and ephrins are essential for the embryonic and postnatal development of epithelial tissues, as well as having an important role in maintaining the epithelial tissue homeostasis in adulthood (Miao and Wang 2009). For example, EphB (EphB2 and EphB3) and ephrin-B2 proteins are involving in the homeostasis of the gastrointestinal tract, where signalling regulates cell sorting in the mature epithelium (see Cell proliferation). There is a number of different cell lineages in the villus region, which are continually renewed and shed into the intestinal lumen (Holmberg et al. 2006). In the mature intestine, four epithelial cell types derive from stem cells in the crypts. Three of the cell types move upward, out of the crypts and onto the villi as they differentiate. The

fourth, the Paneth cell, moves to the base of the crypt. EphB2 and EphB3 forward signalling promotes cell-cycle re-entry of intestinal progenitor cells (Holmberg et al. 2006).

During cell migration, when newly generated epithelial move away from the source of Wnt secreted by surrounding mesenchymal cells at the bottom of the crypt, the epithelial cells gradually lose EphB expression and obtain ephrin-B expression. This generates counter-gradients of EphB and ephrin-B expression along the crypt axis, with high EphB expression at the bottom of the crypts and high ephrin-B expression at the top and in the villi. The canonical Wnt/β-catenin/Tcf signaling pathway is an essential regulator of homeostasis in the intestinal epithelium, mainly by its ability to support the transcription of EphB receptors and inhibit that of ephrin-B ligands (Clevers and Batlle 2006).

Eph and ephrin signalling is implicated also in maintaining skin epithelial homeostasis. In mouse epidermis, EphA2 and ephrin-A1 are expressed in a complementary pattern in the different cell layers as the keratinocytes mature. EphA2 is expressed in a basal to suprabasal gradient with lower expression in the basal layer and higher expression in spinous and granular layers. In contrast, ephrin-A1 expression is mostly limited to the basal layer next to the basement membrane. It is suggested that EphA2/ephrin-A1 interactions in the basal layer serve to regulate cell proliferation taking place only in this location (Miao and Wang 2009). EphB-ephrin-B binding between osteoblasts and osteoclasts has been implicated in the regulation of bone homeostasis in the adult (Zhao, et al. 2006). The communication between osteoclasts and osteoblasts is bidirectional and EphB4 and probably other co-expressed Eph receptors promote the forward signalling thereby maintaining bone homeostasis (Zhao et al. 2006).

Nervous system

Eph receptors and ephrins are abundantly expressed in the developing nervous system in which the organization of neuronal connectivity by guiding axons to the correct target cells and tissues and the formation of synaptic contacts are regulated. The literature in this field is vast, and here only few examples of effects of particular interest to the thesis studies will be given.

EphA4 was originally found to be a fundamental component in the neuronal circuitry controlling movement due to its regulating function in axon guidance and cell migration in the developing central nervous system (Kullander et al. 2001). Corticospinal tract axons need EphA4 to identify the spinal cord midline as an intermediate repulsive target that prevents them from aberrantly recrossing. As a result to this, homozygous *EphA4* knock-out mice display severe motor dysfunctions resulting in reduced and uncoordinated locomotor performance and a kangaroo-like gait (Dottori, et al. 1998; Kullander, et al. 2003). It have been shown that EphA4 has a non-cell-autonomous roll, no EphA4 forward signalling is required, in the axon bundle that connects the two cerebral hemispheres, called the anterior commisure (Cowan et al. 2004; Kullander et al. 2001). EphA4-positive neurons are also important components of the spinal central pattern generator (Kullander et al. 2003). Central pattern generator is a local neuronal network within the spinal cord that generates and coordinates rhythmic muscle activities responsible for coordinated bilateral control over the normal limb alternation that underlies walking (Butt and Kiehn 2003; Kiehn and Kjaerulff 1998).

Topographic mapping of thalamocortical axons requires EphA4 expression. Using genetically modified mice expressing different forms of truncated EphA4 receptors Dufour et al. found that the tyrosine kinase domain of EphA4 is required for the projection of thalamocortical axons, whereas the PBM and the SAM domains are not essential (Dufour, et al. 2006).

Cardiovascular system

Numerous Eph-ephrin interactions have been identified in the developing cardiovascular system, but EphB4 and its ligand ephrin-B2 are the most studied ones. Ephrin-B2 is expressed in arterial endothelial cells, whereas EphB4 is mostly expressed in veins. Both are crucial for angiogenic remodelling and survival of the embryo (Wang, et al. 1998). During angiogenesis, EphB4 and ephrin-B2 interacts in two different ways. First in the borders of the venous and arterial capillaries, it makes sure that arterial capillaries connect only to venous ones. Second, in non-border areas, it ensures that the fusion of capillaries to make larger vessels occurs only between the same types of vessels. Additional information about signalling pathways that interact with EphB4/ephrin-B2 to establish arterial–venous specification is emerging. One is the Notch pathway, the repression of Notch signalling resulted in the loss of ephrin-B2-expressing arteries and their replacement of veins. Conversely, activation of Notch signalling suppressed venous development, causing more arterial cells to form (Lawson, et al. 2002).

EphA4 is expressed on endothelial cells in the vasculature of the developing nervous system (Goldshmit, et al. 2006). This pattern of expression is not retained in the adult animal. Experiments in wild-type mice have shown that after spinal cord injury (causes direct vascular damage and initiates a cascade of events that alters the permeability of the blood–brain barrier) the expression of EphA4 is clearly up-regulated on activated astrocytes, many of which are tightly associated with blood vessels (Goldshmit et al. 2006). In injured spinal cord of EphA4 knock out animals astrocytes were not as tightly associated with blood vessels as the wild-type astrocytes. These results suggest an additional role for the EphA4 receptor in vascular development and repair of the blod brain barrier after spinal cord injury (Goldshmit et al. 2006).

Intestine

The small intestinal epithelium is composed of crypt-villus elements, with the function to absorb water and nutrients. An additional role is to function as a protecting barrier against pathogens. The intestinal epithelium undergoes continuous self-renewal throughout life, and homeostasis is maintained by the balance of cell proliferation, differentiation, and apoptosis. As previously discussed, it is known that signalling between EphB and ephrin-B regulates cell proliferation in the small intestine (Holmberg et al. 2006). This is reflected by the expression pattern along the crypt-villus axis: EphB2 is strongly expressed on cell membranes at the bottom of the crypt, with decreased expression on cells higher in the crypt. In addition, EphB3 is strongly expressed on cells in the crypt base. In contrast, ephrin-B1 and -B2 expression is low on cells at the bottom of the crypt and increased on cells higher in the crypt.

Ephs and ephrins also appear to have an important function during the embryonic development of the small intestine. All family members, with the exception of EphA5, have been shown to be expressed throughout the intestine at all ages although at different levels (Islam et al.). EphA4, EphA8, EphB4, and ephrin-B2 expression are on maximal levels during embryonic life,

suggesting that they may have an important role in intestinal morphogenesis. Sustained expression of other family members in the mature intestine may indicate regulatory roles in enteric differentiation (Islam et al.). For example; Batlle at al. have looked at *EphB2*- and *EphB3*-null mice that demonstrated that the normal upward and downward movement of different cell types in the intestine was disordered (Batlle, et al. 2002). Moreover, it has been reported that signalling between EphB2 and ephrin-B2 regulates cell proliferation in the small intestine (Holmberg et al. 2006), and expression of mouse EphA2 has been identified in E7.5 endoderm and persists through E10.5, suggesting a possible role in early patterning of the gut tube (Moore-Scott, et al. 2007).

Glands

The mammary gland develops mainly postnatally, at difference with most other branching organs that are established during embryogenesis. EphB4 is the most extensively studied Eph receptor implicated in mammary morphogenesis. EphB4 is primarily expressed in myoepithelial cells, and its ligand ephrin-B2 is expressed in luminal epithelial cells, in an estrogen-dependent manner (Nikolova, et al. 1998). The expression of both EphA4 and ephrin-B2 is turned on at the onset of puberty and is differentially regulated during the estrous cycle. During pregnancy both the receptor and its cognate ligand is downregulated and transcriptionally silent for the duration of lactation, after which they are re-induced (Munarini, et al. 2002). Together this suggests that EphB4-ephrin-B2 bidirectional signalling regulates cell proliferation in the mammary gland. Recently, it was shown that also EphA2 and its ligand ephrin-A1 are required for mammary epithelial growth and branching morphogenesis (Vaught, et al. 2009). Loss of EphA2 inhibits the proliferation of the mammary epithelium and delays ductal branching essential for complete fat pad filling (Vaught et al. 2009).

Pancreas develops from the embryonic forgut endoderm. Pancreatic development begins with the formation of ventral and dorsal buds. Differential rotation and fusion of the ventral and dorsal pancreatic buds results in the formation of the definitive pancreas consisting of exocrine and endocrine tissues. During development more or less all ephrins and their receptors are expressed in pancreatic progenitors that are expected to give rise to exocrine acini, but they are hardly detected in the more central parts from which intra-pancreatic ducts and endocrine cells derive (van Eyll, et al. 2006). EphA4 is the only receptor that is not expressed during pancreatic development.

Recent findings indicate that Eph-ephrin bidirectional signalling modulate the function of the insulin-producing β-cells in the adult pancreas. The β-cells adjust their secretion of insulin in response to glucose levels in the blood in order to maintain glucose homeostasis in the body. When the glucose concentration is low, several EphA receptors are tyrosine phosphorylated and initiate forward signalling that holds back insulin secretion. In contrast, at high glucose concentrations reverse signalling mediated by the ephrinAs promotes insulin secretion. In fact, ephrin-A5 knockout animals had a significantly reduced glucose-stimulated insulin secretion (Konstantinova et al. 2007).

Thymus is a specialized organ of the immune system in which the T-lymphocytes mature into immunocompetent cells. Cortical and medullary thymic epithelial cells from both the embryonic and the adult thymus express several Eph and ephrins, in particular those of the B family. EphB2

and ephrin-B2 are expressed on both thymocytes and epithelial cells. Functional studies in *EphA4* knockout mice show defects in thymocyte maturation and a decreased numbers of peripheral T-lympocytes. In addition, the *EphA4*$^{-/-}$ thymus is hypocellular and the size of the gland is very much reduced (Munoz et al. 2006). These defects appear to result from abnormal development of *EphA4*-expressing stromal cells in the thymic cortex leading to impaired thymocyte maturation and survival.

Cancer

Protein tyrosine kinase genes are the largest family of oncogenes. Activating mutations leading to constitutive stimulation of signal transduction pathways that control cell shape, proliferation, differentiation, and migration are key events in carcinogenesis and tumour progression. As Ephs represent the largest family of receptor tyrosine kinases it is not surprising that aberrant Eph signalling is frequently recognized in cancer.

Many Eph receptors are over-expressed in a variety of cancers. In most cases this is associated with tumour-promoting features, although sometimes Ephs may inhibit tumorigenesis. The founding member of the Eph family, EphA1, was isolated from a hepatoma cell-line (Hirai et al. 1987) and is over-expressed in liver, lung, breast and colon carcinomas (Pasquale 2010). In metastatic tumour cell lines the expression levels of both Ephs and ephrins are often higher than in the primary tumours, correlating with the grade of the tumour malignancy and invasiveness. EphB4 present in all examined breast carcinoma cell lines is of particular interest since it has both tumour-suppression and tumour-promoting activities. A hypothesis is that high levels of ephrin dependent EphA2 forward signalling suppress tumorigenesis whereas low levels of forward signalling promote. Indeed, EphB4 forward signalling inhibits breast cancer cell tumorigenicity both *in vitro* and *in vivo* when its tyrosine kinase activity is stimulated by a soluble form of the ligand, ephrin-B2 Fc (Noren and Pasquale 2007). EphB4 can also promote tumorigenesis in breast cancer cell lines, by stimulating ephrin-B2 reverse signalling, inducing angiogenesis (Noren, et al. 2004).

Among the first Eph/ephrin mutations associated with human diseases were identified in prostate cancer cells, comprising the *EPHB2*, *EPHA3* and *ephrin-B1* (*EFNB1*) genes. Huusko et al. have described EphB2-dependent growth inhibition of prostate cancer cells (Huusko et al. 2004) .

Other diseases

Craniofrontonasal syndrome; a developmental disorder mainly characterized by skeletal abnormalities have been linked to heterozygous *EFNB1* mutations that impair the expression and function of ephrinB1 in the affected embryonic tissues (Wieland, et al. 2004). The disease phenotype is probably the result of abnormalities in the formation of embryonic tissue boundaries. *EFNB1* is located on the X-chromosome. Interestingly, the disease is much more severe in females than in males even though only females retain one wild-type *EFNB1* allele. (Twigg, et al. 2004; Wieland et al. 2004). An explanation to this seemingly contradictory finding is the ephrins ability to drive cell sorting. In mouse models of this syndrome, the sorting of ephrin-B1-positive and ephrin-B1-negative cells as a result of X-inactivation in heterozygous females disturbs the patterning of skeletal structures more severely than in males and homozygous females, in which all cells lack ephrin-B1.

EphA4

The thyroid has never before been investigated for Eph and ephrin expression. We found that *EphA4* is detected in the thyroid bud by transcriptome analysis (paper III), and characterized its expression pattern and function in the thyroid both during embryonic development and post-natally (papers III and IV). Although already mentioned and discussed in previous paragraphs, EphA4-mediated effects in mouse will be shortly summarized to finalize the introduction part of the thesis. As EphA4 is one of the most thoroughly studied Eph receptors (comprising 283 hits in PubMed search April 2010) only some of its multiple features and signalling effects will be further commented on.

EphA4 is an exception from the rule that EphA receptors only bind ephrin-A ligands and EphB receptors only bind ephrin-B ligands. Thus, apart from binding to several ephrin-As EphA4 has affinity for all three ephrin-B ligands (Gale, et al. 1996b; Wilkinson 2000). EphA4 binds ephrin-B2 with weaker binding affinity than ephrin-As, depending on a loose fit of the ephrin-B2 G-H loop in the EphA4 ligand-binding channel (Bowden, et al. 2009) , however the biological effects are prominent. Because of the different strategies that EphA4 can use to bind ephrin-A or ephrin-B ligands, it is generally considered that EphA4 achieves the highest promiscuity among the Eph receptors (Qin et al.). Illustrating examples of this are EphA4 interactions with ephrin-B2 (and ephrin-B3) that regulate cell sorting in the rhombomeres and branchial arches of the developing hindbrain (Smith et al. 1997), axon guidance and circuit formation in the developing spinal cord (Kullander et al. 2003; Kullander et al. 2001), somite morphogenesis (Barrios, et al. 2003) and inhibition of axon outgrowth by myelin (Benson, et al. 2005). The restricted expression of EphA4 in rhombomeres 3 and 5, i.e. in between the adjacent ephrin-B expressing rhombomeres number 2, 4 and 6 (Henkemeyer et al. 1994; Nieto et al. 1992), signifies the importance of EphA4 in morphogenetic patterning during embryogenesis. In a later developmental stage this keeps a restricted distribution of neural crest cell (NCC) subpopulations when these migrate from the rhombomeres into the corresponding brachial arches. EphA4-ephrin-B1 binding stabilizes blood clot formation (Prevost, et al. 2005).

Interestingly, in the *EphA4* knock-out mouse Kullander et al. saw that some *EphA4* heterozygous animals show the same phenotype as homozygous animals (Kullander et al. 2003), indicating that both alleles are transcribed and required for function. The central pattern generator controls the alternations that underlie the right and left limb movements in walking. Mice deficient for *EphA4* have lost left-right limb alternations and instead move the limbs in a synchronized manner (Kullander et al. 2003). Looking at EphA4-mediated forward signalling regulation of thalamocortical neuronal projections Dufour et al. recently showed that 43% of the mice deficient for EphA4 have an abnormality in the thalamocortical system whereas the same phenotype is observed in 25% of the heterozygous mutants (Dufour et al. 2006). Surprisingly, in EphA4-EGFP mice, in which the whole intracellular part of the receptor is exchanged for a EGFP molecule, the characteristic mutant abnormalities showed a much higher penetrance in the corresponding homozygous mutants (50% in $EphA4^{EGFP/EGFP}$, 83% in $EphA4^{+/EGFP}$) (Dufour et al. 2006). The phenotype observed in heterozygous animals thus seems to be due to a dominate-negative effect of the truncated EphA4 receptor (Dufour et al. 2006).

AIMS OF THE STUDY

The thesis work concentrated on four separate although partially linked issues of either incomplete, controversial or yet unknown status in the developing thyroid gland. The overall aim was to illuminate and possibly uncover some of the morphogenetic and molecular mechanisms of interest to the understanding of thyroid growth and function and distinct pathologies of the gland.

The specific aims of the included papers were:

I. Provide a detailed map of thyroid morphogenesis in the mouse embryo, focusing on spatiotemporal patterns of growth and relationship to adjacent embryonic tissues and structures (mesoderm, vessels).

II. Identify novel biomarkers of mouse C-cell precursors to better characterize their embryonic origin and transport into the developing thyroid.

III. Investigate the expression pattern of EphA4 in thyroid primordial tissues and putative thyroid phenotypes in mice deficient of EphA4 or expressing a truncated receptor.

IV. Characterize thyroid growth and function responses in *EphA4* mutant mice.

RESULTS AND DISCUSSION

Development of the follicular cell lineage

Origin of progenitor cells

In normal development, the thyroid primordium is first distinguished as a thickening of the anterior foregut endoderm at the base of the prospective tongue. This thickening is termed the thyroid placode, it can be distinguished from the rest of the endoderm by their co-expression of four transcription factors, *Nkx2.1, Foxe1, Hhex, Pax8*. These transcription factors have central functions in other embryonic tissues as well, but it is only in the thyroid the combination of all four can be found. The process leading to the organization of the thyroid placode is termed specification.

Congenital hypothyroidism is the most common congenital endocrine disorder affecting approximately 1 of 3500 newborns (Toublanc 1992), results from a partial or complete loss of thyroid function. To learn about a malfunction, it is important to have a detailed view of the normal process. In **paper I** we investigated the normal thyroid development in mouse embryos. The endoderm outside the placode was found to proliferates, although no BrdU incorporation could be detected in the thyroid primodium. A likely explanation would be that the thickening of the endoderm expands by recruitment of more cells from the neighbouring endoderm. Earlier studies support this. Kinebrew and Hilfers studies (Kinebrew and Hilfer 2001) in chick embryos provides evidences that growth of the thyroid occurs by movements of individual cells and invasion of the nearby pharyngeal cells. The enlargement of the placode and budding to follow takes place in close relationship with the aortic sac (**paper I**), a large vessel that communicates with the primitive heart outflow tract and from which the pharyngeal arch arteries develop.

EphA4 and several *ephrins* were found to be detected in the thyroid bud by transcriptome analysis (**paper III**). The *EphA4* receptor was selected for further investigation. In this paper, we used an EphA4 receptor with the intracellular part exchanged for an EGFP (enhanced green fluorescent protein) molecule for *in vivo* localization of EphA4 protein. EphA4-EGFP was ubiquitously expressed in the anterior foregut endoderm including the thyroid placode in E9.5 embryos. At the budding stage, the thyroid progenitors continued to express EphA4, confirming the results from the transcriptome analysis. The specification and budding process proceed normally even when *EphA4* was deleted (in *EphA4* null mice). The results indicate that EphA4 signalling have no function for these early developmental stages. Another explanation would be that, as previous results have shown, other Eph receptors with similar expression pattern and ligand specificities may have overlapping functions and partially compensate for the loss of the EphA4 receptor so that the thyroid primodium can develops normally (Birgbauer, et al. 2000).

It is generally believed based on the expressions pattern that Foxa2 (the forkhead box transcription factor A2) regulates thyroid development, although this has not been studied in more detail. Since Foxa2 is known to be broadly expressed in the definitive foregut endoderm

(Monaghan et al. 1993; Sasaki and Hogan 1993), it was first important to clarify whether Foxa2 might be expressed also by progenitors of the follicular cell lineage during thyroid organogenesis (paper II). Strong Foxa2 expression was observed as expected in the floor plate and notochord at E9.5. In addition, the anterior endoderm showed Foxa2 immunoreactivity, although it was heterogeneous and preferentially located in the dorsal and ventral aspects of the foregut. Notably, Foxa2 expression was excluded from the zone in the foregut endoderm corresponding to the thyroid placode, identified by the transcription factor Nkx2.1. That the midline thyroid primordium did not express Foxa2 during budding was confirmed by analysing E10 embryos, moreover the detached bud was also lacking Foxa2, suggesting that the early developmental stages of the thyroid are Foxa2 independent.

Embryonic development

During the course of further development, the thyroid bud had assumed an elongated shape with the cranially part attached to the endoderm by the so-called thyroglossal duct. At this stage, the precursor cells are clearly separated from the aortic sac. This is also the first time the cells are proliferating, and detected by BrdU (**paper I**). EphA4-EGFP expression can still be seen in the membrane of all thyroid progenitor cells (**paper III**). The thyroglossal duct disappears at embryonic day 11.5, and the bud is completely detached from the endoderm. Soon after, the developing thyroid starts moving in a caudal direction away from the endoderm, still holding the midline position. At this stage, the earliest sign of vascularisation can be observed in the cup-shaped proliferating primordium that have re-established a close position to the cranial part of the aortic sac. In level with the third pharyngeal arch arteries, the midline thyroid primordium has reached the final position. The fluorescent signal from the EGFP molecule attached to EphA4s extracellular part was strong, even now when the thyroid primodium is a free body (**paper III**).

For the thyroid to develop into its final butterfly-shape, with two lobes located in the anterior neck on the trachea just inferior to the larynx, it has to begin to grow bilaterally. During the bilobation process, the thyroid proliferates and extends laterally along the route of the third pharyngeal arch arteries towards the ultimobranchial bodies. The functional role that the nearness of the aortic sac might have for the developing thyroid is not apparent, although, some possibilities are suggested from data obtained in *Tbx1*$^{-/-}$ embryos. Mice deficient for Tbx1 were observed to have only a single thyroid lobe, this might be because of the lack of the re-established contact between the aortic sac and thyroid primodium. Data suggest that this re-association with the aortic sac guides the thyroid primodium along the third pharyngeal arch arteries, and that this event promote the bilobation process; without this contact in the mutant embryo only one single thyroid diminutive lobe is formed (Fagman, et al. 2007).

At E13.5, the bilaterally outgrowing thyroid surrounds the ultimobranchial bodies and the two anlagen fuse forming the primitive thyroid lobes. Two days later, at E15.5 the growing thyroid lobes consist of cords of follicles in a star like pattern. The organization of the typical thyroid histoarchitecture has started and at E17.5 the thyroid lobes had increased further in size and the cordlike structures has developed into small follicles that accumulated thyroglobulin in the lumen (**paper I**). In the later part of thyroid development EphA4-EGFP was found in most of the expanding parenchyma of the thyroid lobes. However, in the central parts of the lobe known to

harbour not yet scattered C-cells, there were cells undetectable for EGFP (**paper III**); this will be discussed in more detail later on. *EphA4* deficient mice and *EphA4-EGFP* mutants were morphologically investigated. No apparent malformation could be found during the different developmental stages when compared to wild type embryos (**paper III**). The present findings suggest that midline thyroid follicular development is independent of both forward and reverse EphA4 signalling. Foxa2 can still not be detected in progenitors from the lateral thyroid primodium (**paper II**).

Post-natal development

In **paper III** we searched for possible structural abnormalities in the adult thyroid gland in both *EphA4* knockout animals and *EphA4-EGFP* mice in which the intracellular forward-signalling domain is missing. All these animals were clinically euthyroid and the thyroid hormone levels were normal. The gland consisted of two lobes of equal size, and there were no signs of hypoplasia or goiter. The only dissimilarity between the mutant animals and the wild type were at a histological level. The follicular epithelium in *EphA4*$^{-/-}$ and *EphA4*$^{EGFP/EGFP}$ glands was often more flat and the cells displayed a very thin apical cytoplasm with elongated nuclei. Morphometric data over size and numbers of follicles showed that *EphA4*$^{-/-}$ thyroids contained a significantly higher number of small-sized follicles (<1000μm^2) compared to *EphA4*$^{EGFP/EGFP}$ and wild-type mice. The oversized follicles (>3000μm^2), that were in total in minority, were more frequent *in EphA4*$^{EGFP/EGFP}$ glands outnumbering even those in the wild-type. The number of follicles of intermediated size (1000-3000 μm^2) did not differ between genotypes. Total follicular volume estimated from the surface area in serial sections did not significantly differ. In summary, the results demonstrate an altered follicular morphology in mice either lacking EphA4 or in which the forward intracellular signalling domain of the receptor is missing. This indicates that EphA4 regulates follicular cell behaviour cell-autonomously.

When adult wild-type mice were given MMI and ClO_4^- in drinking water for two weeks the intrathyroidal iodine metabolism was blocked and the mice became hypothyroid and thereby the endogenous levels of TSH were increased (**paper IV**). The histology of the gland was fundamentally altered and solid tissues of hyperplastic epithelium replaced the majority of the follicles. This tissue remodelling due to hyperplasia was found to be much less prominent in *EphA4* mutant mice, the difference was most evident in *EphA4* deficient animals. Animals treated with MMI and ClO_4^- obtained increased angiogenesis, however *EphA4* knockout mice that displayed less hyperplasia also exhibited a reduced amount of microvessels than equally treated wild-type mice. Hormonal analysis further suggested that the poor thyroid growth response to goitrogens in *EphA4* mutant mice, which included also haploinsufficient animals, depends on a thyroid defect rather than a pituitary defect (paper IV).

C-cell development

Origin of progenitor cells

In 1962, Copp et al discovered a cell that could produce calcitonin, they named it the C-cell (Copp, et al. 1962). It is generally agreed that C-cells are neuroectodermal emanating from the neural crest, ever since this was first shown in chick-quail chimera experiment (Polak et al. 1974). Fate mapping studies of Wnt1 expressing neural crest progenitors in mice recently challenged this concept (Kameda et al. 2007). Specifically, *Wnt1-Cre/Rosa26R* double heterozygous mutant embryos mapped neural crest-derived cells known to populate the pharyngeal arches but failed to identify any Wnt1 expressing cells in the ultimobranchial bodies or in thyroid co-localizing with the C-cells. Moreover, the C-cells were found to express the epithelial marker, E-cadherin. From this, the authors postulated that C-cells might derive from the endoderm rather than the neuroectoderm. The C-cells origin in higher vertebrates is therefore a controversy. In **paper II**, we used *Tbx1* as a marker of foregut endoderm that is absent in neural crest cells. In recent studies (Fagman et al. 2007), investigation of the midline thyroid primodium revealed no *Tbx1* expression at all in the placode; *Tbx1* would therefore also probably discriminate between different thyroid progenitors. Double heterozygous *Tbx1-CreRosa26R* offsprings was used to trace descending progenitors. *Tbx1* expression detected by X-gal staining could be found in the developing thyroid. As assumed, there were no β-gal activity detected in the thyroid follicular cells. The cells that were expressing *Tbx1-Cre* were mainly located centrally in the lobes and some of them were co-expressing calcitonin. Based on the *Tbx1* results, we further investigated in **paper II** whether members of the Foxa family of forkhead transcription factors known to be expressed in all the endoderm, but excluded from neural crest, could be used to label thyroid progenitor cells and further give strength to the endoderm origin hypothesis of mouse C-cells.

Embryonic development

The lateral thyroid primordia, the paired ultimobranchial bodies, develop from the fourth pharyngeal pouches and transport C-cell precursors to the thyroid. In **paper II** immunohistochemical staining revealed that the budding ultimobranchial bodies were mostly Foxa2 negative, whereas the adjacent endoderm surrounding the pharynx uniformly expressed Foxa2. At the base of the ultimobranchial bud, there was a clear separation between Foxa2 positive and Foxa2 negative epithelium. Foxa2 was increasingly up-regulated in the ultimobranchial bodies between E11.5-13.5 and most cells were expressing Foxa2 immediately before fusion with the midline thyroid, which was still entirely Foxa2 negative.

The ultimobranchial epithelium also gradually acquired Nkx2.1 expression, which in fact preceded the expression of Foxa2 in the same cells. After fusion could cells of the former ultimobranchial bodies be distinguished from the thyroid primodium cells by a weaker Nkx2.1 expression as well as lack of BrdU labelling and Pax8 negativity. This suggested that the growth

properties of the ultimobranchial bodies changed after fusion with the midline thyroid anlage (**paper I**). Until the fusion stage, the ultimobranchial bodies uniformly expressed EphA4 (EGFP-tagged). However, as ultimobranchial bodies approached the median anlage the EGFP expression signal became weaker and eventually undetectable at E13.5. In contrast, EphA4-EGFP was strongly expressed in progenitors of the former median thyroid including those cells that where in immediate contact with the UB border (**paper III**). Possibly, down-regulation of EphA4 in the ultimobranchial bodies is critical for fusion process and delivery of C-cell precursors to the thyroid.

As the developing thyroid lobes enlarge the C-cells increase in number and start to produce calcitonin around embryonic day 15 (**paper I**). Immunolabeling for calcitonin showed that the differentiated C-cells were all EphA4-EGFP negative. The number of C-cells present in the thyroid in late development (E17.5) did not differ between wild-type and *EphA4* mutant embryos, indicating that EphA4 is not required for the propagation of the C-cell lineage in fetal life (**paper III**). Foxa1 and Foxa2 were co-expressed in the central portions of the parenchyma at E13.5. Immunolabeling showed that Foxa2 (and Foxa1) and calcitonin were co-expressed in embryonic C-cells, although Foxa2 positive cells were more numerous presumably representing immature yet undifferentiated C-cell precursors. These observations provide evidence for that mouse C-cells and the ultimobranchial epithelium share the same endoderm markers, indicating that C-cell precursors most likely derive from the foregut endoderm (**paper II**).

Post-natal development

In **paper III** we have revealed that *EphA4*$^{EGFP/EGFP}$ (11020+/-827 (n = 4)) have significantly reduced numbers of C-cells immunoreactive for calcitonin whereas there were no differences between wild-type (26026+/-1670 (n = 6)); and *EphA4*$^{-/-}$ (27590+/-6000 (n = 4)) mice. The number of C-cells were 58% higher in normal animals than in the thyroid of *EphA4-EGFP* homozygous mice. In late development (E17.5) the amount of C-cells present in the thyroid were equal comparing wild-type and *EphA4* mutant embryos. As EphA4 expression was restricted to the follicular epithelium, these observations indicate that EphA4 in thyrocytes cell-non-automously regulates the proliferation of the C-cell after birth and possibly also in the adult gland. An explanation to this may be offered by previously published data on the same mutant mice by Dufour, et al, who discovered that the most sever phenotype in the somatosensory system accounts for homozygous *EphA4-EFGP* mice and not, as one might first assume, *EphA4* null animals (Dufour et al. 2006). It is suggested that EphA4-EGFP is unable to interact with other Eph receptors on the same cell (by cis-interactions) and that this in turn exerts a dominate negative effect.

Lastly, evaluation of a microarray tumor data bank revelaed that *Foxa2* is expressed in medullary thyroid carcinoma (MTC) but not in cancer derived from the follicular epithelium. This suggests that Foxa2 might be a novel biomarker of MTC (**paper II**).

	E9.5	E10.5	E11.5	E12.5	E13.5	E15.5	E17.5
EphA4 Th	+	+	+	+	+	+	+
EphA4 UB			+	+	-	-	-
Foxa2 Th	- (+)	-	-	-	-	-	-
Foxa2 UB		-(+)	+	+	+	+	+
Foxa1 Th							
Foxa1 UB			+	+	+	+	+

Table 1. Summary over EphA4, Foxa2, Foxa1 expression in thyroid midline anlage (Th) and ultimobranchial body (UB) during the different developmental stages. High expression is marked with a +, low –(+), undetectable expressions is marked with -. Empty cells is not experimentally studied.

MAJOR CONCLUSIONS OF THE THESIS PAPERS

Paper I:

- The midline thyroid develops in close proximity to embryonic vessels suggesting these participate as guiding tracks for thyroid organogenesis.

- Progenitor cell proliferation of the follicular lineage is a late event, suggesting that expansion of the specified precursor pool forming the bud occurs by recruitment of cells from other parts of the foregut endoderm.

Paper II:

- Mouse C-cell precursors in the pharyngeal endoderm express Tbx1 and Foxa1/2, arguing against a neural crest origin of C-cells in higher vertebrates.

- Foxa2 is a novel marker of medullary thyroid carcinoma in humans.

Paper III:

- The Eph receptor EphA4 is expressed in thyroid progenitors in early development and in differentiated follicular cells in late development.

- Follicular structure and number of differentiated thyroid C-cells are modulated by EphA4 post-natally.

Paper IV:

- Thyroid growth response to goitrogenic stimulation requires EphA4 presumably by a cell-autonomous mode of action in the follicular cells.

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